Extensive Antibody Cross-reactivity among Infectious Gram-negative Bacteria Revealed by Proteome Microarray Analysis*

Sarah L. Keasey‡‡, Kara E. Schmid‡‡, Michael S. Lee‡‡, James Meegan§§, Patricio Tomass¶¶, Michael Minto¶¶, Alexander P. Tikonov¶¶, Barry Schweitzer¶¶, and Robert G. Ulrich‡‡

Antibodies provide a sensitive indicator of proteins displayed by bacteria during sepsis. Because signals produced by infection are naturally amplified during the antibody response, host immunity can be used to identify biomarkers for proteins that are present at levels currently below detectable limits. We developed a microarray comprising ~70% of the 4066 proteins contained within the Yersinia pestis proteome to identify antibody biomarkers distinguishing plague from infections caused by other bacterial pathogens that may initially present similar clinical symptoms. We first examined rabbit antibodies produced against proteomes extracted from Y. pestis, Burkholderia mallei, Burkholderia cæpacia, Burkholderia pseudomallei, Pseudomonas aeruginosa, Salmonella typhimurium, Shigella flexneri, and Escherichia coli, all pathogenic Gram-negative bacteria. These antibodies enabled detection of shared cross-reactive proteins, fingerprint proteins common for two or more bacteria, and signature proteins specific to each pathogen. Recognition by rabbit and non-human primate antibodies involved less than 100 of the thousands of proteins present within the Y. pestis proteome. Further antigen binding patterns were revealed that could distinguish plague from anthrax, caused by the Gram-positive bacterium Bacillus anthracis, using sera from acutely infected or convalescent primates. Thus, our results demonstrate potential biomarkers that are either specific to one strain or common to several species of pathogenic bacteria. Molecular & Cellular Proteomics 8:924–935, 2009.

Plague is a disease of historical epidemics that remains an important public health problem in limited areas of the world (1). Disease transmission usually occurs through transfer of the bacillus Yersinia pestis by the bite of a flea. However, less frequent direct transfer of viable bacteria by respiratory droplets may result in primary pneumonic infection. A transient intracellular infection of phagocytic cells (2) occurs during the earliest stage of bubonic plague followed by rapid extracellular expansion of bacteria in lymph nodes. The prototypical lymphatic infection of bubonic plague may also progress to bacteremic or pneumatic infection with a very high rate of fatality if there is not rapid intervention by antibiotic treatment (3). Among the reported cases occurring annually in the United States, 15% were fatal in 2006 (4). Although only small numbers of human cases occur each year in North America, a more substantial incidence of plague is found in wild animal populations (5) with seroprevalence rates of up to 100% among mammalian carnivores in endemic areas (6). The geographic range of infection within feral populations is presently unknown but may contribute significantly to the reservoir of potential disease transmission to humans.

Diagnostic tests and prophylactic vaccines or therapies must rapidly distinguish or protect against the many infectious diseases that present similar initial symptoms. Specific diagnostic tests and vaccines for plague are public health priorities primarily because of the threat from potential acts of terrorism. Because human deaths may occur within 48 h of infection (7), delays in proper diagnosis have led to disease complications and fatalities from plague (8). Yet the identification of bacterial sepsis at the earliest stage of clinical presentation is challenging because of the generalized nature of disease symptoms and the difficulty in culturing infectious agents or isolating sufficient material to identify the infectious agent by amplification of genetic markers. Although host antibody responses provide a sensitive indicator of current or past infection, insufficient numbers of validated biomarkers are available, and extensive antibody cross-reactivity among Gram-negative pathogens (9–12) complicates the direct analysis of serum.

Identification of plague-specific antibody interactions is a daunting task because of the complexity of the bacterial proteome encountered by the host during infection. The chromosome of Y. pestis CO92 encodes ~3885 proteins, whereas an additional 181 are episomally expressed by pCD1, pMT1, and pPCP1. For comparison, the proteome of Y. pestis KIM1 contains 4202 individual proteins (13), 87% in common with

---

*This paper is available on line at http://www.mcponline.org

1 The abbreviations used are: KIM, Kurdistan Iran man; LD50, median lethal dose; CaF1, Y. pestis capsular F1.
CO92 (14), and the closely related enteric pathogen Yersinia pseudotuberculosis (15, 16) contains ~4038 proteins (chromosome plus plasmids). Recent technical advances have facilitated the development of microarrays comprising full-length, functional proteins that represent nearly complete proteomes. For example, Zhu et al. (17) reported the development of a proteome microarray containing the full-length, purified expression products of over 93% of the 6280 protein-coding genes of the yeast Saccharomyces cerevisiae, and Schmid et al. (18) described the human antibody repertoire for vaccinia virus recognition by using a viral proteome microarray. This approach opens the possibility of examining the entire bacterial proteome to elucidate proteins or protein pathways that are essential to pathogenicity or host immunity.

We sought to identify biomarkers that could distinguish plague from diseases caused by other bacterial pathogens by measuring host antibody recognition of individual proteins contained within the Y. pestis proteome. The previously reported genomic sequences of Y. pestis strains KIM (13) and CO92 (14), sharing 95% identity, were used for reference. Approximately 77% of the putative Y. pestis proteome can be classified by known homologies. We successfully expressed and purified the majority (70%) of the 4066 ORFs encoded by the chromosome and plasmids of Y. pestis KIM and arrayed these products onto glass slides coated with nitrocellulose. The Y. pestis ORFs subcloned into expression vectors were fully sequenced to confirm quality and identity before use. Different approaches for studying the antibody repertoire for plague in rabbits and non-human primates were compared. Based on results from experiments using the Y. pestis proteome microarray, we identified new candidates for antibody biomarkers of bacterial infections and patterns of cross-reactivity that may be useful diagnostic tools.

