metabolic burden incurred by plasmid-bearing strains both at the intrinsic level of the plasmid DNA sequence and at the level of foreign gene expression.

Reduction of Foreign Antigen Toxicity

It is not enough to optimize in vivo expression levels to secure an appropriate immune response (13). Problems with inherent antigen toxicity may diminish the colonizing ability of live vectors and lower the levels of antigen delivered to immunological inductive sites. In addition, proper folding may be required for conformationally specific epitopes to trigger protective serum antibody responses. For example, neutralizing serum antibodies against the critical 19-kDa carboxyl terminal domain of merozoite surface protein 1 (MSP-1) from *Plasmodium falciparum* are only observed when the six disulfide bridges of the terminal domain are properly folded (33,34); this is unlikely to occur efficiently for antigens synthesized within the reducing environment of the live vector cytoplasm.

In attempts to address potential toxicity and protein folding problems, various antigen export technologies have been developed for surface expression or for the extracellular secretion of foreign proteins. One successful approach utilizes a novel surface display technology based on engineering of expression cassettes derived from the *Pseudomonas syringe* ice nucleation protein (INP); the versatility and promise of this strategy are illustrated by reports of the display of properly folded eukaryotic antigens such as the human immunodeficiency virus type 1 glycoprotein (gp)120 on the surface of *E. coli* (35), and construction of an immunogenic multivalent hepatitis B surface antigen/hepatitis C core protein displayed on the surface of the licensed *S.* Typhi vaccine strain Ty21a (36).

In addition to surface display, secretion of heterologous antigens out of *Salmonella* live vectors has been reported by several groups to enhance the immune response to a foreign protein. Hess et al. (37) reported that cytoplasmic expression of the protective T-cell antigen listeriolysin O (LLO) within recombinant *Salmonella* vaccine strains did not confer protection in mice against lethal challenge with virulent *Listeria monocytogenes*. However, in-frame insertion of LLO within a truncated form of the *E. coli* hemolysin (HlyA) A allowed extracellular secretion of this fusion in the presence of the coexpressed *E. coli* HlyB/HlyD/TolC export apparatus and resulted in protection against lethal challenge with *L. monocytogenes*. Similar results have been reported by other groups using type III secretion systems encoded either by *Salmonella* pathogenicity islands (SPI)-1 (38,39) or by SPI-2 (40) to elicit protection using secreted antigens from both prokaryotic and eukaryotic pathogens.

A novel antigen export system has also been described, which is derived from a cryptic hemolysin encoded by cytolysin A (ClyA) within the chromosome of *S.* Typhi strains, including the clinically proven strains CVD 908-*htrA* (41) and Ty21a (42). The molecular biology of ClyA from *S.* Typhi is well characterized (42–45), and it has been conclusively demonstrated that unfused ClyA is exported out of bacteria via outer membrane vesicles (46). Such a mechanism for vesicle formation raises the intriguing possibility of engineering ClyA to export antigens out from live vectors that are otherwise potentially toxic when expressed cytoplasmically; these vesicles would also carry lipopolysaccharide (LPS), which might improve the immunogenicity of a foreign antigen.

This ClyA-mediated export technology has been successfully applied to the development of *S.* Typhi–based vaccines carrying antigens from prokaryotic and eukaryotic organisms (47–49). The usefulness and versatility of this system has been extensively demonstrated using genetic fusions of ClyA to the cell-binding PA83 subunit of *Bacillus anthracis* anthrax toxin. Delivery of ClyA-PA83 protein fusions by the licensed typhoid vaccine strain Ty21a to mice (48) and by candidate vaccine strain CVD 908-*htrA* to mice and monkeys (49) was shown to elicit high titers of toxin-neutralizing antibodies in animals primed intranasally with live vector and boosted with the licensed BioThrax® anthrax vaccine. These ClyA-PA83 protein fusions were demonstrated to be efficiently exported to the surface of attenuated *S.* Typhi strain CVD 908-*htrA* despite increasing the size of the ClyA export domain from its original 35 kDa to 118 kDa as a fusion protein (Fig. 1). The biological relevance of antitoxin responses against ClyA-PA83 was proven in work carried out with *S.* Typhimurium where mice were protected against a lethal aerosol anthrax spore challenge when oral immunization with live vectors expressed ClyA-PA83 but not when live vectors delivered PA83 fused to *E. coli* HlyA (5). It is, therefore, clear that the regulated expression of foreign

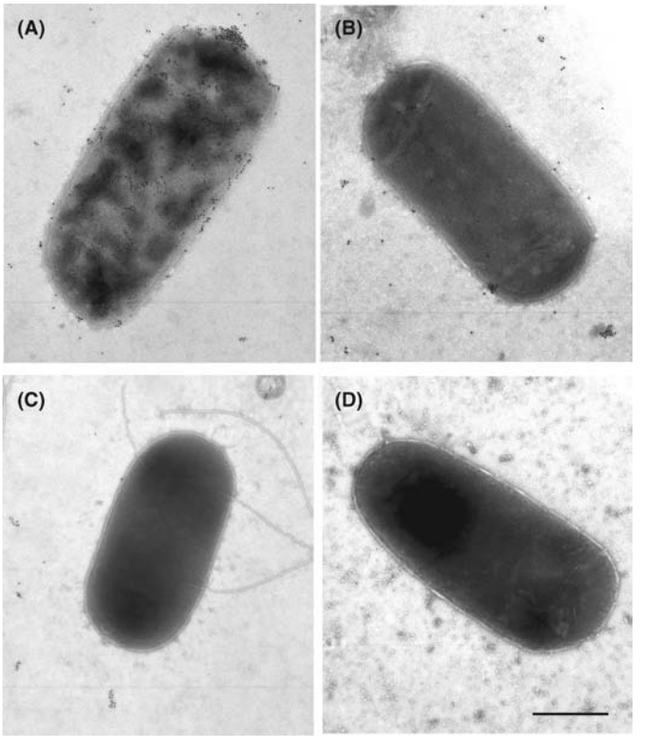

Figure 1 Plasmid-based expression of ClyA-PA83 on the surface of the *Salmonella* Typhi CVD 908-*htrA* live vector, detected by immunogold staining. Immunoelectron micrographs of (**A**) *S.* Typhi CVD 908-*htrA* expressing the ClyA-PA83 protein fusion, (**B**) *S.* Typhi CVD 908-*htrA* expressing unfused PA83 in the cytoplasm, and (**C**) *S.* Typhi CVD 908-*htrA* without any expression plasmid (negative control) incubated with mouse PA-specific antibodies and gold-labeled anti-mouse antibody. (**D**) *S.* Typhi CVD 908-*htrA* expressing the ClyA-PA83 fusion protein, incubated with negative serum and gold-labeled anti-mouse antibody. Bar: 0.25 μm. *Abbreviations*: ClyA, cytolysin A; PA83, Protective Antigen (83 kDa) from anthrax toxin.