Detection and Identification of Virulence Factors in *Yersinia pestis* Using SELDI ProteinChip® System

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ABSTRACT

A rapid method for the detection, purification, and identification of proteins in bacterial extracts was developed using surface enhanced laser desorption/ionization (SELDI) ProteinChip® technology. The effectiveness of this technique for monitoring the expression and identification of temperature- and calcium-regulated virulence factors of *Yersinia pestis*, the bacterium that causes human plague, is demonstrated. *Y. pestis* infection of its mammalian host is thought to be accompanied by rapid up-regulation of a number of genes following a shift from 26°C (the temperature of the flea vector) to 37°C (the temperature of the mammalian host). To model this process, *Y. pestis* cells were grown at 26°C and 37°C in a Ca²⁺-deficient medium. Through an initial protein profiling of the crude bacterial extract on strong anion exchange and copper affinity, ProteinChip arrays detected five proteins that were up-regulated and three proteins that were down-regulated at 37°C. Two of the proteins predominately expressed at 37°C were semi-purified in less than two days. The two proteins were identified as catalase-peroxidase and Antigen 4. Aside from its speed, a salient feature of the SELDI technique is the microgram amounts of crude sample required for analysis.

INTRODUCTION

The explosive progress over the past decade in the fields of protein science, bioinformatics, and genomics has resulted in an increased demand for the development of high-throughput technology that allows for the detection, purification, and identification of proteins at high speed and with high sensitivity. With the wealth of information coming from completed genome projects, the next logical step is proteomic-scale protein analysis. For example, analysis and correlation of mRNA expression with protein expression within an organism require biochemical analysis of thousands of genes and proteins. In the present work, we show that surface enhanced laser desorption/ionization (SELDI) ProteinChip® array technology (6,9) (Ciphergen Biosystems, Palo Alto, CA, USA) is uniquely suited for these kinds of studies, as exemplified by the analysis of several temperature-dependent *Yersinia pestis* virulence factors.

Y. pestis, the bacterium that causes sylvatic and human plague, was used as a model organism to demonstrate the feasibility of the SELDI technique to differentiate proteins expressed under two different growth conditions. Plague is primarily a disease that affects rodents and is transmitted by their associated fleas to humans. During the bacterial residency in the flea, a large number of the proteins involved in mammalian infection are not expressed due to the lower temperature encountered in that host. Expression of virulence factors takes place shortly after *Y. pestis* cells are passed from fleas to

the unique and higher-temperature environment of the mammalian host (10). Temporal induction of selected virulence genes during temperature shift has been previously studied by the direct measurement of the levels of mRNA during this transition (5).

In the current study, we chose the cultivation of yersiniae cells in a Ca^{2+} -deficient media that is associated with the up-regulation of plasmid- and chromosome-encoded virulence factors upon temperature shift from 26°C (temperature encountered in the flea vector) to 37°C (temperature of the mammalian host) (8). Dissecting this mechanism of pathogen adaptation is thus an important element in understanding this process. We demonstrate here that SELDI can be used to monitor protein expression levels at the two temperatures with exquisite sensitivity, allowing facile correlation to mRNA expression levels. This approach will lead to a better understanding of the changes occurring during the temperature induction of virulence genes of *Y. pestis*.

MATERIALS AND METHODS

Bacterial Lysate Preparation

A non-pigmented isolate of *Y. pestis* carrying all three virulence plasmids (pCP1, pMT1, and pPCP1; substrain D27), KIM10 was used to prepare protein lysate samples (4). Cells were grown in Ca^{2+} -deficient WCS, pH 6.8, medium to achieve a low-calcium response as described previously (3). Briefly, yersiniae were grown at 26°C to an absorbance of 0.25 at 620 nm (A_{620}), at which time half of the culture was shifted to 37°C. Both the cells remaining at 26°C and those shifted to 37°C were harvested at 6 h after the temperature shift (at an A_{620} of 1.0–1.2). Cell pellets obtained from 120 mL culture were suspended in 5 mL 20 mM HEPES, pH 7.4, 50 mM NaCl, and disrupted using a French Press (SLM Instruments, Urbana, IL, USA) at 750 psi. The cell debris was removed by centrifugation (20000× *g* for 20 min at 4°C), and the supernatant was stored in aliquots at -70°C. Protein concentration was determined using the BCA kit (Pierce Chemical, Rockford, IL, USA) and adjusted to a concentration of 2 mg/mL before SELDI analysis.

