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Physiological Levels of Glucose Induce Membrane Vesicle Secretion and Affect the Lipid and Protein Composition of *Yersinia pestis* Cell Surfaces

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***Yersinia pestis* grown with physiologic glucose increased cell autoaggregation and deposition of extracellular material, including membrane vesicles. Membranes were characterized, and glucose had significant effects on protein, lipid, and carbohydrate profiles. These effects were independent of temperature and the biofilm-related locus *pgm* and were not observed in *Yersinia pseudotuberculosis*.**

Yersinia pestis, the etiologic agent of plague, is one of the most virulent bacteria identified (1). Phylogenetic analyses indicate that *Y. pestis* evolved relatively recently within the last 20,000 years from *Yersinia pseudotuberculosis*. Although the bacterial genomes of these two species share >90% similarity, the disease transmission, course of infection, morbidity, and mortality are dramatically different (2). *Y. pseudotuberculosis* is an enteropathogen that causes self-limiting infections, while *Y. pestis* is a zoonotic pathogen that causes a systemic disease with very high mortality and morbidity (1). Flea-borne bubonic plague is the primary form of infection that can progress to secondary pneumonic plague. This form of the disease can be transmitted directly between humans without the flea vector, causing pneumonic disease characterized by airborne transmission, rapid disease progression, and mortality rates approaching 100% when untreated (1). Despite these characteristics that highlight the sobering potential for employing *Y. pestis* as a biological warfare agent, there are still no effective commercial vaccines against this pneumonic disease that are available in the United States (3, 4).

Y. pestis successfully proliferates in both flea and mammalian hosts, in which it is exposed to mammalian blood and its components (1). Resistance to complement and the digestive properties of phagocytes are critical for pathogen survival in the bloodstream (1, 5, 6). Recent studies indicate that bacterial passage through the flea vector induces *Y. pestis* resistance to phagocytosis, which, in turn, potentially facilitates its survival and dissemination in the mammalian host (7, 8). Burrows and Bacon found that blood glucose contributes to *Y. pestis* resistance to phagocytosis *in vitro* (9) but did not identify the molecular basis for this effect. Subsequently, we observed that glucose restored the ability of a *Y. pestis* Δ*ail* deletion mutant (6) to autoaggregate when grown in a defined minimal medium (PMH) (51) and enhanced aggregation of the parental strain in both Luria Bertani low-salt (LB) broth and PMH (data not shown). Inasmuch as autoaggregation contributes to virulence in many bacterial pathogens (10–12), including *Y. pestis* (13), we carried out a systematic characterization of the effects of glucose on *Y. pestis*. The goal of this study was to determine glucose-induced changes in bacterial surface composition that may contribute to pathogenicity, survival in the host, and/or identification of novel virulence factors and vaccine targets.

Because enhanced autoaggregation among cultures grown with glucose implied that changes involved *Y. pestis* surface components, field emission scanning electron microscopy (FESEM) was employed to study the external structure of the cells. *Y. pestis* KIM6$^+$ was grown in LB overnight at 28°C with vigorous shaking and transferred into fresh medium with or without 5.5 mM glucose (a physiological blood level [14]). After 24 h of incubation, the cultures were mixed in a 1:1 ratio with fixative solution (2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) and processed for FESEM (5). The samples were analyzed by Quanta 200F (Field Emission Instruments, Hillsboro, OR).

