

Na⁺/H⁺ Antiport Is Essential for *Yersinia pestis* Virulence

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Na⁺/H⁺ antiporters are ubiquitous membrane proteins that play a central role in the ion homeostasis of cells. In this study, we examined the possible role of Na⁺/H⁺ antiport in *Yersinia pestis* virulence and found that *Y. pestis* strains lacking the major Na⁺/H⁺ antiporters, NhaA and NhaB, are completely attenuated in an *in vivo* model of plague. The *Y. pestis* derivative strain lacking the *nhaA* and *nhaB* genes showed markedly decreased survival in blood and blood serum *ex vivo*. Complementation of either *nhaA* or *nhaB* in *trans* restored the survival of the *Y. pestis* *nhaA nhaB* double deletion mutant in blood. The *nhaA nhaB* double deletion mutant also showed inhibited growth in an artificial serum medium, Opti-MEM, and a rich LB-based medium with Na⁺ levels and pH values similar to those for blood. Taken together, these data strongly suggest that intact Na⁺/H⁺ antiport is indispensable for the survival of *Y. pestis* in the bloodstreams of infected animals and thus might be regarded as a promising noncanonical drug target for infections caused by *Y. pestis* and possibly for those caused by other blood-borne bacterial pathogens.

Yersinia pestis, a Gram-negative bacterium, is the causative agent of plague. Because of its high infectivity and lethality, *Y. pestis* is considered a potential bioterrorism weapon. *Y. pestis* is typically introduced into the human body by the bite of an infected flea and is carried to the regional lymph nodes by macrophages. Following replication in the lymph nodes, the bacteria spread throughout the body by the lymph and bloodstream and cause bubonic plague. During the later stages of the disease, the patient usually develops septicemia, disseminated intravascular coagulation, shock, and peripheral gangrene (reviewed in reference 1).

A large number of diverse bacterial species, including pathogens, use sodium as a chemiosmotic coupling ion in addition to, or even instead of, protons (2–5). The sodium motive force (SMF) may be generated either by primary sodium pumps, such as Na⁺-translocating NADH:ubiquinone oxidoreductases (NQR), or by secondary sodium pumps, such as Na⁺/H⁺ antiporters, that are energized by the proton motive force (PMF). Although many pathogenic organisms utilize different elements of the sodium cycle extensively in their physiology, the role of sodium bioenergetics in bacterial virulence in general is not well understood. Since the host presents a potentially unique, and not necessarily energetically favorable, environment for the growth of pathogenic microbes, the capacity to use sodium as a coupling ion can be expected to be of key importance during pathogenesis in a variety of bacterial species (discussed in detail in reference 5).

The reported genome sequence of *Y. pestis* predicts the presence of the primary sodium pump NQR, in addition to four secondary sodium pumps, the Na⁺/H⁺ antiporters NhaA, NhaB, NhaC, and NhaP, which are expected to contribute to SMF generation across the inner membrane of *Y. pestis* (Fig. 1) (4). However, recent detailed biochemical characterization of NhaP-type antiporters NhaP-1 and NhaP-2 from *Vibrio cholerae* revealed that these antiporters mediate K⁺/H⁺ exchange rather than Na⁺/H⁺ exchange under physiological conditions (6, 7). By analogy, we predict that the *Y. pestis* NhaP protein has a similar cation prefer-

ence (Fig. 1). Moreover, NhaC-type antiporters are poorly characterized; *Y. pestis* NhaC (Yp-NhaC) remains a hypothetical protein, and it is not clear at this moment whether it is chemiosmotically active at all. In contrast, one could expect the NhaA-NhaB pair to be the major secondary Na⁺-extruding system of *Y. pestis*, as has been found in many bacterial species possessing the *nhaA* and *nhaB* genes in their genomes (8–10). A comparison of the published genomes of various *Y. pestis* strains (obtained from the databases of the J. Craig Venter Institute [<http://www.jcvi.org/>] and the DOE's Joint Genome Institute [<http://img.jgi.doe.gov/>]) revealed the presence of intact genes for NhaA, NhaC, and NhaP antiporters in all of the strains. Interestingly, the *nhaB* gene of the *Y. pestis* strain KIM contains a frameshift, whereas this gene is intact in all other *Y. pestis* strains; thus, the designations of all *Y. pestis* KIM strains will be followed by “*nhaB*” in parentheses to indicate the presence of this mutation. Furthermore, genes encoding at least 13 putative Na⁺-dependent symporters with various substrate specificities and two gene orthologs of the Na⁺-dependent multidrug efflux pump NorM are predicted to be the consumers of the SMF in the *Y. pestis* genome (Fig. 1) (4).

Importantly, sodium pumps are not only essential for maintaining the SMF as an energy source for Na⁺ symporters; they also protect bacterial cells from Na⁺ toxicity. Irrespective of the pres-

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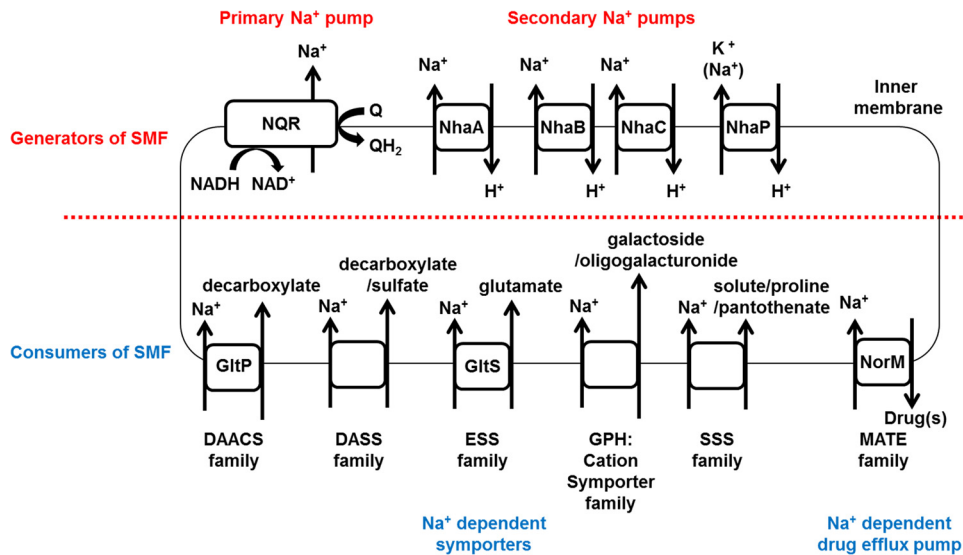


