Proteomics and Bioinformatics Strategies to Design Countermeasures against Infectious Threat Agents

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The potential devastation resulting from an intentional outbreak caused by biological warfare agents such as *Brucella abortus* and *Bacillus anthracis* underscores the need for next generation vaccines. Proteomics, genomics, and systems biology approaches coupled with the bacterial ghost (BG) vaccine delivery strategy offer an ideal approach for developing safer, cost-effective, and efficacious vaccines for human use in a relatively rapid time frame. Critical to any subunit vaccine development strategy is the identification of a pathogen's proteins with the greatest potential of eliciting a protective immune response. These proteins are collectively referred to as the pathogen's immunome. Proteomics provides high-resolution identification of these immunogenic proteins using standard proteomic technologies, Western blots probed with antisera from infected patients, and the pathogen's sequenced and annotated genome. Selected immunoreactive proteins can be then cloned and expressed in nonpathogenic Gram-negative bacteria. Subsequently, a temperature shift or chemical induction process is initiated to induce expression of the ΦX174 *E*-lysis gene, whose protein product forms an *E* tunnel between the inner and outer membrane of the bacteria, expelling all intracellular contents. The BG vaccine system is a proven strategy developed for many different pathogens and tested in a complete array of animal models. The BG vaccine system also has great potential for producing multiagent vaccines for protection to multiple species in a single formulation.

INTRODUCTION

Infectious threat agents include microorganisms such as bacteria, fungi, and viruses or the toxins that they produce, which can be used by terrorists or rogue states with the intent to kill or incapacitate and instill terror in a populace. Infectious agents can also be used for agroterrorism to damage agriculture such as livestock and crops and subsequently disrupt the human food chain.[1] There is an urgent need for the development of next generation vaccines against these infectious agents, particularly after 9/11 and the deliberate contamination of U.S. mail with anthrax spores in late 2001.[2] More recent concerns on the weaponization of these pathogens, particularly anthrax spores, for bioterrorism further heightened this urgency.[3] Because of this underlying urgency, the research and development as well as the production aspects of any vaccine need to be taken into account when designing vaccines against select agents. Thus, the most modern methods for developing select agent vaccines must not only be of a high standard but must also be rapid. The merger of the two relatively new scientific fields of immunoproteomics and bacterial ghost vaccine technology offers a novel and revolutionary approach for the rapid development of nonliving, nonpathogenic, stable, and safe vaccines against these threat agents.

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SUBPROTEOMES OF PATHOGENIC ORGANISMS ARE IDEAL FOR DISCOVERY OF VACCINE CANDIDATE PROTEINS

Each protein fraction, or subproteome, of a pathogen, including the secretome (secreted proteins), membrane proteins, and exosporium, contains many ideal vaccine targets. These subproteomes are often responsible for initial host interactions between the pathogen and host cell or virulence factors that promote pathogen survival. The secretome is of special interest since it contains proteins that can modify the host cell environment and are responsible for some host-cell−bacterial interactions. Since secretome proteins are the most likely of the pathogen's proteins to be taken up by antigen presenting cells, they are leading candidates for both antibody recognition and major histocompatibility complex class II antigen presentation, a response critical to a protective immune response. As a group, membrane proteins promote adherence to host cell surfaces, are responsible for resistance to antibiotics, and promote intercellular communication. Likewise, viral envelope or capsid proteins are usually responsible for adherence to and invasion of host cell surfaces. Both bacterial membrane and viral envelope or capsid proteins often elicit a strong immune response in infected individuals and are ideal targets for a neutralizing immune response. The spore is the infective form of *B. anthracis* and is covered by a spherical transparent mass termed the exosporium, a loose-fitting, balloon-like structure.[4] The exosporium is distinct from the spore coat and is