FESEM revealed a striking difference between *Y. pestis* cells grown with and without glucose. With glucose, there was massive deposition of extracellular material connecting the cells, small spherical structures blebbing or bulging away from ruffled surfaces, and inward bulges with variable diameters (Fig. 1). In contrast, cells incubated without glucose had smooth surfaces and no blebbing and lacked extracellular material deposition (Fig. 1). Some of the features in the glucose-supplemented cultures (outer membrane blebbing, small spherical structures) resembled membrane vesicle (MV) secretion. The MV system has not previously been characterized in *Y. pestis* but is present in multiple pathogenic bacteria (15–18). The induction of this system is related to bacterial stress responses and plays an important role in nutrient acquisition, biofilm development, and modulation of host defense mechanisms during progression of pathogenic lesions (16, 17). Incubation of *Y. pestis* with physiological levels of glucose (5.5 mM) reduced the pH to acidic conditions (data not shown) and

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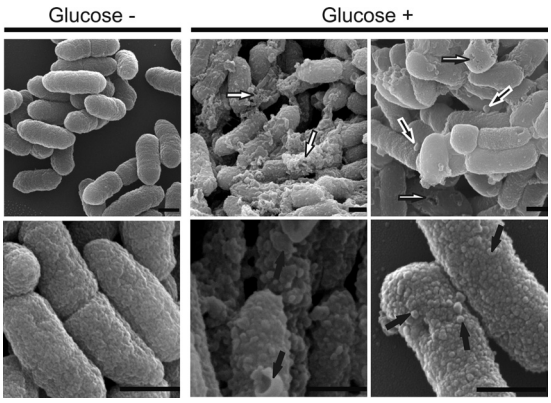

FIG 1 *Y. pestis* phenotypic changes induced by growth with glucose. *Y. pestis* KIM6$^+$ was incubated at 28°C for 24 h in LB medium with or without 5.5 mM glucose. Cells were collected, fixed, and processed for FESEM imaging. White arrows indicate extracellular material deposited on the bacterial cells, arrows that are half white and half black indicate inward buckling, black arrows with a white outline indicate larger membrane vesicles, and black arrows indicate smaller membrane vesicles. Bars, 0.5 μm.

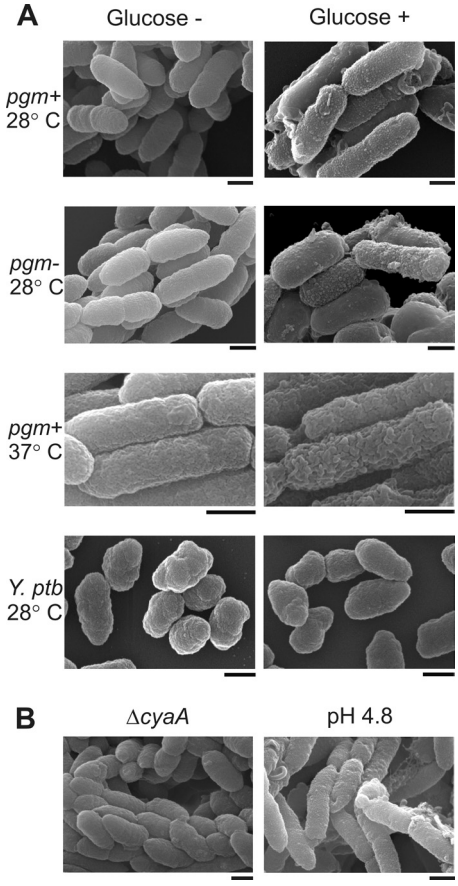

FIG 2 Effect of temperature, species, and strain on *Yersinia* phenotypic differences induced by glucose. (A) *Y. pestis* KIM6$^+$ (*pgm*$^+$), *Y. pestis* KIM6$^-$ (*pgm*$^-$), and *Y. pseudotuberculosis* were incubated at 28°C or 37°C for 24 h in LB medium with or without 11 mM glucose. (B) *Y. pestis* KIM6$^-$ Δ*cyaA* or *Y. pestis* KIM6$^+$ was incubated at 28°C for 24 h in plain LB or HCl-acidified LB (pH 4.8), respectively. Cells were collected, fixed, and processed for FESEM imaging. Bars, 0.5 μm.

was likely an important aspect of the underlying signaling mechanism. To provide a consistent acidic pH (~4.8), 11 mM glucose was used in some experiments.