**EXPERIMENTAL PROCEDURES**

Y. pestis Proteome Microarray—Gateway Entry clones (Invitrogen) of Y. pestis ORFs were obtained from the Pathogen Functional Genomics Resource Center of The Institute for Genomic Research. High throughput methods were used for the subcloning, expression, and purification of GST-tagged proteins derived from the collection of Y. pestis ORF clones as described previously (19, 20) and as described below. The entry clones were subcloned into the pEXP7-DEST expression vector via standard Gateway recombination. The purified entry plasmid DNA was recombined into the destination vector using a 5-μL scale LR reaction. The LR product mixture was used to transform chemically competent Escherichia coli DH10B. Afterward each transformation well was plated onto a Petri dish with medium supplemented with ampicillin and carbenicillin for selection of recombinant bacteria. For each bacterial transformation, four colonies were robotically picked into a 384-well plate with LB-ampicillin/yeast extract/tryptone/antibiotics medium. Plasmid DNA was purified from 1.1-ml cultures of overnight destination clones grown in 2× yeast extract/tryptone medium using an Eppendorf Perfectprep® Plasmid 96 Spin, Direct Bind kit. Final DNA elutions were performed with 2 successive volumes that were combined after each spin through the binding plate, and DNA concentrations were measured directly with the 96-well plates of purified destination plasmid by a Broad Range Quant-IT kit (Invitrogen). A spot check for DNA quality was performed by running at least 24 samples from the assay plate (per plate) on a low resolution agarose E-gel 96 system (Invitrogen). Newly produced destination clones were also evaluated for correct gene identity by performing a single sequencing read on purified plasmid followed by BLAST (Basic Local Alignment Search Tool) analysis. For protein expression and purification, a stock solution of 85 μL of Expressway™ (Invitrogen) reaction mixture composed of E. coli extract, reaction buffer, amino acids, and T7 RNA polymerase enzyme mixture was prepared and dispensed into each well of deep well 96-well plates. A minimum of 500 ng of purified plasmid DNA at 25–200 ng/μL was then robotically dispensed into each well using automated liquid handling equipment. The deep well plate was returned to the 30 °C shaking incubator for 3 h. After centrifugation at 4000 rpm for 5 min, the supernatant was then removed from the incubator and centrifuged briefly (1000 rpm) to collect contents into bottom of wells. One hundred microliters of Expressway feed buffer was then dispensed into each well using automated liquid handling equipment. The deep well plate was then transferred to the 30 °C shaking incubator for 3 h. After centrifugation at 4000 rpm for 5 min, the supernatant was then removed from the incubator and centrifuged briefly (1000 rpm) to collect contents into bottom of wells. One hundred microliters of Expressway feed buffer was then dispensed into each well using automated liquid handling equipment. The deep well plate was returned to the 30 °C shaking incubator for 3 h. After centrifugation at 4000 rpm for 5 min, the supernatant was then removed from the incubator and centrifuged briefly (1000 rpm) to collect contents into bottom of wells. One hundred microliters of Expressway feed buffer was then dispensed into each well using automated liquid handling equipment. A 50% slurry of wash buffer-equilibrated, glutathione-Sepharose was added to the supernatant in each well, and the plate was placed at 4 °C in a shaking incubator set to 200 rpm. The well contents were then transferred to a 96-well filter plate, and the plate was centrifuged for 1 min at 3000 rpm. The resin was then rinsed and washed three times in a HEPES buffer containing 1 M NaCl followed by two washes in a HEPES buffer containing 200 mM NaCl. Bound protein was eluted using a buffer containing 20 mM reduced glutathione during an overnight incubation at 4 °C followed by centrifugation at 4000 rpm for 10 min.

Protein sample sets were electrophoresed on SDS-polyacrylamide gels and immunodetected by Western blot. The immunoblots were electronically captured and processed to generate a table of all the protein molecular weights detected for each sample and uploaded into the database. The protein sizing data for purified protein fractions were automatically scored using an in-house laboratory information management system, ProtoMine™, for the presence or absence of a dominant band at the correct expected molecular weight. Supernatants containing eluted protein were transferred to fresh 96-well plates and stored at ~80 °C.

Recombinant bacterial and control proteins were printed onto glass slides coated with nitrocellulose as described previously for human and vaccinia virus protein arrays (17–22). Protein spot densities of representative slides were measured by using an anti-GST antibody and compared with a dilution series of known quantities of protein that were also printed on each slide. Intraslide and intralot variability in spot intensity and morphology, the number of missing spots, and the presence of control spots were also measured and compared with a defined set of lot release standards before use in any reported studies.

**Antibodies**—Total bacterial proteins were extracted from Y. pestis, Burkholderia mallei, Burkholderia cepacia, Burkholderia pseudomallei, and Pseudomonas aeruginosa using a commercial method (Epícentre Biotechnologies, Madison, WI) and then injected (three times, monthly intervals) into rabbits (n = 1 for each bacterial proteome) for antibody production. Antisera were collected 60 days after inoculation with the extracted proteomes. Rabbit antisera (n = 2) were also produced against intact, γ-irradiated Y. pestis using a method similar to that above. Antisera against Salmonella typhimurium (recognizing a
Antibody Biomarkers for Plague

![Image](https://example.com/image.png)

**Fig. 1. Protein microarray of the Y. pestis proteome.** Western using anti-GST antibody (A) and representative Y. pestis proteins, expressed in vitro, evaluated by SDS-PAGE (B) are shown. C, confocal laser scanner image of proteins spotted in duplicate onto microarray slides and visualized using a rabbit anti-GST antibody bound to Cy5-labeled anti-rabbit antibody.