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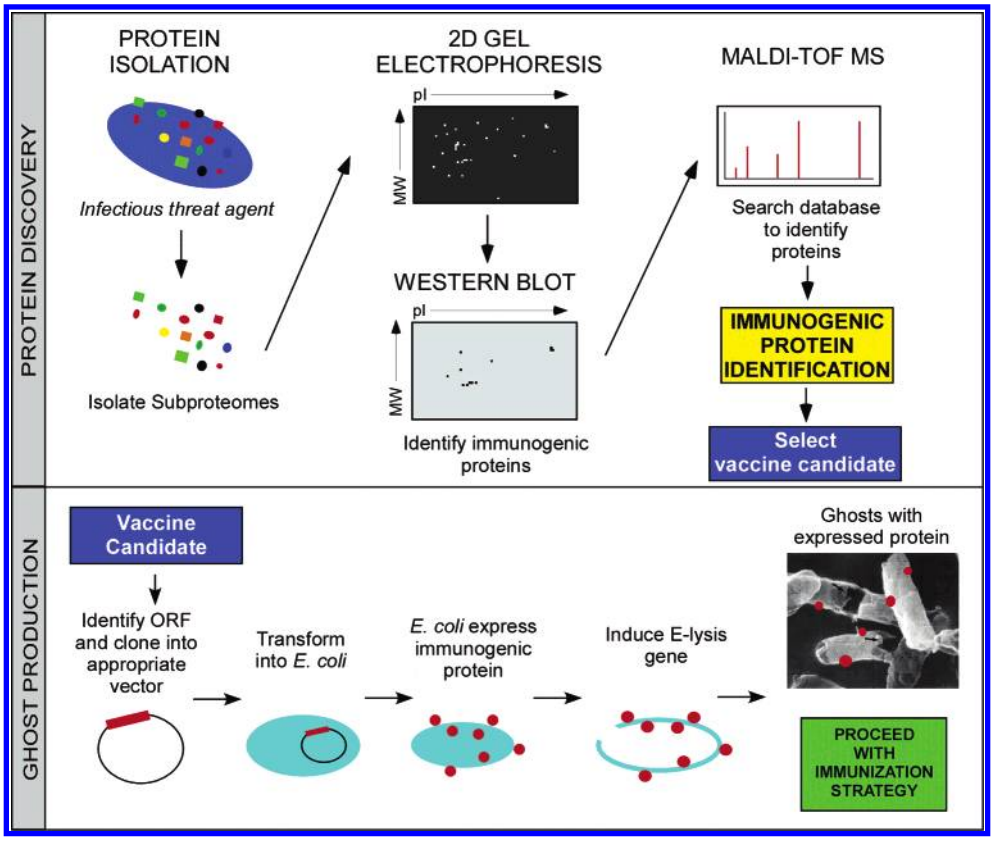

Figure 1. Immunoproteomics-based strategy for identifying immunoreactive proteins from the subproteomes of *B. anthracis*. The ORFs of select immunoreactive proteins are then cloned and expressed in Gram-negative bacteria for production of BG vaccines.

considered the primary physiological barrier between the spore and its environment[5] and may enhance the pathogenicity of the organism.[6,7] Together these three subproteomes represent the initial point of contact between the pathogen and its host and, as such, are prime candidates for vaccine development. In addition to proteins from known pathogens, proteomics may be of value in the identification of immunogenic proteins in emerging or genetically engineered pathogens.

IDENTIFICATION OF PROTEIN TARGETS FOR VACCINE DEVELOPMENT USING IMMUNOPROTEOMICS

Proteomics is the study of the total protein complement expressed by the genome at a given time in the life of an organism. An essential tool in proteomics is two-dimensional electrophoresis (2-DE), in which proteins are separated on the basis of their charge by isoelectric focusing in the first dimension and their mass by SDS−PAGE in the second dimension. Once separated by 2-DE, protein spots are then identified by mass spectrometry (MS).[8,9] MS of proteins results in a peptide mass fingerprint that is characteristic for a particular protein. The identity of an unknown protein can be determined by comparing its peptide mass fingerprint with the theoretical spectrum generated by digestion of each of the proteins in a database by using a search engine such as Mascot from Matrix Science Ltd (http://www.matrixscience-.com).[10] Some of the databases commonly used for protein identification include NCBInr, SWISS−PROT, TrEMBL, and OWL. Alternatively, and more advantageous in terms of speed and accuracy is matching the MS spectra with translations of the nucleotide sequence from an annotated genome of the same organism. This enables the investigator to match expressed proteins to their corresponding open reading frames (ORFs).

Proteins that have the greatest potential of eliciting a protective immune response are collectively referred to as the pathogen's immunome.[11] These immunoreactive proteins are identified by using either Western blot or immunoaffinity chromatography in tandem with other proteomics methods (Figure 1). Specifically, animal or human antisera from pathogen-specific infected individuals can be used to probe Western blots of a pathogen's subproteome for immunoreactive proteins. Once immunoreactive proteins are identified, Western blots can also be performed on different serovars of a pathogen to determine if a candidate vaccine protein has the potential to provide broad-range effectiveness. An example of this workflow is presented in Figure 1, where we have investigated the immunome of select agents *B. abortus* and *B. anthracis* and identified candidate vaccine proteins.