FIG 1 Sodium cycle in energetics in *Y. pestis*. Putative generators and consumers of the sodium motive force (SMF) in *Y. pestis* strain CO92 are shown. The primary Na^+ pump, NQR (YPO3235 to -3240), generates SMF by direct sodium extrusion. The sodium extrusion activity is coupled to NADH oxidation and ubiquinone (Q) reduction. The Na^+/H^+ antiporters NhaA (YPO0470), NhaB (YPO2142), and NhaC (YPO0624), and the predicted $\text{K}^+(\text{Na}^+)/\text{H}^+$ antiporter NhaP (YPO3630), can convert proton motive force into SMF and vice versa. DAACS, dicarboxylate/amino acid:cation (Na^+ or H^+) symporter (YPO05784 and YPO1716); DASS, divalent anion: Na^+ symporter (YPO0759 and YPO2561); ESS, glutamate: Na^+ symporter (YPO0035); GPH, glycoside-pentoside-hexuronide:cation symporter (YPO0995 and YPO1226); SSS, solute:sodium symporter (YPO0251, YPO1853, YPO3002, and YPO3657); MATE, multidrug and toxic compound extrusion (YPO1571 and YPO2392).

ence of functional NQR, Na^+/H^+ antiporters have been shown to play a leading role in the Na^+ resistance of bacteria (9, 10). Among the Na^+/H^+ antiporters examined, antiporters of the NhaA type are usually extremely kinetically competent, fast, and pH dependent, displaying maximal activity at alkaline pHs, and are indispensable for survival in sodium-rich environments, especially alkaline environments (8–10). Like that of *Escherichia coli* NhaA, the activity of *Y. pestis* NhaA (Yp-NhaA) is regulated by pH and selectively exchanges Na^+ and Li^+ for H^+ (11; J. Winogradzki and P. Dibrov, unpublished data). NhaB-type antiporters are much less active, “housekeeping” cation exchangers that normally play an auxiliary role. Like NhaA, they exchange sodium ions for protons in an electrogenic manner (3 H^+ per 2 Na^+), and, unlike NhaA, they are expressed constitutively (9, 10, 12). Although the activity of *E. coli* NhaB does not show pH sensitivity (13), *Vibrio alginolyticus* NhaB shows pH-dependent activity, reaching its maximum at alkaline pHs (14). To date, no biochemical analyses of the Yp-NhaB protein have been reported.

Blood-borne bacterial pathogens can survive in blood, and thus, they can cause disease. To survive in blood, bacterial pathogens must be able to resist the multiple innate defense systems present in blood. In *Y. pestis*, a type III secretion system (T3SS) and secreted Yop effector proteins, encoded on a *Yersinia* virulence plasmid, are known to prevent phagocytosis and other protective innate immune responses. An outer membrane protein, Ail, is also known to protect *Y. pestis* cells against complement-mediated lysis (15, 16). In addition to the known essential factors for survival in blood, *Y. pestis* has to be able to tolerate the salt concentrations in blood in order to establish an infection. Thus, sodium pumps may play a role in its pathogenesis. To date, however, there is no report investigating the roles of the various *Y. pestis* sodium pumps in the context of its pathogenic potential.

In this study, we investigated the roles of the primary sodium

pump, NQR, and the two main secondary sodium pumps, the Na^+/H^+ antiporters NhaA and NhaB, in *Y. pestis* virulence. It was found that the loss of both NhaA and NhaB, but not NQR, abolishes *Y. pestis* virulence, presumably because these antiporters are essential for protecting *Y. pestis* from Na^+ toxicity in blood.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains were kept at -80°C in 20% glycerol stocks. *Y. pestis* strains were grown in LB media at 30°C unless otherwise noted. Media were supplemented with antibiotics as appropriate, as follows: streptomycin (Str), 100 $\mu\text{g}/\text{ml}$; ampicillin (Amp), 100 $\mu\text{g}/\text{ml}$; kanamycin (Km), 50 $\mu\text{g}/\text{ml}$.

Construction of *Y. pestis* strains carrying deletions in *nqrABCDEF*, *nhaA*, and *nhaB*. To construct the *nqrABCDEF* and *nhaA* deletion mutant strains of *Y. pestis* KIM5-3001 (*nhaB*), PCR of a kanamycin resistance (Km^r) cassette flanked by the FLP recombination target (FRT) sites and homologous regions of the target gene was performed essentially as described previously (17) by using primer pairs listed in Table 2 ($\Delta\text{nhaA-KIM-P1}$ and $\Delta\text{nhaA-KIM-P2}$, $\Delta\text{nqr-P1}$ and $\Delta\text{nqr-P2}$). The ΔnhaA mutant was also constructed in *Y. pestis* KIM5, using different sets of primers ($\Delta\text{nhaA-KIM-P1B}$ and $\Delta\text{nhaA-KIM-P2B}$), also listed in Table 2. *Y. pestis* strains containing plasmid pKD46 were grown first in heart infusion broth (HIB) at 28°C to an optical density at 620 nm (OD_{620}) of 0.5 and then for 2 h with 0.2% L-arabinose. Electrocompetent cells were prepared as described previously (18) and were electroporated with 2 μl of the purified PCR product. Gene replacements were confirmed by PCR analysis. The Km^r cassettes were removed using the temperature-sensitive plasmid pCP20, which encodes the FLP recombinase. After FLP/FRT-mediated site-specific recombination, Km -sensitive colonies containing a single FRT site were identified by replicate plating and PCR analysis. Temperature-sensitive plasmids pKD46 and pCP20 were cured from the *nqrABCDEF* and *nhaA* deletion mutants by overnight growth at 38°C . The ΔnhaA and $\Delta\text{nhaA} \Delta\text{nhaB}$ mutant strains of *Y. pestis* CO92 were constructed by similar lambda Red recombinase mutagenesis strategies (19),

