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

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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 cepecia*, *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 pneumonic 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* KIM[1] contains 4202 individual proteins (13), 87% in common with

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[1] The abbreviations used are: KIM, Kurdistan Iran man; LD_{50}, 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/carbenicillin medium. Size validations of destination clones were performed by PCR amplification of DNA extracted from bacterial colonies and assessed by capillary electrophoresis on a Caliper AMS90 DNA chip. One of four destination colonies that matched the expected insert size was selected and rearrayed into deep well plates with 2× 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 of 92 wells. Two wells received an expression-verified positive control expression plasmid (pEXP-GST-CALML3). The plates were sealed and placed into a shaking incubator set to 30 °C, 300 rpm for 1 h. The deep well plate 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 transferred to a fresh deep well plate 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 retained 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 immunoblot images 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 cepecia*, *Burkholderia pseudomallei*, and *Pseudomonas aeruginosa* using a commercial method (Epicentre 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

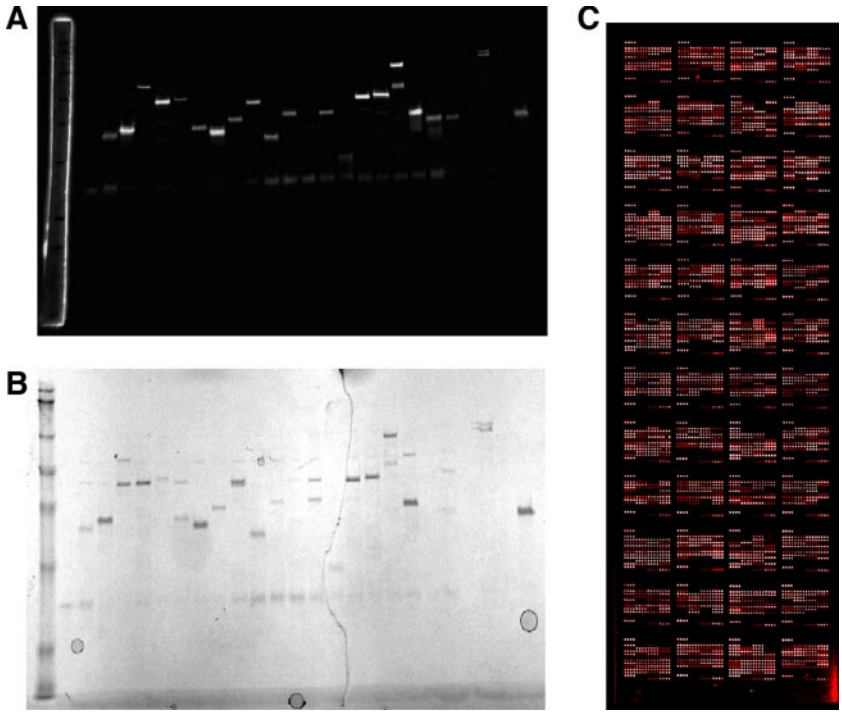

FIG. 1. **Protein microarray of the *Y. pestis* proteome.** Western using anti-GST antibody (*A*) and representative *Y. pestis* proteins, expressed *in vitro*, evaluated by SDS-PAGE (*B*) are shown. *C*, confocal laser scanner image of proteins spotted in duplicate onto microarray slides and visualized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

broad range of O and H strains), *Shigella flexneri* (recognizing *Shigella dysenteriae*, *Shigella boydii*, and *S. flexneri*), and *E. coli* were commercially obtained (Fitzgerald Industries, Concord, MA).