***Brucella abortus* Membrane Proteins.** Brucellosis is a major zoonotic disease caused by members of the genus *Brucella*. The human form of the disease is characterized by undulant fever, arthritis, and dementia. In domesticated animals, brucellosis is characterized by abortion and sterility. Different species of *Brucella* have been classified as potential biological weapons of mass destruction because of their highly infectious nature. Among the various nomen species, *B. melitensis*, *B. abortus*, and *B. suis* are the main causes of brucellosis.[12,13] Knowledge of membrane proteins that are induced during infection and those that contribute to pathogenicity would aid in the design of safe and efficacious vaccines. Using antisera from human patients post-infected

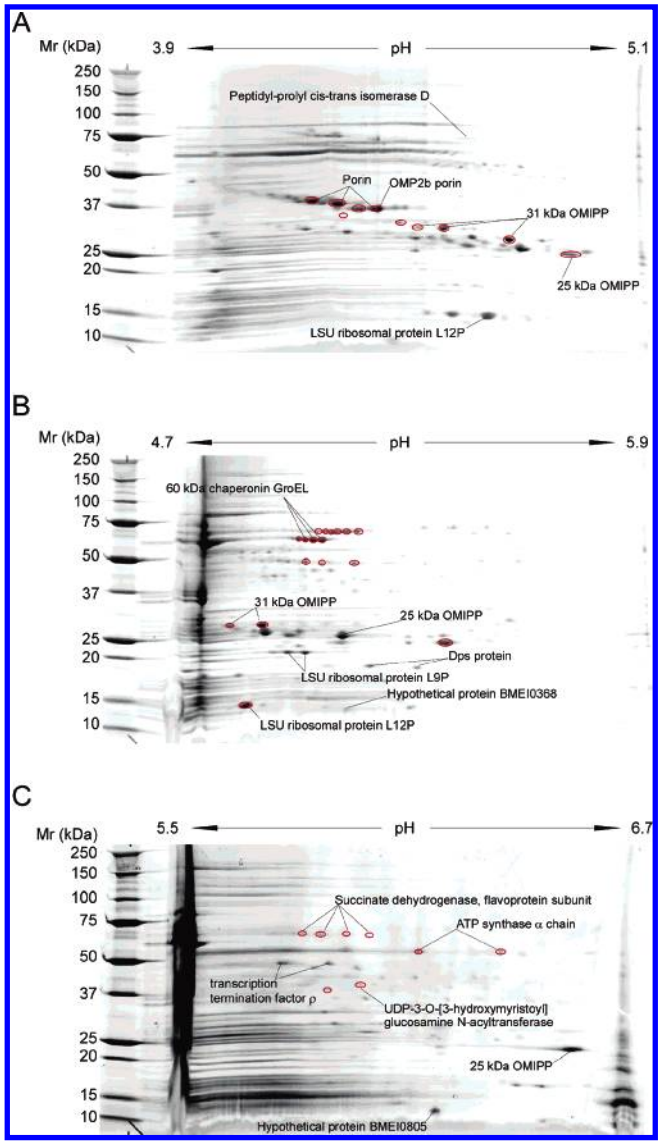

Figure 2. Immunogenic membrane proteins (encircled) of *Brucella abortus* strain 2308 at pH ranges 3.9−5.1 (A), 4.7−5.9 (B), and 5.5−6.7 (C). The 2D gels shown above were stained with SYPRO Ruby, but the colors were reversed during imaging. Immunogenic proteins were determined by Western blot analysis and are matched with the corresponding protein spots on SYPRO Ruby-stained average gels. An average gel (composed of three replicate gels) was analyzed using Phoretix 2D Expression software (Nonlinear Dynamics, Newcastle upon Tyne, U. K.).

with brucellosis, the immunogenic membrane proteins from the virulent strain of *B. abortus* 2308 were determined using 2D Western blot analysis at narrow pH ranges (i.e., 3.9−5.1; 4.7−5.9, and 5.5−6.7). Some of the identified immunogenic proteins include various protein isoforms of porin, 31 kDa outer membrane immunogenic protein precursor (OMIPP), and 60 kDa chaperonin, OMP2b porin, 25 kDa OMIPP, succinate dehydrogenase flavoprotein subunit, ATP synthase α chain, and UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase (Figure 2A−C).