We investigated whether the glucose-induced changes in the cell were part of previously identified growth phenomena associated with biofilm formation in flea hosts. To determine if the deposition of the extracellular material induced by glucose was dependent on the biofilm-related locus (*pgm*), KIM6$^-$, a strain lacking that chromosomal fragment, was tested. Like KIM6$^+$, abundant deposits of extracellular material were visible on KIM6$^-$ cells incubated with glucose, demonstrating that the glucose effect was *pgm* independent (Fig. 2A). Additionally, the glucose effect was observed in cells grown at either 28°C or 37°C, indicating that it was not temperature dependent (Fig. 2A). Also, changes were not in response to osmolarity, since neither 5.5 and 11 mM xylitol nor 5.5 and 11 mM ribose induced them (data not shown). To discriminate whether the phenotype was dependent on the catabolite repression or simply induced by the acidic conditions resulting from glucose metabolism, two approaches were taken. When glucose causes catabolite repression, cyclic AMP (cAMP) levels are reduced (19). With that in mind, an adenylate cyclase deletion mutant (Δ*cyaA*, *y0382*) was engineered to mimic catabolite repression by removing this key enzyme responsible for cAMP synthesis. Similarly to the parental strain grown with glucose, the Δ*cyaA* strain demonstrated slower growth and a slight increase in autoaggregation (data not shown); however, FESEM imaging of the mutant strain revealed few vesicles and little deposition of extracellular material (Fig. 2B). The pH-dependent changes were investigated by growing *Y. pestis* KIM6$^+$ in LB medium acidified with HCl to pH 4.8. Growth in acidic medium was heavily impaired, and cultures reached a maximum optical density at 600 nm (OD_{600}) of ~0.2 instead of 2.2 or more in neutral medium and showed increased autoaggregation. Under acidic conditions, *Y. pestis* KIM6$^-$ elongated and some membrane vesicle secretion and extracellular material deposition was observed (Fig. 2B), but this treatment did not reproduce the dramatic changes of glucose-grown cells. Together, these results showed that the glucose-induced phenotype was not induced by acidic conditions alone but was from the additive effects of glucose metabolism and low pH. Finally, the effect of glucose was unique to *Y. pestis*. The phenotype was not observed in *Y. pseudotuberculosis*, and the MV secretion was a feature that discriminated the highly infectious vector-borne systemic pathogen from its enteric self-limiting disease predecessor (Fig. 2A). *Y. pseudotuberculosis* cultures showed no surface changes when incubated with glucose and maintained the culture medium at pH 8.

To characterize the membranes and extracellular deposits, material was isolated from cells grown with and without glucose, and protein, lipid, and carbohydrate fractions were extracted and analyzed. In order to avoid interference from *pgm*-related biofilm production by KIM6$^+$, a KIM6 strain lacking *pgm* was used. To avoid interference from capsule production (Caf1 antigen), the bacteria were grown at 28°C. After 24 h of incubation with or without glucose, cells were collected, washed once with water, resuspended in water, and vigorously vortexed for 5 min. Next the cells were centrifuged, the supernatants were collected and filtered (0.2 μm), and the bound material was lyophilized. For protein analysis, the lyophilizates were resuspended in sample buffer and separated on 12.5% SDS-PAGE and the differing bands were cut