Processing and Analysis of ProteinChip Arrays

The SELDI analysis was performed using 10 µg crude cytosol extract of *Y. pestis* grown at 26°C and 37°C, respectively. A strong anion exchange ProteinChip array (SAX-2; CIPHERGEN Biosystems) was employed to capture negatively charged proteins, and an immobilized copper affinity array (IMAC- Cu^{++} ; CIPHERGEN Biosystems) was used to capture proteins with affinity for copper to select for a specific subset of proteins from the *Y. pestis* crude extracts. Captured proteins were directly detected using a PBSII ProteinChip Array reader (CIPHERGEN Biosystems), a laser desorption/ionization time-of-flight mass spectrometer.

Before loading the cell lysate, the SAX-2 array was equilibrated two times with 5 µL 20 mM HEPES, pH 7.4 (5 min each). After removing the equilibration buffer, 5 µL cell lysate was added and incubated for 15 min on a rocker. Cell lysate was removed, and the surface was washed three times with the equilibration buffer (5 min each). A quick final rinse with water was performed. The surface was allowed to dry,

followed by two additions of 0.4 µL saturated sinapinic acid (SPA; CIPHERGEN Biosystems) in 50% acetonitrile and 0.5% trifluoroacetic acid (TFA). Captured proteins were detected using the ProteinChip Reader.

The same procedure was followed for the IMAC- Cu^{++} array with the following modifications. The surface was first loaded with 5 µL 50 mM copper sulfate two times for 5 min, followed by a quick rinse with water to remove the unbound copper. The surface was then equilibrated two times with 5 µL PBS (5 min each). For the NP-2 chips (Normal Phase array; CIPHERGEN Biosystems), samples were applied directly to the surface.

Tryptic Digestion and Peptide Mapping

The two bands of interest (up-regulated proteins) were excised from the SDS-PAGE gel. An in-gel digest with 1 pmol trypsin was performed (11). The tryptic digestion was done overnight at 37°C in a reaction volume of 30 µL 50 mM ammonium bicarbonate buffer, pH 8.0. Subsequently, 2 µL of the digestion mixture were analyzed on NP-2. Alpha-cyano-4-hydroxy cinnamic acid (CIPHERGEN Biosystems) was used to detect peptides generated in the tryptic digest. The masses of the tryptic digested peptides were measured on a PBSII system that had been calibrated using internal standards. Molecular weights of the peptides were used for protein identification using the ProFound Database, with a mass tolerance of 1 Da and a maximum missed cut of 1.

RESULTS AND DISCUSSION

Differential expression of proteins in *Y. pestis* grown at 37°C and 26°C was examined by SELDI mass spectrometry

Figure 1. SELDI capture of proteins from bacterial cell lysate onto SAX-2 ProteinChip array. Top and bottom panel are different molecular weight regions of the same spectra. Ten micrograms of crude cytosolic extracts of *Y. pestis* grown at 26°C versus 37°C were analyzed on SAX-2 chip. The 14.9- and 78.8-kDa proteins were expressed only at 37°C. The experiment was repeated five times.

Figure 2. Partial purification scheme (developed at CIPHERGEN Biosystems, by T. Pham) of the two temperature-inducible proteins using spin columns.

Figure 3. Final purification for tryptic mapping. (A) Separation on SDS-PAGE gel for tryptic digestion. 140 μ L of pH 6.0 and pH 5.0 fractions of the anion exchange column from both 26°C and 37°C lysate were loaded on the gel. The gel was stained with Coomassie® Blue. (B) Analysis of the peptides generated by tryptic digestion on SELDI time-of-flight mass spectrometry. Two microliters of the 30 μ L trypsin digestion mixture were analyzed on an NP-2 array.

Table 1. Identification of (A) the 78.8-kDa Protein and (B) the 14.9-kDa Protein by Comparison of Peptide Maps to the NCBI Protein Database

A.			
Rank	Probability	Protein Description	MW (kDa)
1	1.0e+00	Catalase/oxidase- <i>Yersinia pestis</i>	81
2	3.5e-31	Transferrin-binding protein2- <i>Haemophilus influenza</i>	70
B.			
Rank	Probability	Protein Description	MW (kDa)
1	5.0e-01	Antigen 4 (adhesin)- <i>Yersinia pestis</i>	15
2	6.9e-04	<i>E. coli</i> hypothetical protein	16

using SAX-2 and IMAC-Cu⁺⁺ (figure not shown) ProteinChip arrays. Specifically, 10 µg total protein lysate were used to capture a subset of proteins based on the affinity of the bacterial proteins for the two ProteinChip surfaces used. Captured proteins were then detected directly from the ProteinChip surface by time-of-flight mass spectrometry using the SELDI system. This process was completed in less than 1 h by analyzing multiple samples in parallel on the same ProteinChip array. Two proteins of mass 14.9 and 78.8 kDa in samples from bacteria grown at 37°C for 6 h (Figure 1), which were not seen in samples from bacteria grown at 26°C, bound to the SAX-2 ProteinChip. Both proteins bound at pH