Rhesus macaques were vaccinated (intradermal vaccination) three times at 1-month intervals with protein subunit vaccines against anthrax (*Bacillus anthracis* recombinant protective antigen; List Biologics, Wako, TX) and plague (*F1*-V; kindly provided by Vicki Pearson, National Institute of Allergy and Infectious Diseases). Sera were collected 1 month after aerosol challenge with either *B. anthracis* spores (n = 6; average 377 × LD₅₀) or *Y. pestis* (n = 1; average 124 × LD₅₀) and γ-irradiated before subsequent analyses. Antibody titers were monitored by ELISA before and after irradiation, and these results confirmed that there was no significant loss in antibody activity. Non-vaccinated cynomolgus macaques (n = 10) were also challenged in a manner similar to that above. Seven of the 10 animals were rescued from lethal infection by treating with either doxycycline (n = 5) or doxycycline plus simvastatin (n = 2), and convalescent sera were collected 42 days postchallenge. The remaining three animals did not receive antibiotic treatment, and acute phase sera were collected on a daily basis until euthanasia of moribund individuals. Prechallenge sera were retained from all animals used in our studies.

**Western Blots—Proteomes** (total protein extracts) were obtained from bacteria as described above and separated by 10% PAGE, loading 10 µg of protein/lane. The electrophoresed proteins were transferred to nitrocellulose membranes (Invitrogen), blocked with 0.2% casein in PBS (12 h at 4 °C), and probed with a 1:500 dilution of rabbit sera containing antibodies against the bacterial proteomes. Bound antibody was detected with a 1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) and a chemiluminescent substrate (GE Healthcare).

**Antibody Interactions with Microarrayed Proteins—** All proteome microarray manipulations were performed at room temperature. Microarray slides were incubated with blocking buffer (PBS, pH 7.4, containing 1% BSA and 0.1% Tween 20) for 1 h. Serum samples were diluted to optimize the signal to noise background before recording data from final concentrations. Sera were diluted in a probe buffer consisting of PBS, pH 7.4, 5 mM MgCl₂, 0.05% Triton X-100, 1% glycerol, and 1% BSA. Based on preliminary optimization of results, the following final dilutions of sera were used for microarray binding: rabbit, 1:1000; non-human primate, 1:50. The slide surface was overlaid with 100 µl of diluted serum, covered with a glass coverslip, and incubated (1 h at 22 °C) in a humid (80%) chamber. The coverslips were removed, and the arrays were washed three times with probe buffer. Antibodies binding to arrayed proteins were detected by incubation (1 h at 22 °C) with goat anti-rabbit IgG (heavy + light) labeled with Alexa® Fluor 647 (Invitrogen) and diluted to 1 µg/ml. The arrays were washed three times and allowed to dry (15 min at 22 °C) before analysis. Microarray slides were imaged using a GenePix 4000B scanner (MDS Analytical Technologies, Toronto, Canada), and image analysis was performed using GenePix Pro 6.0 software (MDS Analytical Technologies). Raw pixel counts were generated by scanning arrays at 635 nm using a photomultiplier tube gain setting of 500 and a power setting of 100%. These settings minimized background signals and were optimal for detecting fluorescence from specific antibody binding events. The fluorescence signal of each spot was measured and subtracted from the local background median intensity. The signals from each protein, spotted in duplicate, were then averaged. Acquired data were analyzed using ProtoArray Prospector v3.1 (Invitrogen) in Immune Response Profiling mode. Data were analyzed by calculation of Chebyshev’s inequality p value and Z-score. All recorded positive binding events had Z-scores >3.5 and Chebyshev’s inequality p values <0.0003623 (equal to 1/total samples on array).

**RESULTS**

**Y. pestis Proteome Microarray—** Entry ORF clones for *Y. pestis* (KIM) were subcloned into the pEXP7-DEST expression vector (20) and used to transform *E. coli* DH10B for preparation of plasmids. All clones were characterized by PCR amplification to confirm correct size and sequenced to confirm identity. The *Y. pestis* proteins were expressed using Expressway (Invitrogen), an *in vitro* transcription/translation system.
based on extracts from *E. coli*, a similar Gram-negative bacterium, to increase the likelihood of maintaining native protein modifications. All proteins were affinity-purified and characterized by sodium dodecylsulfate gel electrophoresis and Western blot (Fig. 1, A and B) to confirm correct size and purity after purification. Soluble proteins passing quality control criteria were robotically arrayed on glass slides coated with nitrocellulose, and representative slides from each printing lot were further characterized by incubating with rabbit anti-GST antibody and a Cy5-labeled anti-rabbit antibody (Fig. 1 C).

Each slide was also printed with control proteins (Fig. 2) and a dilution series of known quantities of GST that was used to calculate a standard curve for normalization of data. The intraslide and intralot variability in spot intensity and morphology, the number of missing spots, and the presence of control spots were also measured by an automated algorithm (Proto-Mine, Invitrogen). Slides that passed the above quality control criteria were then stored at −20 °C until use. Of the 4066 ORFs encoded by *Y. pestis*, 2760 proteins (~70%) were arrayed on slides used for the experiments described.

**Antibody-Protein Interactions Measured by Proteome Microarray**—We first examined antibody interactions with the arrayed proteins using rabbit hyperimmune sera produced against the extracted *Y. pestis* proteome (Fig. 2A). The rabbit anti-*Y. pestis* IgG bound to a discrete set of proteins on the microarray (Fig. 2, B and C), and binding was not detectable if the antibodies were preincubated with total protein extracted from *Y. pestis* (data not shown). The majority of proteins recognized by antibodies against the extracted *Y. pestis* proteome were also recognized by IgG from rabbits vaccinated with intact, irradiated *Y. pestis* (Table I). In general,
these bacterial antigens represented a variety of functional classes of intrabacterial and surface proteins. These data suggested that IgG directed against proteomes extracted from Y. pestis could be used to predict the antibody response to intact bacteria. In considering the specificity of the signals obtained by microarray, it was possible that small amounts of E. coli proteins carried over from the in vitro expression were also present in the affinity-purified products printed on the slide surfaces, and these may have contributed to the binding data. Some of the weaker spot intensities were further reduced by including 1–5% (w/v) proteins extracted from E. coli. However, we found that further dilution of the rabbit antisera minimized any potential background antibody interactions. A distinct pattern of antibody interactions with the arrayed proteins was observed with final dilutions of hyperimmune sera, whereas significant binding to the microarray was not detected with the same dilutions of control preimmune sera (data not shown).