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
Strains		
<i>Y. pestis</i>		
KIM5-3001	NhaB ⁻ pCD1 ⁺ pPCP1 ⁺ pMT1 ⁺ Pgm ⁻ Str ^r	11, 24
KIM5-3001 Δnqr	KIM5-3001 $\Delta nqrABCDEF$	This study
KIM5-3001 $\Delta nhaA$	KIM5-3001 $\Delta nhaA$	This study
KIM5	NhaB ⁻ pCD1 ⁺ pPCP1 ⁺ pMT1 ⁺ Pgm ⁻	G. Plano lab collection
KIM5 $\Delta nhaA$	KIM5 $\Delta nhaA$	This study
CO92	NhaB ⁺ pCD1 ⁺ pPCP1 ⁺ pMT1 ⁺ Pgm ⁺	36
CO92 $\Delta nhaA$	CO92 $\Delta nhaA$; Km ^r	This study
CO92 $\Delta nhaA \Delta nhaB$	CO92 $\Delta nhaA \Delta nhaB$; Km ^r Amp ^r	This study
<i>E. coli</i>		
Top10	Cloning host strain	Invitrogen
EP432	<i>melBLid</i> $\Delta nhaA1::kan$ $\Delta nhaB1::cat$ $\Delta lacZY thr1$	12
Plasmids		
pCR2.1-TOPO	Cloning vector	Invitrogen
pKD4	Km ^r ; template for PCR amplification for lambda Red recombinase-mediated recombination	17
pCP20	Amp ^r Cm ^r ; temperature-sensitive replicon; FLP recombinase	17
pWKS130	pSC101-based low-copy-number vector; Km ^r <i>lacZ'</i>	37
pWKS130-nhaA	<i>nhaA</i> ORF and promoter region cloned into pWKS130	This study
pBADTOPO	Cloning vector with pBAD promoter	Invitrogen
pBAD-nhaA	<i>nhaA</i> ORF cloned into pBADTOPO under the control of the pBAD promoter	This study
pET6×HN-C	Expression plasmid with T7 <i>lac</i> promoter	Clontech Laboratories
pET-nhaB	<i>nhaB</i> ORF cloned into pET6×HN-C under the control of the T7 <i>lac</i> promoter	This study

in which 974 bp of *nhaA* was replaced with a Km^r cassette and 1,453 bp of *nhaB* was replaced with an Amp^r cassette. The primers used for these constructs ($\Delta nhaA$ -CO-P1 and $\Delta nhaA$ -CO-P2, $\Delta nhaB$ -CO-P1 and $\Delta nhaB$ -CO-P2) are also listed in Table 2.

Cloning of the *nhaA* and *nhaB* genes. For the complementation analyses, we cloned the *nhaA* gene as follows. The DNA fragment, which contained the *nhaA* promoter region and open reading frame (ORF), was amplified by PCR using chromosomal DNA of *Y. pestis* KIM5-3001 (*nhaB*) as a template. The primers used for this construct, *nhaA*-pWKS-P1 and *nhaA*-pWKS-P2, included SacI and BamHI restriction sites, respectively, and are listed in Table 2. The PCR products obtained were digested with SacI and BamHI, gel purified, and then ligated into the

SacI and BamHI sites of vector pWKS130. pWKS130 has a pSC101 origin of replication and is stably maintained along with the native plasmids. The *nhaB* expression plasmid was constructed as follows. The *nhaB* ORF was amplified by using primers *nhaB*-pBAD-P1 and *nhaB*-pBAD-P2 (Table 2), with chromosomal DNA of *Y. pestis* KIM5-3001 (*nhaB*) as a template, and was cloned into pBADTOPO. Because *nhaB* from KIM5-3001 (*nhaB*) contained a frameshift mutation, we performed site-directed mutagenesis to restore the function of the gene by using primers *nhaB*-pBAD-SDM-P1 and *nhaB*-pBAD-SDM-P2 (Table 2), a high-fidelity DNA polymerase, KOD -Plus-, version 2 (Toyobo), and DpnI (Invitrogen). The site-directed mutagenesis was confirmed by sequencing. The restored *nhaB* gene was amplified by PCR using primers *nhaB*-pET-P1 and *nhaB*-pET-P2

TABLE 2 Primers used in this study

Primer	Sequence (5' to 3') ^a
$\Delta nhaA$ -KIM-P1	GATATACCAGTCTCGATAAAAAATAGCTTCGCTGGATATCTGTGTAGGCTGGAGCTGCTTC
$\Delta nhaA$ -KIM-P2	CAGTAAAGCAATAGCAGCCGACGAATACCCAAAGATTGCATATGAATATCCTCCTTAGT
$\Delta nhaA$ -KIM-P1B	GGGCTGGAAGTTAAACGTGAGTTGATGGAAGGCTCTTTGTGTGTAGGCTGGAGCTGCTTC
$\Delta nhaA$ -KIM-P2B	CCTAGCAATATCCCCTAGTTTGGAAATAGGTCGTACAGAGCGCATATGAATATCCTCCTTAGT
Δnqr -P1	AAAGGGCTAGATCTTCCCATTGCTGGAGCCCCGTTTCAGTGTGTAGGCTGGAGCTGCTTC
Δnqr -P2	ATAAAATTCACAATCCTCTGGAGCCGGGTGATCTTTCAGCATATGAATATCCTCCTTAGT
$\Delta nhaA$ -CO-P1	GCTGCCGCTATCGCCTTGTGTATGGCGAACAGCGCCCTACAAGGGTTTATCGTTGTGGGAAGATGCGTG
$\Delta nhaA$ -CO-P2	CAGTAAGCTGTAACCCACAACAGCCGCTGTGGTAGAGCCTAGCAATATCCCCGGCTCGTATGTTGTGTGG
$\Delta nhaB$ -CO-P1	GGACATTACCAACAGACAAGCTGTACTCAAGAATTTTCTTGGTAACTCTCCGATCTTTTCTACGGGGTCTGACG
$\Delta nhaB$ -CO-P2	CCGTTTGTGATGTGTGGCAACTGATCCATCCAGCCTGAGTCAGCCACTCCTTTTCGGGGAAATGTGCGG
<i>nhaA</i> -pWKS-P1	GGGGGAGCTCATGCTGGTTAATTAGCAGCTAGCC
<i>nhaA</i> -pWKS-P2	GGGGGGGATCCTTACCTTACGTTAACAGCCTTCCGCCG
<i>nhaB</i> -pBAD-P1	GAGGAATAATAAATGGACATTACCAACAGACA
<i>nhaB</i> -pBAD-P2	TCAAGTGTGGGATGGCAACCCCG
<i>nhaB</i> -pBAD-SDM-P1	GTCTCGTTACAACAATTTGAATTTGTTGGCGGTCGCC
<i>nhaB</i> -pBAD-SDM-P2	GGCGACCGCCAACAATTCAAATTTGTTGAACGAGAC
<i>nhaB</i> -pET-P1	GACATTACCAACAGACAAGCTGTA
<i>nhaB</i> -pET-P2	GAAGAGAATTCGTGTGGGATGGCAACCCCGTTTGT

^a Restriction sites are underlined.