7.4, suggesting that each had a pI less than 7.4. In addition, the 78.8-kDa protein also bound to the IMAC-Cu⁺⁺ ProteinChip, suggesting the presence of at least one free histidine imidazole group on the surface. In contrast, the 14.9-kDa protein bound to the SAX-2 ProteinChip but not to the IMAC-Cu⁺⁺ ProteinChip, suggesting the absence of a free metal binding motif. The experiments were repeated five times, and in all repeats the two proteins were detected only in samples from bacteria grown at 37°C.

To identify the 78.8- and 14.9-kDa proteins, a partial purification scheme was devised (Figure 2). Since both proteins apparently had a pI lower than 7.4 while differing in size, 600

µg total bacterial lysate were first separated by molecular weight using a K30 size selection spin column (Ciphergen Biosystems). Five 30-µL fractions were collected, of which 1 µL each fraction was spotted on an NP-2 ProteinChip to monitor the elution of the 78.8-kDa protein (data not shown). The first three fractions, which contained the protein, were pooled and passed over an anion exchange spin column (Ciphergen Biosystems). Using a step pH gradient from 9.0 to 3.4, the proteins were further separated on the basis of their pI. Eight 150-µL fractions were collected, of which 1-5 µL each fraction were spotted on an NP-2 ProteinChip to monitor the elution of the 78.8-kDa protein (data not shown). A 140-µL aliquot of fractions that contained the 78.8-kDa protein was concentrated and run on a 4%–20% SDS-PAGE gradient gel (Figure 3A). Surprisingly, a fraction of the 14.9-kDa protein eluted in the high molecular weight fraction from the K30 size exclusion column as well, suggesting that it might be a component of a larger complex. Further purification of the 14.9-kDa protein on the anion exchange column revealed that the protein eluted at pH 6.0 (Figure 3A).

After partial fractionation of both proteins, which largely eliminated other proteins that comigrate on the SDS-PAGE gel, the protein bands were cut from the gel for subsequent trypsin digestion. Following tryptic digestion of the proteins, the peptide fragments were analyzed by SELDI, which revealed 47 peptides for the 78.8-kDa protein. A search of the NCBI protein Database by ProFound was performed that included all kingdoms and proteins with a mass range from 10 to 100 kDa. Out of these 47 peptides, 35 matched with the catalase/peroxidase KatY protein (also known as Antigen 5 or p70) of *Y. pestis* (Table 1A). The remaining unmatched peptides could be from contaminants such as keratin and trypsin. Several additional lines of evidence support the notion that this protein is indeed KatY. First, expression of KatY is known to be induced at 37°C. Second, the enzyme is expressed as an 81.4-kDa protein containing a leader signal sequence of 2.6 kDa (2,4), which upon processing leaves a 78.8-kDa protein, matching the molecular mass determined by SELDI. Third, the predicted pI of KatY is 6.4, explaining its elution off the anion exchange spin column at pH 6.0 and its affinity for the SAX-2 ProteinChip at neutral pH. Finally, amino acids 96–109 and 255–268 are active site motifs encoding proximal and distal histidine imidazoles, which presumably facilitated binding to the IMAC-Cu⁺⁺ ProteinChip.

Trypsin digestion of the 14.9-kDa protein generated eight peptides (Figure 3B), three of which matched the Antigen 4 protein (also known as pH 6 antigen) of *Y. pestis* (Table 1B). Antigen 4, like KatY, is known to be induced at 37°C (1) and in conditions of low calcium (8). In addition, Antigen 4 possesses a fibrillar structure required for full virulence (7), which may explain its elution in the higher molecular weight fraction on the K30 size exclusion column under the native conditions used in these experiments.

As described here, two virulence factors, KatY and Antigen 4, of *Y. pestis* were identified using the SELDI ProteinChip technology by measuring protein expression levels at 37°C and 26°C. In support of the findings reported, expression of both genes encoding for KatY and Antigen 4 was also observed at the described conditions using mRNA analysis by dot-blot hybridization (5). Importantly, as we have shown, the entire process of profiling protein expression patterns to the identification of a specific protein can be executed in only three days.

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