Antibody Biomarkers for Plague

Antibody Recognition of Proteomes from Gram-negative Pathogens—We next examined a panel of rabbit antisera

| ORF | Protein name | Protein extract | Bacteria |
|-----|--------------|-----------------|----------|
| 0042 | Putative transposase, NP_667386 | + | + |
| 0071 | sopAB transcription repressor, NP_857962 | + | + |
| 0165 | Repressor of treA,B,C, NP_667508 | + | + |
| 0281 | Hypothetical protein, NP_667622 | + | + |
| 0417 | ATP-binding component of a membrane-associated complex, NP_667755 | + | + |
| 0496 | Hypothetical protein, NP_667814 | + | + |
| 0523 | Hypothetical protein, NP_667860 | + | + |
| 0539 | ATP-binding protein of ATP-binding cassette transporter, NP_667874 | + | + |
| 0565 | Hypothetical protein, NP_667902 | + | + |
| 0609 | GroEL protein, NP_667946 | + | + |
| 0913 | Hypothetical protein, NP_668244 | + | + |
| 1001 | Putative transposase, NP_668336 | + | + |
| 1043 | Acyl-CoA thioesterase II, NP_668373 | + | + |
| 1054 | Transposase, NP_857837 | + | + |
| 1094 | Putative transcriptional regulator, NP_668421 | + | + |
| 1121 | Putative transposase, NP_668448 | + | + |
| 1270 | Deoxyribozyme primidinoproteinase, NP_668592 | + | + |
| 1299 | recO protein, NP_668622 | + | + |
| 1349 | Nucleoside-diphosphate kinase, NP_668671 | + | + |
| 1415 | Hypothetical protein, NP_668736 | + | + |
| 1437 | Hypothetical protein, NP_668758 | + | + |
| 1516 | Putative oxidoreductase component, NP_668836 | + | + |
| 1596 | Putative transcriptional regulator, NP_668914 | + | + |
| 1773 | Hypothetical protein, NP_669090 | + | + |
| 1941 | Putative transposase, NP_669256 | + | + |
| 2187 | Hypothetical protein, NP_669497 | + | + |
| 2225 | Putative transposase, NP_669534 | + | + |
| 2375 | Putative amidotransferase, NP_669682 | + | + |
| 2378 | Hypothetical protein, NP_669685 | + | + |
| 2385 | Putative Coenzyme A transferase, NP_669692 | + | + |
| 2398 | Arac-type transcriptional regulator, NP_669705 | + | + |
| 2707 | Putative acyl transferase, NP_670009 | + | + |
| 2725 | Putative oxidoreductase, NP_670026 | + | + |
| 2806 | Leucyl, phenylalanyl-tRNA-protein transferase, NP_670106 | + | + |
| 2849 | ATP-binding component of putrescine ABC, NP_670149 | + | + |
| 2882 | pH 6 antigen fimbrial subunit, NP_670182 | + | + |
| 3096 | Hypothetical protein, NP_670395 | + | + |
| 3140 | 2,3,4,5-Tetrahydropyridine-2-carboxylate N-succinyltransferase, NP_404654 | + | + |
| 3504 | Suppressor of ftsl, NP_670801 | + | + |
| 3701 | Putative regulator, NP_670998 | + | + |
| 3799 | Putative transposase, NP_671093 | + | + |
| 3904 | Hypothetical protein, NP_671199 | + | + |
| 3906 | Hypothetical protein, NP_671197 | + | + |
| 4092 | Hypothetical protein, NP_671384 | + | + |
| YPKp01 | Transposase, NP_857780 | + | + |

TABLE I
Rabbit antibody response to extracted Y. pestis proteome and intact bacteria
produced against proteomes of other pathogenic, Gram-negative bacteria to determine relative amounts of antibody cross-reactivity. Complex patterns of extensive cross-reactivity among these antisera were visible by Western blots (Fig. 3) using proteomes extracted from Y. pestis and E. coli. Preimmune sera from rabbits also contained antibodies that bound to proteins extracted from E. coli, whereas no binding to Y. pestis proteins was observed on the Western blots with these antisera (Fig. 3).

Binding of the anti-Gram-negative IgG to the microarrayed Y. pestis proteins was examined, and these interactions were readily grouped according to specific observed patterns (Fig. 4). First, analysis of the antibody recognition profile resulted in identification of proteins that bound antibody against all Gram-negative pathogens examined (Fig. 4A, red): a putative transposase; repressor protein of treA, B, and C; uroporphyrinogen decarboxylase; phenylalanine-tRNA synthetase; a putative aminotransferase; putative coenzyme A transferase; and a protein of unknown function. All of the cross-reactive proteins were conserved within the examined group of Gram-negative bacteria (Table II), suggesting that antibody interactions with these antigens were based on similarities in protein structure. Further, we also noted proteins recognized by antibodies in combinations that were unique to each pathogen, and we refer to these as fingerprints (Fig. 4B, blue). The number of fingerprint proteins shared between the different bacteria may reflect the degree of conservation of antibody-accessible surfaces within the proteomes. For example, Y. pestis and B. mallei appeared to share the most fingerprint proteins. Finally, a small group of proteins was uniquely recognized by antibodies against only one pathogen; hence these were designated as signature proteins (Fig. 4B, blue).