(Table 2). Primer nhaB-pET-P2 included an EcoRI restriction site. The PCR products obtained were digested with EcoRI, gel purified, and ligated into the StuI and EcoRI sites of the pET6×HN-C plasmid, which is located under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible T7 *lac* promoter.

Mouse models of septicemic and bubonic plague. *Y. pestis* strains were inoculated from frozen glycerol stock to 5 ml brain heart infusion (BHI) and were grown overnight at 28°C without aeration. Overnight cultures were diluted 1:100 into 10 ml Luria-Bertani (LB) medium and were incubated again overnight (16 to 18 h) without aeration, at 37°C. The overnight LB cultures had a density of about 1×10^8 CFU/ml as determined by a Petroff-Hausser chamber count and were diluted in phosphate-buffered saline (PBS). The LB cultures of the mutant and parent strains achieved the same density. Eight- to 12-week-old female Rocky Mountain Laboratory (RML) Swiss Webster mice were used. Groups of 5 to 10 mice were injected intravenously in the tail vein (septicemic-plague model) with 100 μl of PBS containing 1,000 bacteria (KIM5 strains) or intradermally in the right lower lumbar region (bubonic-plague model) with 25 μl containing 10 or 100 bacteria (CO92 strains). The number of bacteria injected was confirmed by a CFU count of the inoculum preparations. Infected animals were observed three times daily and were euthanized upon signs of terminal plague (evidence of lethargy, hunched posture, and reluctance to respond to external stimuli). Heart blood, spleens, or both were collected immediately after euthanasia. Dilutions of blood and triturated spleen were plated onto blood agar containing 1 μg/ml Irgasan to verify plague and to determine the bacterial loads in these tissues. These studies were conducted at biosafety level 3 and were approved by the Rocky Mountain Laboratory Animal Care and Use Committee in accordance with NIH NIAID guidelines.

Isolation of membrane vesicles. For the functional expression of Yp-NhaA, EP432, a Na⁺/H⁺ antiporter-deficient strain of *E. coli* kindly provided by E. Padan (Hebrew University of Jerusalem, Jerusalem, Israel), was used. EP432 is a K-12 derivative (*melBLid ΔnhaA1::kan ΔnhaB1::cat ΔlacZY thr1*) (12). EP432/pBAD-YpNhaA transformants were grown in LBK medium (modified LB medium in which NaCl was replaced with KCl) supplemented with 100 μg/ml ampicillin, 30 μg/ml kanamycin, 34 μg/ml chloramphenicol (Cm), 25 mM LiCl, and 0.01% arabinose. Cells were harvested at an OD₆₀₀ of 1.5 to 1.8 and were immediately used for the isolation of inside-out membrane vesicles as described previously (20). Briefly, overnight cultures of EP432 transformants were grown in LBK medium containing the antibiotics listed above. These cultures were then used to inoculate the growth medium at a concentration of 1:100. After harvesting, the cells were washed three times in a buffer containing 200 mM sorbitol, 10% (wt/vol) glycerol, and 20 mM Tris-H₂SO₄ (pH 7.5). After the last wash, the bacterial pellet was resuspended in the same buffer containing 1 mM 1,4-dithiothreitol (DTT), 1 μg/ml pepstatin-A, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and approximately 5 mg/liter DNase. The bacterial suspension was then passed twice through a French press (Aminco), and the unbroken cells were pelleted. The membrane fraction was collected by ultracentrifugation (Optima LE-80K ultracentrifuge), resuspended, stored in the same buffer containing all the additions except DNase, and assayed for cation/proton antiport activity as described previously (20–23).

Measurement of transmembrane ΔpH. For measurements of the transmembrane pH gradient (ΔpH), aliquots of vesicles (200 μg of protein) were added to 2 ml of a buffer containing 200 mM sorbitol, 25 mM KCl, 5 mM MgCl₂, 10% (wt/vol) glycerol, 15 μM acridine orange, and 50 mM bis-tris propane (BTP)-HCl adjusted to pH 8.0. The Na⁺(Li⁺)/H⁺ antiport activity was then registered using the acridine orange fluorescence-quenching/dequenching assay. Respiration-dependent generation of ΔpH was initiated by the addition of 10 mM Tris-D-lactate, and the resulting quenching of acridine orange fluorescence was monitored in a Shimadzu RF-1501 spectrofluorophotometer (excitation at 492 nm; emission at 528 nm). Antiport activity was estimated based on the ability to dissipate the established ΔpH in response to the addition

of NaCl or LiCl at the indicated concentrations (see Fig. 7 and 8). A 10 mM concentration of each was used in the determination of the pH profile of activity, and 0.1 mM to 20 mM was used in the determination of apparent *K_m*. The antiport activities are expressed as percentages of restoration of lactate-induced fluorescence quenching. All assays were performed at room temperature.

Blood and blood serum survival assay. Defibrinated sheep blood and sheep serum (Colorado Serum Company) were used for the animal blood and serum survival assays. Human blood was purchased from the Biological Specialty Corporation. Human serum was purchased from Lonza. Bacterial strains were grown overnight in LB medium at 30°C, washed with PBS, and suspended in PBS. Approximately 10⁵ or 10⁶ CFU of the diluted bacteria was inoculated into 4 ml of the blood or blood serum and was shaken for 18 h at 30°C. CFU counts were determined before and after shaking in defibrinated sheep blood or sheep blood serum by plating on LB agar and incubating plates for 2 days.