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 $377 \times LD_{50}$) or *Y. pestis* ($n = 1$; average $124 \times LD_{50}$) 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 $MgCl_2$, 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

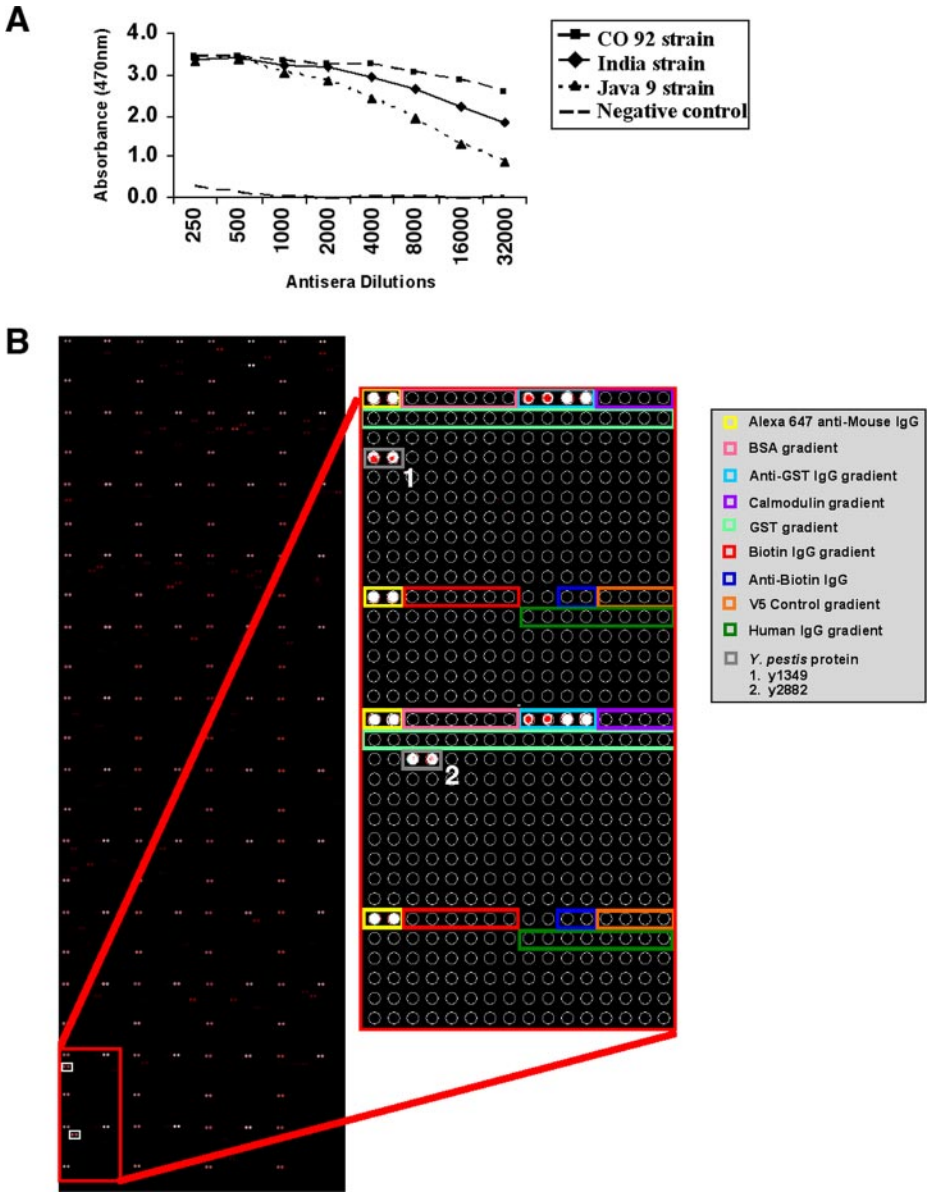

FIG. 2. **Rabbit anti-*Y. pestis* antibody interactions with proteome microarray.** *A*, rabbit antisera produced against the *Y. pestis* CO92 proteome recognized diverse *Y. pestis* strains (India, CO92, and Java 9) by ELISA. *B*, binding of anti-proteome IgG. The microarray was incubated with rabbit hyperimmune sera against the extracted *Y. pestis* proteome (diluted 1:1000), and bound IgG was detected with an Alexa 647-labeled goat anti-rabbit antibody and a laser confocal scanner. *C*, enlarged image of two subgrids from the microarray illustrating control proteins (*colored boxes*) and representative antibody binding to arrayed *Y. pestis* proteins (*gray boxes*).