Antibody Response to Plague—Among potential animal hosts, pulmonary plague in non-human primates most closely recapitulates human pathology (23). Therefore, we examined antibody responses to infection by first comparing convalescent sera collected from plague and anthrax survivors. Anthrax is caused by the Gram-positive B. anthracis, and these data allowed us to measure specificity of antibody binding using the protein microarray. Serum was collected from a surviving rhesus macaque that was vaccinated with an experimental plague vaccine consisting of a single recombinant fusion of CaF1 and Y. pestis low Ca²⁺ response V-antigen proteins (24) and then aerosol challenged with Y. pestis (CO92) bacteria. Also included in this plague group were seven cynomolgus macaques that were aerosol-challenged with Y. pestis and then treated with antibiotics. In addition, sera were also obtained from rhesus monkeys vaccinated with the anthrax vaccine B. anthracis recombinant protective antigen and aerosol-challenged with B. anthracis (Ames) spores. Although other antibody isotypes were detectable, measuring IgG levels provided the most consistent results and therefore was the focus of our studies. Convalescent plague antibodies detected at least 46 Y. pestis proteins (Fig. 5), whereas five proteins were recognized only by antibodies from animals surviving anthrax: a putative Leucine-responsive regulatory protein-like transcriptional regulator, two hypothetical proteins, imidazole glycerol phosphate synthase subunit, and outer membrane porin A. We next examined binding of acute phase antibodies from non-vaccinated cynomolgus macaques collected daily after Y. pestis or B. anthracis spore challenge (Table III) and compared the results with those obtained from vaccinated or antibiotic-rescued monkeys surviving pulmonary plague or anthrax (Fig. 5).

Non-vaccinated animals were septic with culturable bacteria within 3–4 days and died within 4–6 days of infection. The pattern of binding to the Y. pestis proteome microarray indicated no similarities between antibodies obtained from moribund and surviving individuals. These results suggested that few neutralizing antibodies of convalescence sera were detectable in animals that later succumbed to lethal disease. However, specific antibody signatures were detectable in sera from acutely infected individuals within 48 h of infection and before detectable sepsis (Table III). It was important to under-
stand how antibody responses to the extracted bacterial proteome compared with antibodies recovered from plague survivors. For this purpose, we compared the results obtained from convalescent non-human primate antibodies with results from rabbit antibodies to intact or proteome-extracted *Y. pestis* (Fig. 5). Approximately 40% of the *Y. pestis* antigens were detected by antibodies that were cross-reactive (red) or fingerprints (combination of proteins unique to one pathogen but individual protein recognition can be shared; green). A signature proteins (unique to one pathogen; blue).
TABLE II

| Protein ID | Description | Protein Data Bank homology | B. mallei (ATCC 23344) | B. pseudomallei K92243 | P. aeruginosa PAO1 | E. coli APEC 01 | S. flexneri 2a str. 301 | Salmonella enterica (SC-B67) | B. cepecia phage Bcepa Nazgul |
|------------|-------------|---------------------------|------------------------|-------------------------|-------------------|-----------------|------------------------|-----------------------------|-----------------------------|
| y0166      | Trehalose repressor | YP_104561.1 | YP_108430.1 | NP_250639.1 | YP_859910.1 | NP_709960.1 | YP_219298.1 | NP_919020.1 |
| y0496      | Uroporphyringen decarboxylase | YP_104466.1 | YP_109984.1 | NP_253721.1 | YP_859852.1 | NP_709791.1 | YP_219035.1 | NP_919004.1 |
| y1299      | DNA repair protein recO | YP_102341.1 | YP_109019.1 | NP_249463.1 | YP_859582.1 | NP_708417.1 | YP_217561.1 | NP_919018.1 |
| y2375      | Transcriptional regulator | YP_103544.1 | YP_107705.1 | NP_253725.1 | YP_859852.1 | NP_709791.1 | YP_219035.1 | NP_919004.1 |
| y2385      | Acetyl-CoA hydrolase | YP_106223.1 | YP_111645.1 | NP_254132.1 | YP_859026.1 | NP_709205.1 | YP_218045.1 | n.m. |

* Y. pestis proteins that bound antibodies from all antisera tested.

b Expectation value from Smith-Waterman sequence alignments. Below significance, bold.

Plague before isolating cultivable bacteria. It may be possible to develop preclinical diagnosis patterns may still allow identification of the correct bacterium by eliciting an antibody response. The absence of somatic and body biomarkers may result from inclusion of proteins that may not reach detectable levels before onset of life-threatening disease. Thus, increased assay specificity and sensitivity may allow for more accurate identification of the correct bacterium.

**DISCUSSION**

We identified several putative antibody biomarkers for plague by using a microarray encompassing the majority of proteins from several Gram-negative bacteria recognized in all Y. pestis-antibody microarrays: 1) Y. pestis proteins that clustered into three general categories: proteins recognized in all Y. pestis-antibody microarrays; 2) Y. pestis proteins that were recognized by antibodies from plague survivors (31–32). This observation is noteworthy because antibody reactivity with both species involved less than 10% of the thousands of proteins present within the Y. pestis proteome, and the rabbit data did not involve an actual infection.

*Y. pestis* proteins that bound antibodies from all antisera tested.