Growth assay. The artificial serum medium Opti-MEM I Reduced Serum Medium (referred to below as Opti-MEM) (Invitrogen) was used for *in vitro* growth assays. LB medium (Difco) buffered with BTP (Sigma) at pH 6.5 or pH 7.4 (LBB) was also used for growth assays. The pH of LB medium was adjusted by the addition of HCl or NaOH. Bacterial strains were grown overnight in LB medium at 30°C, washed with PBS, and suspended in PBS to obtain an OD₆₀₀ of 1.0. Portions (1 μl and 10 μl) of the diluted bacteria were inoculated into 5 ml of Opti-MEM or LB medium and were shaken at 30°C for 24 h. Bacterial growth was evaluated by measuring the OD₆₀₀.

RESULTS

Na⁺/H⁺ antiporters are essential for *Y. pestis* virulence. To investigate the roles of sodium pumps in the virulence of *Y. pestis*, we constructed mutant strains with deletions of the primary sodium pump, NQR, or the predicted main secondary sodium pump, the Na⁺/H⁺ antiporter NhaA from *Y. pestis* KIM5-3001 (*nhaB*) (11, 24). We tested the virulence of these mutant strains by using an established mouse model of septicemic plague (25). While the KIM5-3001 (*nhaB*) Δ*nqr* strain showed a level of virulence similar to that of the parent strain (Fig. 2A), the KIM5-3001 Δ*nhaA* (*nhaB*) strain was highly attenuated when tested in this model (data not shown). To further confirm these results, a new *nhaA* deletion mutant strain was constructed in *Y. pestis* KIM5. Again, high attenuation was observed for this *Y. pestis* KIM5 Δ*nhaA* (*nhaB*) strain in mice. These attenuated phenotypes were partially reversed by providing the *nhaA* gene carrying its native promoter on a low-copy-number plasmid, pWKS130, to the KIM5-3001 Δ*nhaA* (*nhaB*) (data not shown) and KIM5 Δ*nhaA* (*nhaB*) (Fig. 2B) strains.

Because the *nhaB* gene of *Y. pestis* KIM strains contains a frameshift, our *nhaA* deletion variants in the KIM5-3001 (*nhaB*) and KIM5 backgrounds lack both functional NhaA and functional NhaB. Thus, we next asked whether both NhaA and NhaB are essential for *Y. pestis* virulence. To test this, we used a fully virulent *Y. pestis* strain, CO92 that has three intact Na⁺/H⁺ antiporters, *nhaA*, *nhaB*, and *nhaC* (Fig. 1). We constructed single (*nhaA*) and double (*nhaA nhaB*) mutant derivatives of *Y. pestis* CO92 and tested their virulence in an intradermal mouse model of bubonic plague. Unlike the KIM5-3001 Δ*nhaA* (*nhaB*) or KIM5 Δ*nhaA* (*nhaB*) strain, the *Y. pestis* CO92 Δ*nhaA* strain showed full virulence, similar to that of the parent strain (Fig. 2C). Since the *Y. pestis* CO92 Δ*nhaA* Δ*nhaB* double mutant was fully attenuated, like the KIM5-3001 Δ*nhaA* (*nhaB*) or KIM5 Δ*nhaA* (*nhaB*) strain (Fig. 2D), these data suggested that only the loss of both NhaA and NhaB results in an attenuated phenotype.

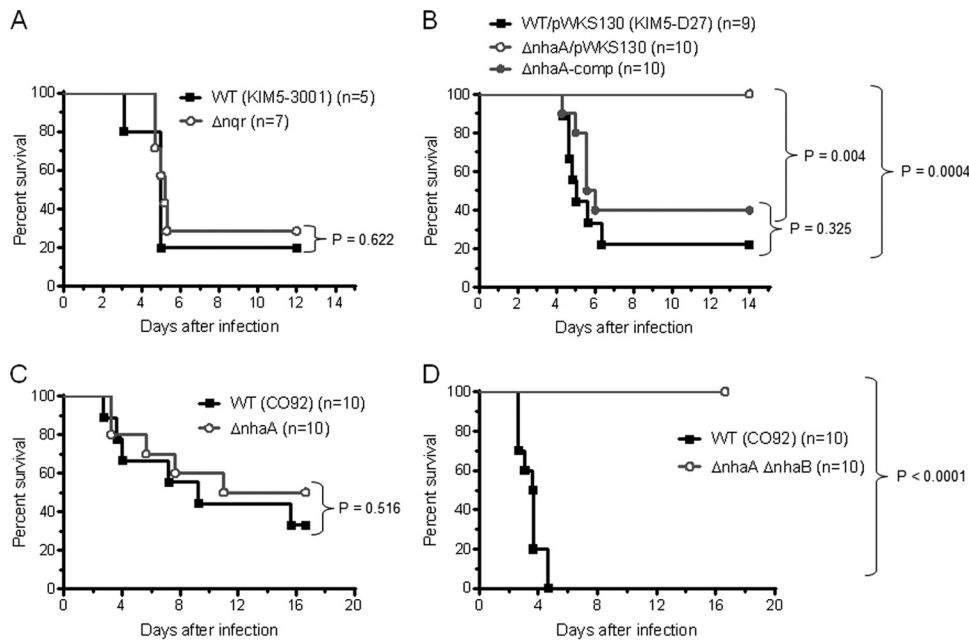


FIG 2 Effects of *Y. pestis* sodium pump mutations on *Y. pestis* virulence. (A and B) The parent *Y. pestis* strain KIM5-3001 [WT (KIM5-3001)] and its $\Delta nqrABCDEF$ derivative (A) or the parent strain *Y. pestis* KIM5 harboring pWKS130 (WT/pWKS130), a $\Delta nhaA$ derivative harboring pWKS130, and the $\Delta nhaA$ strain carrying plasmid pWKS130 containing the intact *nhaA* gene and its native promoter ($\Delta nhaA$ -comp) (B) were assessed by intravenous injection of groups of mice with 1,000 CFU. (C and D) The parent *Y. pestis* strain CO92 [WT (CO92)] and its *nhaA* (C) or *nhaA nhaB* (D) deletion derivative were assessed by intradermal injection of groups of 8-week-old female RML mice. A total of 10 CFU (C) or 100 CFU (D) was injected. *P* values were calculated by the log rank test.