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 (ProtoMine, 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. These antibodies recognized diverse strains of *Y. pestis* by ELISA (Fig. 2*A*). 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,

TABLE I
Rabbit antibody response to extracted Y. pestis proteome and intact bacteria

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_667876	+	+
0565	Hypothetical protein, NP_667902	+	+
0609	GroEL protein, NP_667946	+	+
0913	Hypothetical protein, NP_668244	+	+
1006	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	Deoxyribodipyrimidine photolyase, 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 aminotransferase, 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 ftsI, 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	+	−

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

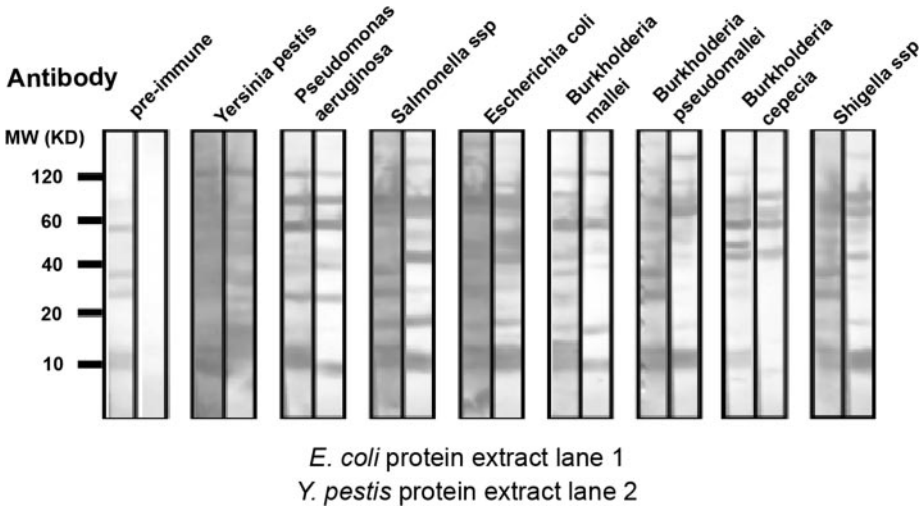

FIG. 3. **Antibody recognition of proteins extracted from Gram-negative bacteria.** Equal amounts (per lane) of total proteins extracted from *Y. pestis* and *E. coli* were electrophoresed on 10% polyacrylamide gels, transferred to nylon membranes, and probed with a 1:500 dilution of the indicated antisera. Bound antibody was detected with goat anti-rabbit IgG conjugated to horseradish peroxidase using chemiluminescence.

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. 4*A*, *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. 4*A*, *green*). 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. 4*B*, *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 Ca^{2+} 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-