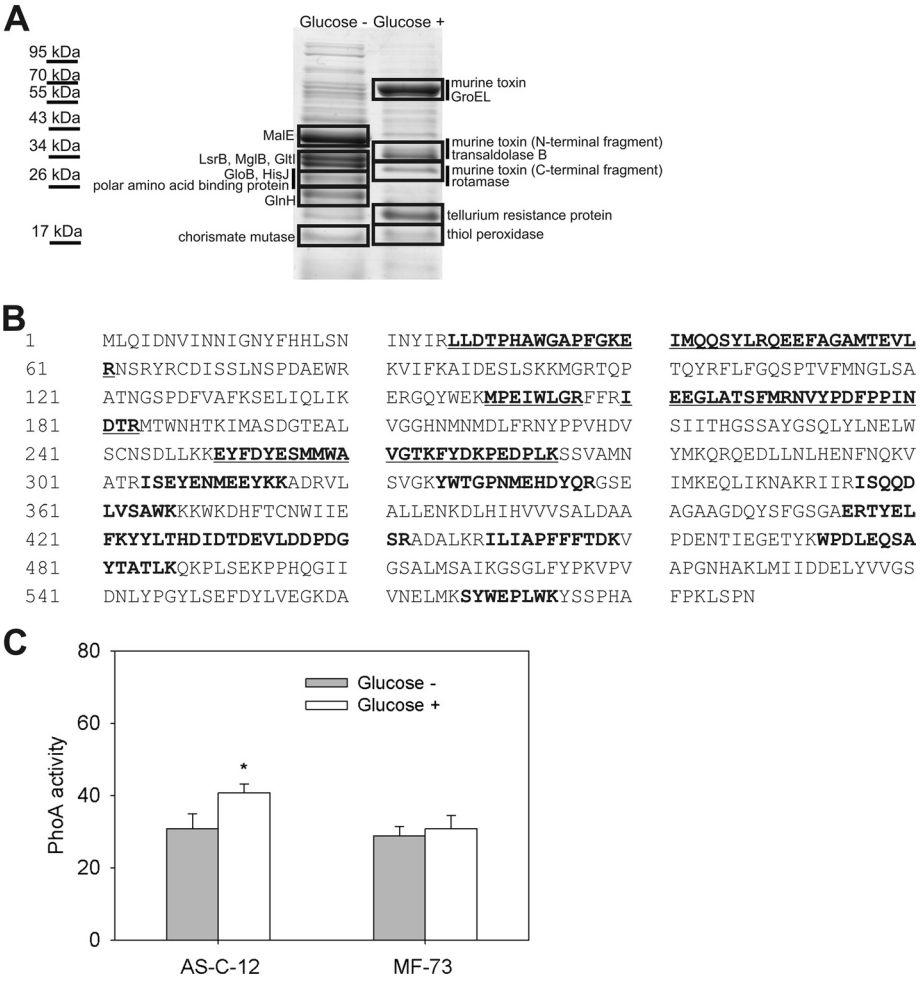

FIG 3 Effect of glucose on proteins in the membrane-associated fractions from *Y. pestis*. (A) *Y. pestis* KIM6$^-$ was incubated for 24 h in LB medium with or without 11 mM glucose, collected, washed, centrifuged, and resuspended in water. Cells were vigorously vortexed and centrifuged, the supernatant was collected and filter sterilized, and the membrane-associated fraction was lyophilized. Proteins were extracted, separated on SDS-PAGE, and stained with Coomassie blue. Molecular masses were estimated with the prestained PageRuler protein standard. Indicated protein bands were cut from the gel and identified by LC-ESI MS/MS (see Table S1 in the supplemental material). (B) Patterns of the murine toxin (MT) peptides detected by MS analysis in the protein bands: ~34 kDa (peptides marked with bold and underlined font) and ~30 kDa (peptides marked with bold font) suggest that MT was processed from its native form (found in the protein band of ~63 kDa). (C) Two (AS-C-12 and MF-73) *Y. pestis* KIM6$^+$ *phoA* fusion constructs with the mini-Tn*phoA* insertions in the murine toxin gene were incubated with and without 11 mM glucose as described above. Alkaline phosphatase was measured in the cultures after 24 h. The asterisk (*) indicates a statistically important difference (Mann-Whitney test; $P < 0.01$) in the alkaline phosphatase activity between the AS-C-12 cultures grown with and without sugar; no statistically important difference between the conditions was found for the MF-73 strain (Mann-Whitney test; $P = 0.668$).

from the gel, trypsin digested, and subjected to mass spectrometry (MS) identification (6). For lipid analysis, lyophilizates were subjected to fatty acid methyl ester (FAME) and phospholipid fatty acid (PLFA) gas chromatography (GC) analyses. The service was performed by Midi Labs (Newark, DE) according to the company's protocols. For carbohydrate analyses, the lyophilized lysates were acid hydrolyzed, derivatized with either *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) or *N*-trimethylsilylimidazole (TMSI), and separated by GC/MS.