Na⁺/H⁺ antiporters are essential for *Y. pestis* survival in sheep blood and sheep serum. Since survival in blood is key to *Y. pestis* virulence, we tested the survival of the *nhaA* (*nhaB*) mutant, the *nqr* mutant, and the parent strain in animal blood. For this purpose, we grew the *Y. pestis* KIM5-3001 (*nhaB*), KIM5-3001 Δnqr (*nhaB*), and KIM5-3001 $\Delta nhaA$ (*nhaB*) strains in defibrinated sheep blood and monitored bacterial survival by measuring the CFU after 18 h. Whereas the KIM5-3001 (*nhaB*) parental strain and the KIM5-3001 Δnqr strain showed significant increases in the CFU after 18 h in sheep blood, the KIM5-3001 $\Delta nhaA$ (*nhaB*) strain showed a significant decrease in the CFU after 18 h in sheep blood (Fig. 3A). Like the *Y. pestis* KIM5-3001 (*nhaB*) derivative, the KIM5 $\Delta nhaA$ (*nhaB*) strain also showed decreased survival in sheep blood (Fig. 3B). Provision of the *nhaA* gene in *trans* allowed the KIM5 $\Delta nhaA$ (*nhaB*) strain to grow in blood similarly to the parent strain (Fig. 3B), confirming that the *nhaA* gene is required for the survival of *Y. pestis* KIM (*nhaB*) strains in blood. However, our *in vivo* data suggested that either NhaA or NhaB is essential for *Y. pestis* virulence (Fig. 2C and D). To confirm that either *nhaA* or *nhaB* is required for *Y. pestis* survival in blood, we cloned the *nhaB* gene into a pET expression vector under the control of its IPTG-inducible hybrid T7 *lac* promoter. The resultant plasmid was transformed into the KIM5 $\Delta nhaA$ (*nhaB*) mutant, and the resultant strain was tested for survival in sheep blood. Expression of the *nhaB* gene by adding IPTG restored the survival of the KIM5 $\Delta nhaA$ (*nhaB*) strain in sheep blood to parental-strain levels (Fig. 3C), suggesting that only the loss of both antiporters produces a survival defect. The CFU assays for KIM5 $\Delta nhaA$ (*nhaB*) survival in sheep blood were also performed by plating on brain heart infusion agar plates, an optimal growth medium for *Y. pestis*, instead of LB agar plates;

however, similar defects in recovered CFU were observed on the two types of plates.

We next tested the survival of the $\Delta nhaA$ (*nhaB*) mutant, the Δnqr mutant, and the parent strain in animal serum. As in the blood survival assay, the *Y. pestis* KIM5-3001 $\Delta nhaA$ (*nhaB*) strain showed significantly decreased CFU, whereas the KIM5-3001 (*nhaB*) parent strain and the KIM5-3001 Δnqr (*nhaB*) strain showed significantly increased CFU, after 18 h in sheep serum (Fig. 4). The *Y. pestis* KIM5 $\Delta nhaA$ (*nhaB*) strain also showed decreased survival in sheep serum, and providing the *nhaA* gene in *trans* completely restored the ability of the KIM5 $\Delta nhaA$ (*nhaB*) strain to survive in sheep serum (data not shown). These data indicate that the combined loss of two Na⁺/H⁺ antiporters, NhaA and NhaB, strongly inhibited *Y. pestis* growth in blood, suggesting that reduced survival in blood might be responsible for the attenuated *in vivo* phenotype of the *nhaA nhaB* mutants. Since a similar survival defect phenotype of the *nhaA nhaB* double mutant was also observed in blood serum, the immune cells in blood are clearly not responsible for the survival defect of the *nhaA* mutant in blood. It should be noted that the *Y. pestis* double mutant did not show a survival defect phenotype in blood and serum when we used a higher inoculation dose (>10⁶ CFU) (data not shown).

***Y. pestis* Na⁺/H⁺ antiporter mutants show a growth defect in an artificial serum medium.** Since serum has complement-mediated lysis and antimicrobial peptide-mediated killing activities, we next tested whether such antimicrobial activities in serum are responsible for the decreased survival rates of the *Y. pestis* antiporter mutants in blood and serum. For this purpose, we tested the survival of the *Y. pestis* KIM5-3001 $\Delta nhaA$ (*nhaB*) mutant in heat-inactivated sheep serum. The heat-inactivated serum should not contain active complement but might retain heat-stable antimi-