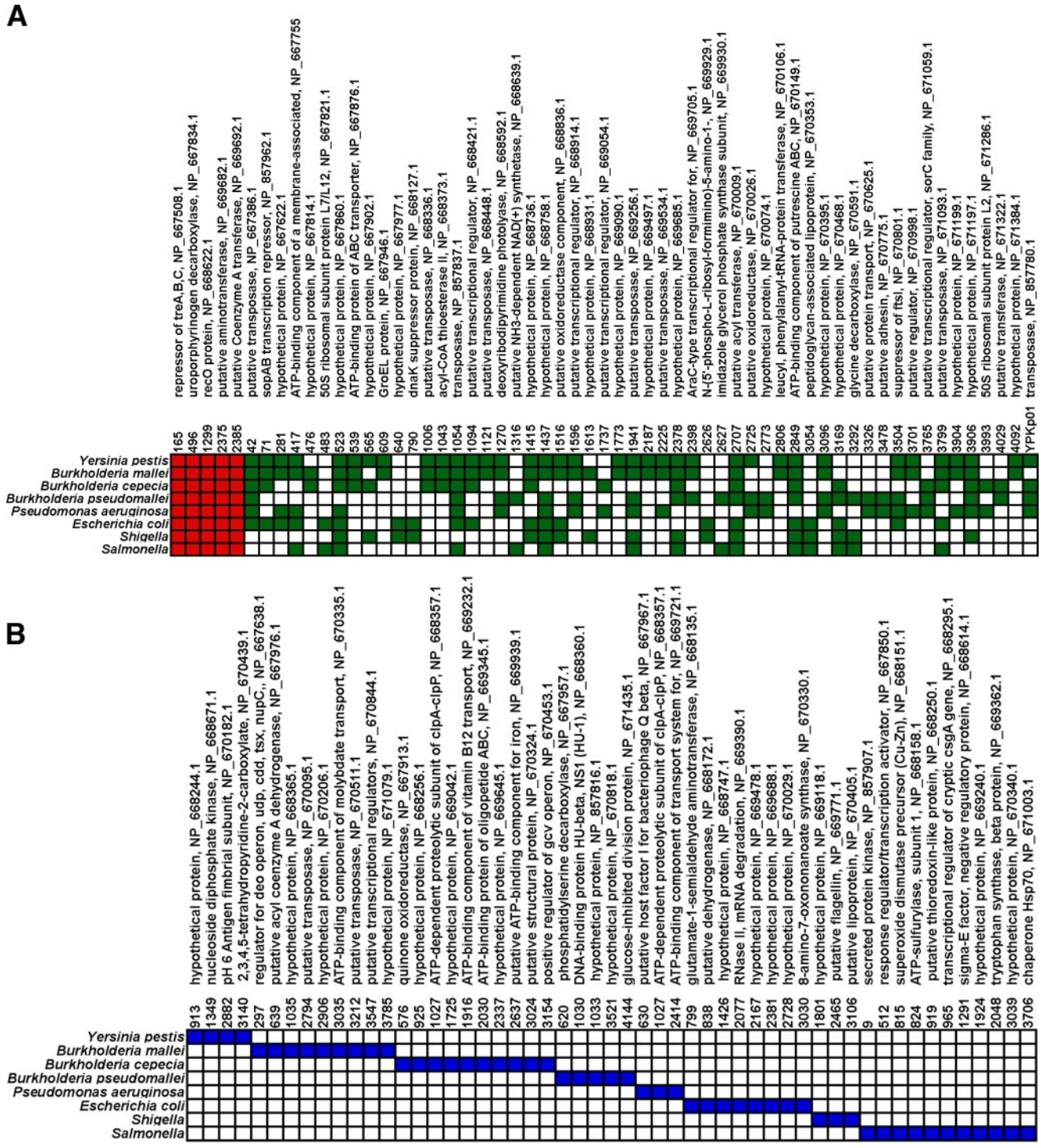

FIG. 4. **Profile of rabbit antibody response to Gram-negative pathogens.** Rabbit hyperimmune sera against each bacterial proteome were diluted 1:1000. After incubation with primary sera, binding of IgG was detected with an Alexa Fluor 647-labeled goat anti-rabbit antibody and a laser confocal scanner. *A*, proteins recognized by antibodies that were cross-reactive (*red*) or fingerprints (combination of proteins unique to one pathogen but individual protein recognition can be shared; *green*). *B*, signature proteins (unique to one pathogen; *blue*).

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

TABLE II
Similarites among cross-reactive proteins from Gram-negative pathogens

n.m., no match; str., strain.

Protein ID[a]	Description	Protein Data Bank homology	*B. mallei* (ATCC 23344)	*B. pseudomallei* K96243	*P. aeruginosa* PAO1	*E. coli* APEC 01	*S. flexneri* 2a str. 301	*Salmonella enterica* (SC-B67)	*B. cepecia* phage BcepNazgul
y0165	Trehalose repressor	1byk, 45%	YP_104561.1	YP_108430.1	NP_250639.1	YP_859910.1	NP_709960.1	YP_219298.1	NP_919020.1
			3.90e–16[b]	5.00e–20	1.10e–20	1.50e–81	6.60e–82	2.80e–82	**0.19**
y0496	Uroporphyrinogen decarboxylase	1uro, 47%	YP_104466.1	YP_109984.1	NP_253721.1	YP_859582.1	NP_709791.1	YP_219035.1	NP_919004.1
			4.30e–92	1.90e–91	1.30e–117	4.30e–146	2.00e–148	2.80e–155	**0.35**
y1299	DNA repair protein recO	1u5k, **12%**	YP_102341.1	YP_109019.1	NP_249463.1	YP_853699.1	NP_708417.1	YP_217561.1	NP_919018.1
			1.10e–26	3.50e–26	6.90e–29	9.60e–89	1.70e–91	1.30e–89	**0.11**
y2375	Transcriptional regulator	1x0m, 32%	YP_103544.1	YP_107705.1	NP_250345.1	YP_852595.1	NP_708249.2	YP_216582.1	n.m.
			1.20e–48	1.40e–47	8.10e–78	5.20e–20	5.90e–09	3.50e–20	
y2385	Acetyl-CoA hydrolase	1xr4, **17%**	YP_106223.1	YP_111645.1	NP_254132.1	YP_859026.1	NP_709205.1	YP_218045.1	n.m.
			5.30e–108	1.40e–106	1.70e–13	**0.16**	**0.15**	5.30e–152	