***Bacillus anthracis* Secretome and Exosporium Proteins.** *B. anthracis* is the causal agent of anthrax. Its most common form is cutaneous, which is acquired directly through the skin after handling infected animal tissue or contaminated animal products. Inhalation anthrax, on the other hand, is caused by inhaling an aerosol of anthrax spores into the respiratory tract. This form was the one used in deliberately

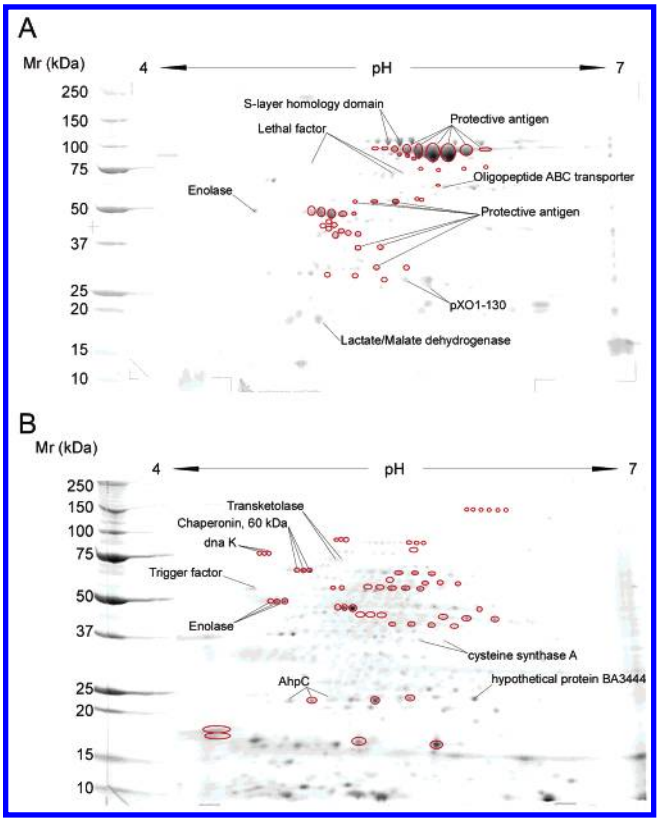

Figure 3. Immunogenic secretome (A) and exosporial (B) proteins (encircled) of *B. anthracis*. The analysis of immunogenic proteins on 2D Western blots was described in Figure 2.

exposing the civilian population in late 2001[3]. Although an anthrax vaccine called AVA is currently available, its efficacy is far below that of the Sterne live spore vaccine in small animal studies.[14] The development of an improved anthrax vaccine and the limitations of using the currently licensed AVA vaccine have been the subject of recent reviews.[2,15] Other studies also suggested that additional spore or vegetative antigens may be used as vaccine enhancers for full protection against anthrax.[16−18] The immunogenic proteins from the secretome and exosporium of *B. anthracis* that were identified using 2D Western blot analysis are shown in Figure 3A and B, respectively. The major immunogenic protein identified in the secretome is the protective antigen (PA) and its various protein isoforms (Figure 3A). In the exosporium, some of the identified immunogenic proteins include dnaK, 60 kDa chaperonin, enolase, and alkyl hydroperoxide subunit C (AhpC) (Figure 3B).

Prior to further vaccine development in our laboratory, immunoreactive proteins are scored for their potential as vaccine candidates using a multifaceted algorithm consisting of the following criteria:

1. The immunogenic protein is located in multiple subproteomes (i.e., membrane, secretome, and exosporium) within the pathogen and, thus, may be a better candidate than those found in only one subproteome.

2. The immunogenic protein's degree of immunogenicity as reflected by the total spot volume on a Western blot versus the total spot volume of the protein observed on a 2D gel.

3. The immunogenic protein's lack of homology to corresponding human proteins (i.e., an ideal candidate would have low to no protein homology to humans).

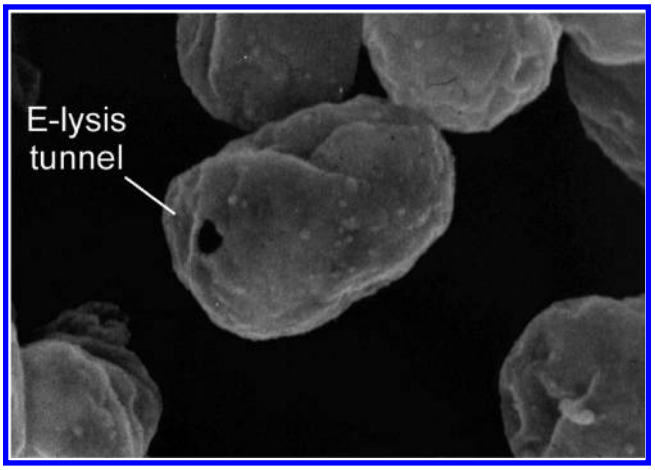

Figure 4. Scanning electron micrograph of an *E. coli* BG with an *E*-lysis tunnel through which the cytoplasmic content is expelled.