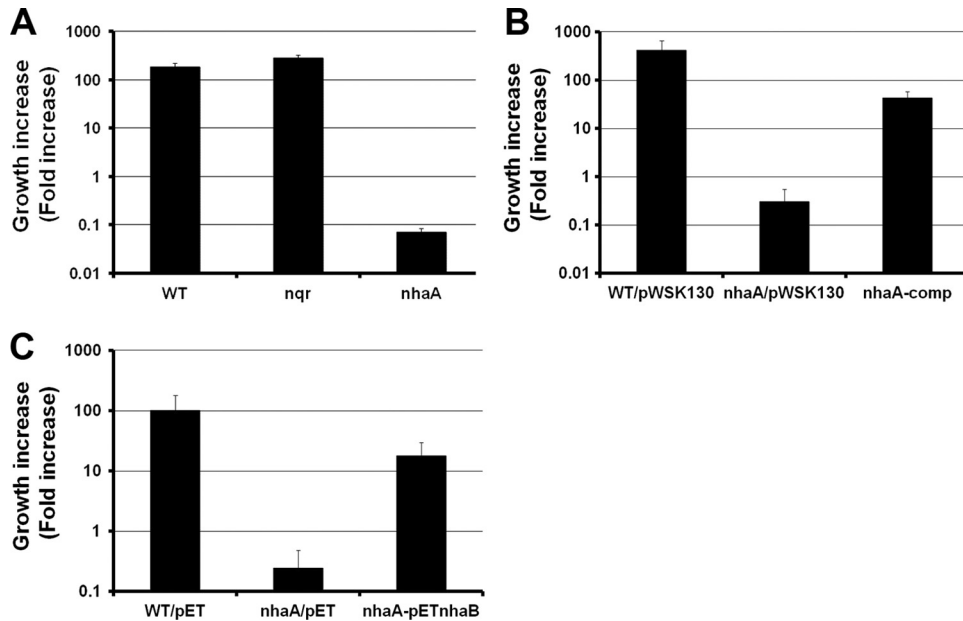


FIG 3 Survival of the *Y. pestis* sodium pump mutants and parent strains in defibrinated sheep blood. Approximately 10^5 CFU of bacteria was inoculated into 4 ml of defibrinated sheep blood, and the mixture was shaken at 30°C for 18 h. The numbers of surviving bacteria (CFU) in each sample were determined by plating on LB agar plates and incubating the plates at 30°C for 2 days. The growth increase is the ratio of inoculated bacteria to bacteria recovered after the 18-h shaking. (A) The parent strain *Y. pestis* KIM5-3001 (WT) and its *nqrABCDE* and *nhaA* deletion derivatives were tested. (B) The parent strain *Y. pestis* KIM5 carrying an empty plasmid (WT/pWSK130), its *nhaA* deletion derivative carrying an empty plasmid (*nhaA*/pWSK130), and the *nhaA* deletion strain carrying plasmid pWSK130 containing the intact *nhaA* gene (*nhaA*-comp) were tested. (C) The parent strain *Y. pestis* KIM5 (WT/pET), its *nhaA* deletion derivative (*nhaA*/pET), and the *nhaA* deletion strain carrying plasmid pET containing the intact *nhaB* gene under the control of the T7 *lac* promoter (*nhaA*/pET-*nhaB*) were tested. IPTG was added at a final concentration of 1 mM. The error bars indicate standard deviations.

crobal peptides. *Y. pestis* KIM5-3001 (*nhaB*) showed increased CFU counts after 18 h in heat-inactivated sheep serum, whereas the KIM5-3001 Δ *nhaA* (*nhaB*) strain showed decreased CFU counts in heat-inactivated sheep serum (data not shown). This suggested that the complement system present in serum is not responsible for the decreased CFU count of the antiporter mutant in serum. To eliminate any role of heat-resistant antimicrobial peptides, we next investigated the growth of our *Y. pestis* strains in the artificial serum medium Opti-MEM I Reduced Serum Medium (Opti-MEM). Strain *Y. pestis* KIM5 (*nhaB*) grew relatively

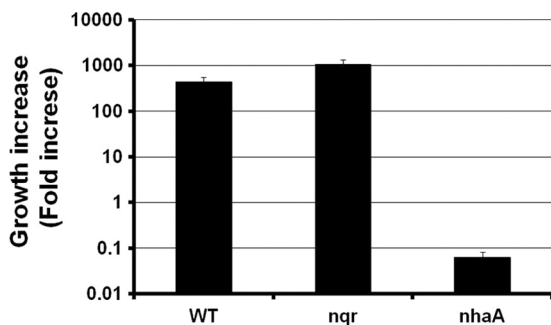


FIG 4 Survival of *Y. pestis* sodium pump mutants and parent strains in sheep serum. Approximately 10^5 CFU of the parent strain *Y. pestis* KIM5-3001 (WT) or its *nqr* or *nhaA* deletion derivative was inoculated into 4 ml of sheep serum, and the mixture was shaken at 30°C for 18 h. The number of surviving bacteria (CFU) in each sample was determined by plating onto LB agar plates and incubating the plates at 30°C for 2 days. The growth increase is the ratio of inoculated bacteria to bacteria recovered after the 18-h shaking. The error bars indicate standard deviations.

well in Opti-MEM, whereas the KIM5 Δ *nhaA* (*nhaB*) strain showed a severe growth defect in Opti-MEM at 24 h after inoculation (Fig. 5A). However, at 48 h after inoculation, the KIM5 Δ *nhaA* (*nhaB*) strain showed a growth yield similar to that of the parent strain (data not shown). This growth phenotype of the KIM5 Δ *nhaA* (*nhaB*) strain in Opti-MEM could be complemented by expressing either *nhaA* or *nhaB* in *trans* (Fig. 5A and B). Since Opti-MEM contains no biological factors (e.g., complement or antimicrobial peptides) to kill bacterial cells, these data indicated that the various antimicrobial activities found in serum are not responsible for the growth defect of the *Y. pestis* antiporter mutant in blood and serum.

Na^+ concentrations in blood, serum, and Opti-MEM are maintained at relatively high levels (135 to 145 mM). Since our data suggested that the growth defect of the *Y. pestis* antiporter mutant is not due to antimicrobial factors found in blood and serum, we hypothesized that Na^+/H^+ antiporters may be essential for bacterial survival in such relatively high salt concentrations. Consistent with this idea, the *Y. pestis* KIM5 Δ *nhaA* (*nhaB*) strain showed slower growth than the parent strain in LB medium containing 140 mM NaCl, buffered at pH 7.4 [LBB (pH 7.4)] (Fig. 6). Such growth retardation was specific to NaCl and was not observed in LBB (pH 7.4) containing 140 mM KCl (Fig. 6). Moreover, the KIM5 Δ *nhaA* (*nhaB*) strain showed no growth defect phenotype in LBB (pH 6.5) containing 140 mM NaCl (data not shown), indicating that such a growth defect phenotype is pH dependent. As in the blood and blood serum survival assays, we again noticed that the KIM5 Δ *nhaA* (*nhaB*) strain showed a growth defect phenotype in Opti-MEM and LBB (pH 7.4) con-