There were profound differences in the membrane-associated and blebbed proteins between bacteria incubated with and without glucose (Fig. 3A). These biochemical differences were not seen until the glucose-grown cultures showed a drop in pH (data not shown). Two prominent protein bands, one doublet with a mass of ~63 kDa and one lower protein band with a mass of ~20 kDa, constituted major fractions of the membrane-associated proteins of the cells cultured with glucose. Additionally, three other unique bands were found in this fraction (~34, 30, and 18 kDa). Protein identification by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) analysis (see Table S1 in the supplemental material) revealed known virulence or pathogenic factors common to many human pathogens, including pneumonic pathogens. Noteworthy among these factors were *Y. pestis* murine toxin (MT), GroEL, rotamase, and scavengase (20–24). Among them, MT and GroEL are also pathogenic factors during the bacterial infection of insects. MT is involved in *Y. pestis* survival in the insect vector (25), and GroEL possesses insecticidal properties (26). Potential preventative or therapeutic treatment has been associated with (i) GroEL that has been successfully tested as a vaccine or is a vaccine candidate against numerous pathogens (27–29) and (ii) scavengase that is a target of antivirulence compounds that reduce the expression of a type III secretion

system (T3SS) (19). Additionally, three proteins are associated with infection of and survival in macrophages (GroEL [30], tellurium resistance protein [31], and scavengase [23, 32]). Moreover, the profile of membrane-associated proteins from *Y. pestis* grown with glucose corresponded to that of proteins associated with MVs in other pathogens (phospholipase, adhesins, and GroEL) (16, 18, 26). In contrast, identification of nine proteins (with band sizes of ~40, 34 [three proteins], 30 [three proteins], 27, 25, and 18 kDa) in cultures grown without glucose revealed that most were involved in catabolism (regulators or transporters) and that their activation either downregulates virulence (33, 34) or has not been described. Only autoinducer 2 (AI-2) binding protein (35) and chorismate mutase (36) have the potential to regulate host-pathogen interactions (see Table S1 in the supplemental material). Notably, 44% of proteins (encoded by *y0028*, *y2662*, *y1189*, and *y1607*) found in the cultures grown without glucose and only one protein (encoded by *y1990*) found in the cultures grown with glucose were identified to belong to the Crp (catabolite regulation protein) regulon (RegPrecise [37]).

To investigate if the presence of unique proteins found on the surface of bacteria incubated with glucose was due to *de novo* gene expression or to increases in protein secretion, promoter activity of a representative protein, murine toxin, was assayed in the two different conditions. This protein was selected as it encompassed a major constituent of the extracellular proteins and has been shown to have very important biological significance for *Y. pestis* transmission and pathogenesis (25). Two *Y. pestis* KIM6$^+$ mini-Tn*phoA* mutants (AS-C-12 and MF-73) (38) with insertions in the murine toxin gene were grown with and without 11 mM glucose for 24 h as described above. Alkaline phosphatase was then assayed in the permeabilized cultures as described previously (39) with a small modification of 1 M Tris buffer, pH 8.5. A small, 1.3-fold increase (Mann-Whitney test; $P < 0.01$) in the alkaline phosphatase activity was found in the AS-C-12 cultures grown with glucose compared to unsupplemented LB; however, no such difference was found in the MS-73 strain (Mann-Whitney test; $P = 0.668$) (Fig. 3C). Additional controls showed that activity was not masked by the low pH of the medium (data not shown). Considering the magnitude of the change in the murine toxin concentration on the surface of bacteria grown with glucose and the inadequate promoter activation to account for this, we concluded that its presence in the membrane-associated fraction was due primarily to increased secretion.