[a] *Y. pestis* proteins that bound antibodies from all antisera tested.
[b] Expectation value from Smith-Waterman sequence alignments. Below significance, bold.

that were recognized by antibodies from plague survivors were also observed with antibodies from rabbits. This observation is noteworthy because antibody recognition by both species involved less than 100 of the thousands of proteins present within the *Y. pestis* proteome, and the rabbit data did not involve an actual infection.

DISCUSSION

We identified several putative antibody biomarkers for Gram-negative pathogens by using a microarray encompassing the majority of proteins from the *Y. pestis* proteome. Rabbit antibodies produced against the proteomes extracted from several Gram-negative bacteria recognized *Y. pestis* proteins that clustered into three general categories: proteins recognized in all Gram-negative species examined (cross-reactive proteins), combinations of proteins unique to one pathogen (fingerprint), and proteins unique to only one pathogen (signature). Antibodies from non-human primates that recovered from a potentially lethal aerosol challenge with *Y. pestis* recognized several proteins in common with antibodies produced against the extracted bacterial proteome, supporting the importance of these proteins as potential disease biomarkers. Additional unique biomarkers of disease were identified in sera from vaccinated or acutely infected non-human primates prior to overt sepsis.

The most useful sepsis biomarkers should enable diagnosis at the earliest stage of infection and provide a means for identifying the specific etiological agent. Our results illustrate the application of proteome microarrays for the discovery of new diagnostic biomarkers. There are previously reported tests for diagnosing plague in the field and clinic that should be noted. For example, an assay based on the release of the capsular protein CaF1 into serum during infection was used to confirm epidemic cases (25). Further, the detection of soluble CaF1 in sputum of patients provided a presumptive diagnosis of pneumonic plague as early as the 2nd day after the onset of the symptoms of plague (26), although seroconversion occurs 7 days after disease onset (27). The low Ca^{2+} response V-antigen of *Y. pestis* was also used in diagnostic assays (28), and an assay based on detection of the plasminogen activator protein of *Y. pestis* was reported (29). However, these previously described diagnostic tests for plague relied on antigens that are not expressed by all isolates (30) or that may not reach detectable levels before onset of life-threatening disease. Thus, increased assay specificity and sensitivity may result from inclusion of the provisional antibody biomarkers we report here. The absence of some markers may still allow identification of the correct bacterium by pattern recognition of fingerprint or signature proteins (31, 32). Because we were able to detect antibody responses to plague before isolating culturable bacteria, it may be possible to develop proteomics assays that are sensitive enough to allow presymptomatic diagnosis.