b Expectation value from Smith-Waterman sequence alignments. Below significance, bold.
Our results provide a partial window into the host antibody response to infection and vaccination. Similar to previous observations with the human antibody response to vaccinia virus (18), proteins recognized by antibodies directed against *Y. pestis* constituted only a small percentage of the total bacterial proteome. The extensive antibody cross-reactivity noted was presumably the result of similarities in protein structures. At present we cannot identify any commonality among the *Y. pestis* proteins that were recognized by the antibody response to plague to suggest why these particular products were antigenic. In general, prediction of antibody recognition remains enigmatic (33). Because a limited number of individual animals were examined, we anticipate that including a larger population in the future will allow more robust conclusions. We also noted the recognition of a minor number of *Y. pestis* proteins by antibodies of either control sera or sera from animals infected with *B. anthracis*. These appear to represent legitimate binding events, perhaps a result of antibody cross-reactivity. Additional factors may also alter the proteins recognized during infection. For example, the bacterial proteome may vary according to the strain of pathogen. Strains are classified into one of three original *Y. pestis* biovars (Antiqua, Medievalis, and Orientalis) based on phenotypic differences, epidemiology, and chromosome location of...
the IS100 insertional element (34), and additional variation in proteome content among other plague isolates is expected. Thus, there does not appear to be a simple relationship between a small number of pathogenic proteins and the more virulent phenotype but rather multiple, perhaps subtle differences in proteomes. For example, loss of the lipopolysaccharide O-antigen found in *Y. pseudotuberculosis* appears to be essential for function of the plasminogen activator (35), another virulence factor uniquely acquired by *Y. pestis* and encoded on pPCP1. Furthermore plague bacteria have evolved to survive in burrows inhabited by infected rodents, within the flea gut or phagocytes of mammalian hosts, and finally as an extracellular infection. These different environmental demands are anticipated to evoke unique bacterial proteomes. The pMT-encoded phospholipase D is essential for bacterial survival within the flea (36, 37). The ability of *Y. pestis* to cause disease in mammalian hosts is conferred, in part, by components of the coordinately regulated low Ca\(^{2+}\)/H1001 response stimulon encoded on a common 70-kb virulence plasmid (pCD1). The pCD1-encoded poly-peptides include the *Yersinia* outer proteins and a specialized type III secretion system for controlled delivery of these virulence factors to infected cells during infection (38). Although the antibody response to CaF1 is a sensitive indicator of plague, expression of this capsular protein is initiated after bacterial growth in host temperatures of 37 °C, likely accounting for a delay in detection of days subsequent to infection.

A previous study by Li *et al.* (39) examined rabbit antibody responses to a *Y. pestis* live vaccine using a microarray comprising 149 proteins (less than 4% of the entire proteome). The pH 6 antigen of *Y. pestis* was recognized by rabbit antibodies

| ORF | Protein name | Plague | Anthrax |
|-----|--------------|--------|---------|
| 0008 | Hypothetical protein, NP_857906 | + | – |
| 0036 | Hemolysin co-regulated protein, NP_667380 | + | – |
| 0140 | UDP-N-glucosamine 1-carboxyvinyltransferase, NP_667483 | + | – |
| 0498 | Hypothetical protein, NP_667836 | + | – |
| 0838 | Putative dehydrogenase, NP_668172 | + | – |
| 1012 | Putative oxidoreductase, NP_668342 | + | – |
| 1028 | ATP-dependent specificity component of clpP, NP_668358 | + | – |
| 1029 | DNA-binding ATP-dependent protease La, NP_668359 | + | – |
| 1038 | Putative LRP-like transcriptional regulator, NP_668368 | + | – |
| 1406 | Phosphoribosylglycinamide formyltransferase 1, NP_668727 | + | – |
| 1437 | Hypothetical protein, NP_668758 | + | – |
| 1789 | Hypothetical protein, NP_669106 | + | – |
| 1792 | Hypothetical protein, NP_669109 | + | – |
| 1801 | Hypothetical protein, NP_669118 | + | – |
| 1953 | Hypothetical protein, NP_669268 | + | – |
| 2274 | Putative oxidoreductase component, NP_669583 | + | – |
| 2290 | Peptide chain release factor RF-1, NP_669598 | + | – |
| 2757 | Nicotinate phosphoribosyltransferase, NP_670058 | + | – |
| 3124 | (3R)-Hydroxyimyristoyl acyl carrier protein, NP_670423 | + | – |
| 3125 | UDP-3-O-(3-hydroxyimyristoyl)-glucosamine, NP_670424 | + | – |
| 3169 | Hypothetical protein, NP_670468 | + | – |
| 3581 | Hypothetical protein, NP_670878 | + | – |
| 3873 | Regulator of gluconate (gnt) operon, NP_671167 | + | – |
| 3993 | 50 S ribosomal subunit protein L2, NP_671286 | + | – |
| 4089 | Glycine-tRNA synthetase, β subunit, NP_671381 | + | – |
| 0041 | Putative transposase, NP_667385 | – | + |
| 0165 | Repressor of treA,B,C, NP_667508 | – | + |
| 0270 | Hypothetical protein, NP_667612 | – | + |
| 0290 | Hypothetical protein, NP_667631 | – | + |
| 0390 | Transcriptional regulator, NP_667728 | – | + |
| 0483 | 50 S ribosomal subunit protein L7/L12, NP_667821 | – | + |
| 0677 | Transcriptional regulatory protein, NP_668014 | – | + |
| 0832 | L-Isoaspartate protein carboxylmethyltransferase, NP_668166 | – | + |
| 1216 | Putative transposase, orf2 protein, NP_668541 | – | + |
| 2062 | Hypothetical protein, NP_669375 | – | + |
| 2627 | Imidazole glycerol phosphate synthase subunit, NP_669930 | – | + |
| 3077 | Hypothetical protein, NP_670376 | – | + |
| 3520 | 3,4-Dihydroxy-2-butanone-4-phosphate synthase, NP_670817 | – | + |

* Three cynomolgus macaques challenged with *Y. pestis* and three challenged with *B. anthracis*. Pre- and postchallenge sera were examined individually on the *Y. pestis* array.
in both studies, whereas antibody binding to the outer membrane porin A protein observed by Li et al. (39) was only detectable with sera from rhesus anthrax survivors in our study. Based on our results, we suggest that a subset of *Y. pestis* proteins may be useful for diagnosing plague, for developing new vaccines, and as targets for potential therapies. Because proteome microarrays allow the simultaneous screening of an extensive number of potential targets using only small amounts of biological samples, it may now be possible to examine large populations to identify and validate biomarkers for diagnosis of infectious diseases. Future applications for microarrays of pathogen proteomes may also include discovery of other biomarkers for diagnosis of infectious disease before the appearance of clinical symptoms.