4. The immunogenic protein's potential ability to confer protection as previously reported in the literature for similar proteins in other pathogens.

5. Proteins that are immunogenic in all serovars of a species will be better candidates since they will confer immunoprotection across multiple strains of a species.

6. The immunogenic protein contains high-scoring class-I and class-II MHC binding peptides as predicted by bioinformatics analyses.

This method is used to eliminate potentially disadvantageous proteins such as GroEL, which may prove to be either problematic (e.g., possible induction of autoimmunity because of its amino acid similarity to human proteins) or immunogenic but not protective.

Bacterial Ghost (BG) Vaccines. Once identified, select immunoreactive proteins are chosen for subsequent incorporation into BGs. BGs are nonliving and nonpathogenic envelopes of Gram-negative bacteria that are produced by the expression of the cloned *E* gene.[19] Individual *E* gene subunits combine with each other and fuse with the inner and outer membranes of Gram-negative bacteria, forming an *E*-lysis tunnel through which the cytoplasmic content is expelled (Figure 4). In addition, *Staphylococcus aureus* endonuclease can be expressed during the BG production process to digest chromosomal and plasmid DNA, curbing any concerns over cross-species transfer of genetic material to the vaccinated organism. Once produced, BGs are devoid of nucleic acids, ribosomes, and other cytoplasmic components, yet the inner and outer membrane constituents such as LPS, pili, and flagella are maintained. After *E*-mediated cell lysis, each BG production batch is freeze-dried and contains no viable cells. A variety of BG have been produced and tested in many animal models.[20,21] Figure 5 shows a typical example of BG protein extract from a BG containing the highly immunogenic PA from *B. anthracis*. Using PA in a vaccine formulation has been previously shown to confer protection, and thus, the BG-PA vaccine is also expected to induce a protective immune response in upcoming animal trials.

BGs are ideal alternatives to recombinant protein, live attenuated, and heat- or chemically killed vaccines because they meet the following criteria:

•They are flexible in the types of BG vaccines that may be produced since potential vaccine candidate protein(s) from any species can be expressed in the Gram-negative *E. coli* before the creation of BG.

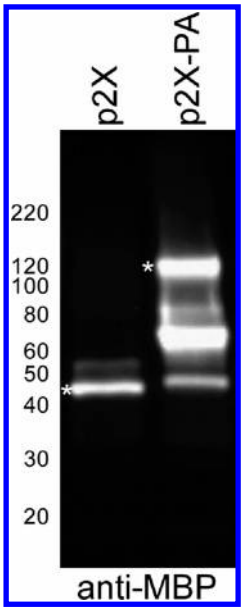

Figure 5. Western blot analysis of total protein extracts from bacterial ghosts containing p2X vector encoding a maltose binding protein (MBP) (control) or p2X-PA vector encoding the MBP protective antigen (PA) fusion protein. The blot was probed with anti-MBP, and the overexpressed MBP−PA fusion protein with the correct predicted molecular weight is indicated (*). The numbers on the left are the molecular weights in kDa of the protein standards used.

•They are nonliving and, thus, pose no pathogenic threat. Although BGs contain the intact LPS, studies have shown that the dosage of BG needed for inducing efficient immune responses can be administered without leading to endotoxin-related side effects.

•They are natural adjuvants and retain many of the immune stimulating proteins, lipids, sugars, and membrane-associated structures of their living counterparts.

•They are efficient antigen delivery systems since their surface properties facilitate recognition by primary-antigen-presenting cells by pattern-recognition and toll-like receptors.

•They are produced quickly in large quantities by fermentation.

•They are stable for long periods of time at ambient temperature as freeze-dried material and, thus, do not require cold storage. This eliminates the costs associated with cold chain transport and warehousing and increases the geographical range of use.

•They are able to be self-administered through noninvasive and needle-free means.

•They are capable of delivering DNA-encoding vaccines.

•They are devoid of all genetic material as a result of expression of the *S. aureus* endonuclease in the production phase; therefore, each batch contains no living cells, and the chance of horizontal gene transfer from the BG vaccine to natural human flora is eliminated.

CONCLUSIONS

Vaccines provide the most important mechanism for protecting human populations against infectious diseases. A novel vaccine that can elicit a rapid protective immune response; improves the clinical course of an exposed person;

has a very high benefit-to-risk ratio; is easily manufactured, distributed, and administered; and is stable for long-term stockpiling is essential for combating weaponized threat agents. A proteomics-based BG vaccine meets these requirements for modern day biodefense vaccines.

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