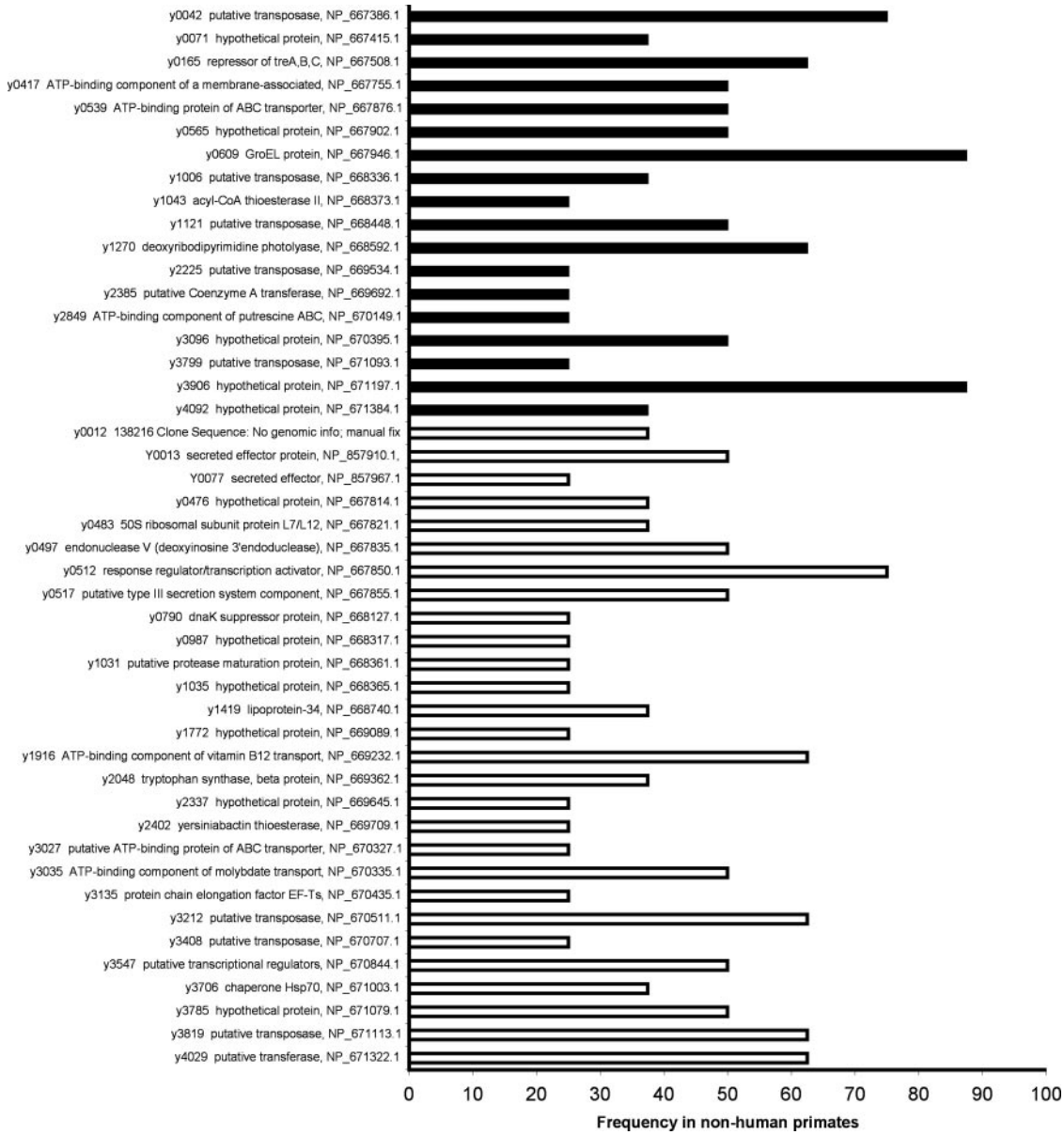

FIG. 5. **Convalescent antibody response of plague survivors.** Sera from vaccinated (rhesus macaque; $n = 1$) and non-vaccinated, antibiotic-rescued (cynomolgus macaque; $n = 7$) non-human primates were examined. *Shaded bars*, proteins recognized by antibodies from both non-human primates and rabbits vaccinated with a proteome extract or intact bacteria. *Open bars*, proteins recognized by antibodies only from non-human primate sera.

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

TABLE III
Y. pestis proteins recognized by serum antibodies from acutely infected non-human primates

ORF	Protein name	Plague[a]	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	(3*R*)-Hydroxymyristoyl acyl carrier protein, NP_670423	+	−
3125	UDP-3-*O*-(3-hydroxymyristoyl)-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	−	+

[a] 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.

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+} response stimulon encoded on a common 70-kb virulence plasmid (pCD1). The pCD1-encoded polypeptides 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

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.

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