Acknowledgments—We acknowledge Bret K. Purcell and Garry L. Morefield (United States Army Medical Research Institute of Infectious Diseases (USAMRIID)) for supplying sera from non-human primates; Patrick Burr (Pathogen Functional Genomics Resource Center) for supplying data and the *Y. pestis* entry clone collection; Ricky Ulrich (USAMRIID) for preparing Gram-negative proteome extracts; Michael Salcius, Diane Buhr, and Gregory A. Michaud (Protein Array Center, Invitrogen) for expression and purification of recombinant proteins; Shannon Beatty (Invitrogen, Frederick, MD) for assistance in assay development; and Lisa Freeman-Cook and Arman Bashirova for a critical reading of the manuscript. All animals used in this research project were cared for and used humanely according to the following policies: the United States Public Health Service Policy on Humane Care and Use of Animals (1996), the Guide for the Care and Use of Laboratory Animals (1996), and the United States Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (1985). All USAMRIID animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal use was approved by the Institutional Animal Care and Use Committee and conducted in accordance with Federal Animal Welfare Act regulations.

* This work was supported by Defense Threat Reduction Agency Contracts B.10002 (to R. G. U.) and W81XWH-05-2-0077 (to J. M.).

To whom correspondence should be addressed: Laboratory of Molecular Immunology, USAMRIID, 1425 Porter St., Frederick MD 21702. Tel.: 301-619-4232; Fax: 301-619-8334; E-mail: robert.ulrich@amedd.army.mil.

REFERENCES

1. Boisier, P., Rahalison, L., Rasolomaharo, M., Ratsitorahina, M., Mahafaya, M., Razafimahena, M., Duplantier, J. M., Ratsifasanaomanana, L., and Chanteau, S. (2002) Epidemiologic features of four successive annual outbreaks of bubonic plague in Mahajanga, Madagascar. Emerg. Infect. Dis. 8, 311–316

2. Strely, S. C., and Harmon, P. A. (1984) *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. *Infect. Immun.* 45, 649–654

3. Inglesby, T. V., Dennis, D. T., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Etzen, E., Fine, A. D., Friedlander, A. M., Hauer, J., Kasper, J. F., Layton, M., McDade, J., Osterholm, M. T., O'Toole, T., Parker, G., Perl, T. M., Russell, P. K., Schoch-Spana, M., and Tonat, K. (2000) Plague as a biological weapon: medical and public health management. *J. Am. Med. Assoc.* 283, 2281–2290

4. Centers for Disease Control and Prevention (2006) Human plague—four states. 2006. *MMWR Morb. Mortal. Wkly. Rep.* 55, 940–943

5. Wild, M. A., Shenk, T. M., and Spraker, T. R. (2008) Plague as a mortality factor in Canada lynx (*Lynx canadensis* reintroduced to Colorado. *J. Wildl. Dis.* 42, 646–650

6. Saitkeld, D. J., and Stapp, P. (2006) Seroprevalence rates and transmission of plague (*Yersinia pestis*) in mammalian carnivores. *Vector Borne Zoonotic Dis.* 6, 231–239

7. Mittal, V., Rana, U. V., Jain, S. K., Kumar, K., Pal, I. S., Arya, R. C., Iloymar-Armani, R. L., Lal, S., and Agarwal, S. P. (2004) Quick control of bubonic plague outbreak in Uttar Kashi, India. *J. Commun. Dis.* 36, 233–239

8. Crook, L. D., and Tempest, B. (1992) Plague: a clinical review of 27 cases. *Arch. Intern. Med.* 152, 1253–1256

9. Nielsen, K., Smith, P., Yu, W. L., and Halbert, G. (2007) *Salmonella* enterica serotype urbana interference with brucellosis serology. *J. Immunol. Serology* 28, 289–297

10. Rahman, M. Z., Sultana, M., Khan, S. I., and Birkeland, N. K. (2007) Serological cross-reactivity of environmental isolates of *Enterobacter*, *Escherichia*, *Stenotrophomonas*, and *Aerococcus* with *Shigella* spp.-specific antisera. *Curr. Microbiol.* 54, 63–67

11. Chart, H., Okubadejo, O. A., and Rowe, B. (1992) The serological relationship between *Escherichia coli* O157 and *Yersinia enterocolitica* O9 using sera from patients with brucellosis. *Epidemiol. Infect.* 106, 77–82

12. McCabe, W. R., Brunis, D., Green, D. E., and Johns, H. M. (1977) Cross-reactive antigens: their potential for immunization-induced immunity to Gram-negative bacteria. *J. Infect. Dis.* 136, (suppl.) S161–S166

13. Deng, W., Burland, V., Plunkett, G., III, Boutin, A., Mayhew, G. F., Liss, P., Perna, N. T., Rose, D. J., Mau, B., Zhou, S., Schwartz, D. C., Fetherston, J. D., Lindler, L. E., Brubaker, R. R., Plano, G. V., Straley, S. C., McDonough, K. A., Nilles, M. L., Matson, J. S., Blattner, F. R., and Perry, R. D. (2002) Genome sequence of *Yersinia pestis* KIM. *J. Bacteriol.* 184, 4601–4611

14. Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaiha, M., James, K. D., Churcsher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeño-Tarragà, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karyasheva, A. V., Leather, S., Moxon, S., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrett, B. G. (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* 413, 523–527

15. Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L., Brubaker, R. R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Danchex, D., Elliott, J. M., Derbise, A., Hausser, L. J., and Garcia, E. (2004) Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13826–13831

16. Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A., and Carniel, E. (1999) *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14043–14048

17. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, J., Bertone, P., Lan, N., Jansen, R., Bidlemaner, S., Houfek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstlein, M., and Snyder, M. (2001) Global analysis of protein activities using proteome chips. *Science* 293, 2101–2105