Profound qualitative changes after growth with glucose were also found in the lipid fraction (Table 1). Not only did the fatty acid and phospholipid composition differ, but more importantly, higher percentages of unsaturated fatty acids were measured in cultures grown with glucose (PFLA, 41.67% versus 23.85%; FAME, minimum of 25.97% versus 9.03%). The presence of unsaturated fatty acids is well known to be associated with enhanced virulence and resistance to low pH among diverse pathogens, such as *Vibrio* and *Streptococcus* (40, 41). This observation warrants further investigation into the context of *Y. pestis* pathogenesis. Carbohydrate analysis revealed significantly fewer species and no unique representative in the membrane-associated fraction after incubation with glucose (data not shown).

Previously it was suggested that the bloodstream environment induces bacterial resistance to phagocytosis by suppression of *Y. pestis*-specific antigens (42). We hypothesized that glucose may induce phenomena previously unrecognized in *Y. pestis*, such as the observed changes in lipid and protein compositions and/or the induction of the MV secretion system. We suggest that many of the changes in protein populations may result, at least in part, from the increased activity of an MV secretion system. This unique bacterial secretion system is used by many Gram-negative bacteria to combat host defense through delivery of large numbers of toxic components into host cells or into the extracellular milieu (15–18). This secretion was specifically induced by physiological levels of glucose and was not observed in the closely related enteric *Y. pseudotuberculosis*. It has been known for a long time that *Y. pestis* metabolizing glucose significantly lowers the pH of the medium (9). While it is unlikely that this effect would occur in the blood of the mammalian host, even during septicemia, whether the pathogen can affect the pH in the flea vector is unknown (8). The pH in the flea midgut is normally between 6 and 7 (43).

TABLE 1 Identification of lipids differentially expressed in the presence or absence of glucose

Fatty acid	% of each fatty acid in the presence or absence of glucose	
	Absence	Presence[a]
PFLA[b]		
$C_{14:1}$ ω8c	7.15	
$C_{14:0}$	3.72	1.51
$C_{16:4}$ ω3c		12.33
$C_{16:0}$ N alcohol		4.36
$C_{16:1}$ ω7c		23.04
$C_{16:0}$	53.20	35.48
$C_{17:1}$ ω7c 10-methyl	6.35	
$C_{17:0}$ cyclo		9.58
$C_{18:3}$ ω3c	10.35	
$C_{18:1}$ ω7c		6.30
$C_{18:0}$	19.23	7.41
Total % of unsaturated acids	23.85	41.67
FAME[c]		
$C_{9:0}$	5.77	
$C_{10:0}$	1.45	
Summed feature 2[d]		13
$C_{12:0}$	9.23	9.35
$C_{14:0}$	1.60	1.38
$C_{16:0}$ N alcohol	44.99	3.81
Summed feature 3[d]	1.66	20.01
$C_{16:0}$	20.39	30.67
$C_{17:0}$ cyclo		8.29
$C_{18:3}$ ω6c (6,9,12)	4.37	
Summed feature 5[d]		1.06
Summed feature 8[d]		5.96
$C_{18:1}$ ω9c	3	
$C_{18:0}$	7.53	6.48
Total % of unsaturated acids	9.03	25.97[e]