18. Schmid, K. E., Keasey, S. L., Pettman, P., Emerson, G. L. M., Meegan, J., Tikhonov, A. P., Chen, G., Schweitzer, B., and Ulrich, R. G. (2006) Antigens of the human plague vaccine *Y. pestis* KIM and sex inactivation by use of a novel protein microarray suggests that antibodies recognize less than 10% of the total viral proteins. *Proteomics Clin. Appl.* 2, 1528–1538

19. Schweitzer, B., Predki, P., and Snyder, M. (2003) Microarrays to characterize protein interactions on a whole-proteome scale. *Proteomics* 3, 2190–2199

20. Michaud, G. A., Salcius, M., Martone, R., Buhr, D., Duarte, J. C., McCague, J. E., Liu, X., Samuels, M., Stalder, C., Ball, J., Tikhonov, A., Keppetipola, S., Kudlicki, W., Meegan, J., and Schweitzer, B. I. (2007) Cell-free protein synthesis for protein microarrays, in *Cell-Free Protein Expression* (Kudlicki, W., Katzen, F., and Bennett, R., eds) pp. 134–144, landes Bioscience, Austin, TX

21. Schweitzer, B., and Kingsmore, S. F. (2002) Measuring proteins on microarrays. *Curr. Opin. Biotechnol.* 13, 14–19
22. Zhu, H., Klemic, J. F., Chang, S., Bertone, P., Casamayor, A., Klemic, K. G., Smith, D., Gerstein, M., Reed, M. A., and Snyder, M. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet.* **26**, 283–289
23. Swearengen, J. R. (ed) (2006) *Biodefense: Research Methodology and Animal Models*, p. 331, CRC Taylor & Francis, Boca Raton, FL
24. Heath, D. G., Anderson, G. W., Jr., Mauro, J. M., Welkos, S. L., Andrews, G. P., Adamovicz, J., and Friedlander, A. M. (1998) Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine. *Vaccine* **16**, 1131–1137
25. Splettstoesser, W. D., Rahalison, L., Grunow, R., Neubauer, H., and Chanteau, S. (2004) Evaluation of a standardized F1 capsular antigen capture ELISA test kit for the rapid diagnosis of plague. *FEBS Immunol. Med. Microbiol.* **41**, 149–155
26. Ratsitorahina, M., Chanteau, S., Rahalison, L., Ratsifasoamanana, L., and Boisier, P. (2000) Epidemiological and diagnostic aspects of the outbreak of pneumonic plague in Madagascar. *Lancet* **355**, 111–113
27. Rasoamanana, B., Leroy, F., Boisier, P., Rasolomaharo, M., Buchy, P., Carniel, E., and Chanteau, S. (1997) Field evaluation of an immunoglobulin G anti-F1 enzyme-linked immunosorbent assay for serodiagnosis of human plague in Madagascar. *Clin. Diag. Lab. Immun.* **4**, 587–591
28. Gomes-Solecki, M. J., Savitt, A. G., Rowehl, R., Glass, J. D., Bliska, J. B., and Dattwyler, R. J. (2005) LcrV capture enzyme-linked immunosorbent assay for detection of *Yersinia pestis* from human samples. *Clin. Diag. Lab. Immunol.* **12**, 339–346
29. Mahesh, S., Shukla, J., Tutela, U., and Batra, H. V. (2005) Molecular detection of *Yersinia pestis* isolates of Indian origin by using Pla specific monoclonal antibodies. *Comp. Immunol. Microbiol. Infect. Dis.* **28**, 131–144
30. Friedlander, A. M., Welkos, S. L., Worsham, P. L., Andrews, G. P., Heath, D. G., Anderson, G. W., Jr., Pitt, M. L., Estep, J., and Davis, K. (1995) Relationship between virulence and immune response in recent studies of the F1 capsule of *Yersinia pestis*. *Clin. Infect. Dis. Suppl.* **2**, S178–S181
31. Shibata, D. (1999) Pattern recognition and arrays. The times are a-changing. *Am. J. Pathol.* **154**, 979–980
32. Weljie, A. M., Dowlatabadi, R., Miller, B. J., Vogel, H. J., and Jirik, F. R. (2007) An inflammatory arthritis-associated metabolite biomarker pattern revealed by 1H NMR spectroscopy. *J. Proteome Res.* **6**, 3456–3464
33. Ponomarenko, J. V., and Bourne, P. E. (2007) Antibody-protein interactions: benchmark datasets and prediction tools evaluation. *BMC Struct. Biol.* **7**, 64
34. Motin, V. L., Georgescu, A. M., Elliott, J. M., Hu, P., Worsham, P. L., Ott, L. L., Slezek, T. R., Sokhansanj, B. A., Regala, W. M., Brubaker, R. R., and Garcia, E. (2002) Genetic variability of *Yersinia pestis* isolates as predicted by PCR-based IS100 genotyping and analysis of structural genes encoding glycerol-3-phosphate dehydrogenase (glpD). *J. Bacteriol.* **184**, 1019–1027
35. Kukkonen, M., Suomalainen, M., Kylonen, P., Lahteenmaki, K., Lang, H., Virkola, R., Helander, I. M., Holst, O., and Korhonen, T. K. (2004) Lack of O-antigen is essential for plasminogen activation by *Yersinia pestis* and *Salmonella enterica*. *Mol. Microbiol.* **51**, 215–225
36. Hinnesbusch, B. J., Perry, R. D., and Schwan, T. G. (1996) Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. *Science* **273**, 367–370
37. Hinnesbusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G., and Forsberg, A. (2002) Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296**, 733–735
38. Cornelis, G. R. (2000) Molecular and cell biology aspects of plague. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 8778–8783
39. Li, B., Jiang, L., Song, Q., Yang, J., Chen, Z., Guo, Z., Zhou, D., Du, Z., Song, Y., Wang, J., Wang, H., Yu, S., Wang, J., and Yang, R. (2005) Protein microarray for profiling antibody responses to *Yersinia pestis* live vaccine. *Infect. Immun.* **73**, 3734–3739