[a] 11 mM glucose.
[b] PLFA, phospholipid fatty acid; values are percentages of total fatty acids.
[c] FAME, fatty acid methyl ester; values are percentages of total fatty acids.
[d] Summed features are groups of fatty acids that cannot be separated by gas liquid chromatography (GLC) with the Midi system and are as follows: summed feature 2, comprising any combination of $C_{14:0}$ 3OH, $C_{16:1}$ iso I, $C_{12:0}$ aldehyde, and an unknown fatty acid of equivalent chain length 10.9525; summed feature 3, comprising any combination of $C_{16:1}$ ω7c and $C_{16:1}$ ω6c; summed feature 5, comprising any combination of $C_{18:0}$ ante and $C_{18:2}$ ω6,9c; summed feature 8, comprising any combination of $C_{18:1}$ ω7c and $C_{18:1}$ ω6c.
[e] The minimum total percentage of unsaturated acids was calculated with the assumption of 100% participation of saturated acids in summed features 2 and 5.

However, measurements were not taken when *Y. pestis* infected and colonized the insect. Indirect evidence, based on the comparison of the gene expression profiles of *Y. pestis* in the colonized flea and under *in vitro* conditions, suggests that the bacterium likely encounters a low pH. For example, increased expression of the acidic pH-inducible PhoPQ system and pH 6 antigen fimbrial usher protein (PsaC) (8) and induction of a phagocytosis-resistant phenotype after transit through the flea vector itself (7, 8) are just a few changes indicating that *Y. pestis* may be exposed to low pH in the flea before entering a mammalian host. Glucose in the flea blood meal would be supplied to *Y. pestis* every few days. Studies by Vadyvaloo et al. showed that the glucose phosphotransferase system (PTS) was indeed slightly upregulated in the flea relative to that under *in vitro* conditions (8). Another source of glucose in the flea digestive tract may be its derivative, *N*-acetylglucosamine. We found (data not shown) that incubation of *Y. pestis* with 11 mM *N*-acetylglucosamine reduced the pH to the same level as incubation with glucose (pH 4.8). *N*-acetylglucosamine is a component of chitobiose and chitin present in the flea's proventriculus spines, in which *Y. pestis* forms biofilms (8). Significantly upregulated genes encoding chitobiose PTS uptake and utilization systems (*chbBC*, *chbF*) were found in *Y. pestis* inside the flea (8). It has been suggested that chitobiose and *N*-acetylglucosamine may be available for *Y. pestis* (8) and for *Borrelia burgdorferi* (44), another vector-borne pathogen, during chitin turnover in the insect.

Gene expression analysis of the bacterium in the flea environment identified insecticide-like toxin proteins to facilitate resistance to phagocytosis (8); however, other factors have yet to be determined. Here we indicated that *Y. pestis* can activate a secretion mechanism allowing for efficient export of many identified pathogenesis factors to the bacterial surface and operating independent of change in exported protein expression. We showed it using phospholipase D, a toxin for mice and rats (45, 46) that is also required for insect infection (25). We suggest that the phenotype seen on the surface of glucose-grown bacteria is evidence of an MV phenomenon (47) capable of enhancing macromolecular secretion as described here. MVs are known to vary in size and biochemical properties depending on the bacterial genus, growth conditions, and lipopolysaccharide (LPS) component (15, 18, 48, 49). Although Gram-negative bacterial MVs contain primarily periplasmic and outer membrane proteins, some are associated with cytoplasmic and inner membrane proteins, indicating that the vesicles can originate from the inner as well as the outer membrane and engulf whatever proteins are found there (50).

While this system may lack specificity, it compensates by exporting large amounts of toxins quickly. Many of the identified MV proteins are common virulence or pathogenic factors. Moreover, ones like GroEL (27–29) and scavengase have been successfully tested as vaccines or as vaccine candidates against numerous pathogens. Therefore, our findings may provide (i) novel vaccine candidates for plague, (ii) new insight into the mechanisms by which vector-borne *Y. pestis* efficiently evades the host immune system (7, 8), a trait that separates it from its less-virulent precursor, the enteric pathogen *Y. pseudotuberculosis*, and (iii) evidence of an alternative delivery mechanism of *Y. pestis* virulence factors to target host cells if the MVs subsequently fuse with their membranes.

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