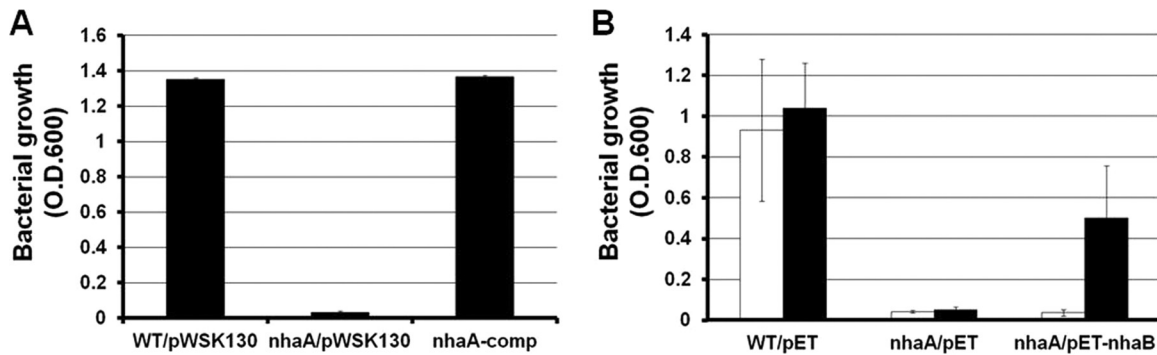


FIG 5 Growth of *Y. pestis* in Opti-MEM. Approximately 10^5 CFU of bacteria was inoculated into 5 ml of Opti-MEM, and the mixture was shaken at 30°C for 24 h. Bacterial growth was measured by the OD_{600} . (A) The parent strain *Y. pestis* KIM5 carrying an empty plasmid (WT/pWSK130), its *nhaA* deletion derivative strain carrying an empty plasmid (*nhaA*/pWSK130), and the *nhaA* strain carrying plasmid pWSK130 containing the intact *nhaA* gene (*nhaA*-comp) were tested. (B) The parent strain *Y. pestis* KIM5 carrying an empty plasmid (WT/pET), its *nhaA* deletion derivative strain carrying an empty plasmid (*nhaA*/pET), and the Δ *nhaA* strain carrying plasmid pET containing the intact *nhaB* gene under the control of the T7 *lac* promoter (*nhaA*/pET-*nhaB*) were tested. IPTG was added at a final concentration of 1 mM. The error bars indicate standard deviations.

taining 140 mM NaCl at 24 h, but not at 48 h, after inoculation (data not shown). Also as in the blood and blood serum survival assays, the KIM5 Δ *nhaA* (*nhaB*) strain showed no growth defect in Opti-MEM or LBB (pH 6.5) containing 140 mM NaCl when the inoculation size was greater than 10^6 CFU (data not shown).

Pharmacology of Na^+ (Li^+)/ H^+ antiporter catalyzed by *Y. pestis* NhaA. To probe the pharmacological profile of Yp-NhaA, Yp-NhaA activity was measured directly in everted membrane vesicles. For this purpose, we cloned the *Y. pestis* *nhaA* gene into the pBAD-TOPO vector and transformed the resultant pBAD-YpNhaA plasmid into the antiporter-deficient *E. coli* strain EP432 (12). In the presence of arabinose, Yp-NhaA restored the ability of strain EP432 to grow at high concentrations of LiCl and NaCl, verifying the functional expression of this antiporter in the heterologous host *E. coli* (data not shown). Further, we isolated sub-bacterial membrane vesicles from EP432/pBAD-YpNhaA cells and analyzed the pH profile and activity of Yp-NhaA, as well as its affinities for Na^+ and Li^+ , as in a previous study (11). The results obtained for LiCl⁺ and NaCl⁺ were essentially the same. In both cases, the antiporter was inactive at pHs below 6.5 and displayed manifold alkaline activation, reaching maxima for Na^+ / H^+ and Li^+ / H^+ exchange at pH 8.0. We also confirmed that Yp-NhaA has

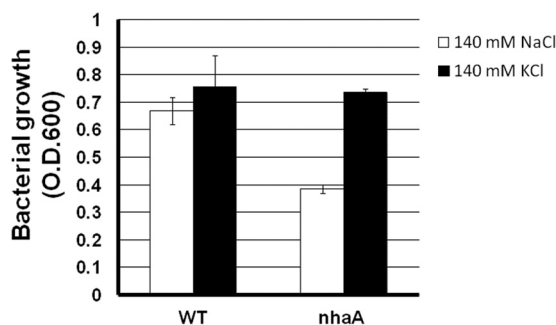


FIG 6 Growth of a *Y. pestis* mutant strain in LBB (pH 7.4) with either 140 mM NaCl or 140 mM KCl. Approximately 10^5 CFU of the parent strain *Y. pestis* KIM5 (WT) or its *nhaA* deletion derivative was inoculated into 5 ml of LBB (pH 7.4) containing 140 mM NaCl (open bars) or KCl (filled bars), and the mixture was shaken at 30°C for 24 h. Growth was measured by the OD_{600} . The error bars indicate standard deviations.

an almost 5-fold higher affinity for Li^+ than for Na^+ , as was found in reference 11, although our measurements yielded Na^+ and Li^+ concentrations required for half-maximal response of 3.5 mM (Fig. 7, top) and 0.7 mM (Fig. 7, bottom), respectively. These

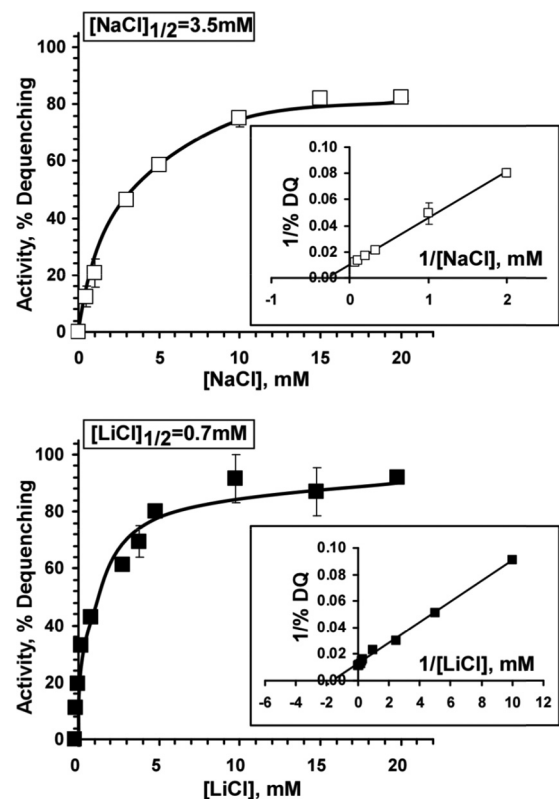


FIG 7 Determination of the concentration of Na^+ or Li^+ required for a half-maximal response of Yp-NhaA ($[NaCl^+]_{1/2}$ and $[LiCl^+]_{1/2}$) in inside-out membrane vesicles derived from EP432/pBAD-YpNhaA cells. Assays were performed in a buffer adjusted to pH 8.0 using 50 mM BTP- H_2SO_4 with varying concentrations of NaCl and LiCl as described in Materials and Methods. $[NaCl^+]_{1/2}$ and $[LiCl^+]_{1/2}$ of 3.5 mM and 0.7 mM, respectively, were determined. The average results for two separate isolations are plotted. Error bars indicate standard deviations. DQ, dequenching.

numbers are somewhat higher than those reported for Yp-NhaA by Arkin and collaborators (0.86 mM and 0.18 mM) (11). A probable reason for these differences is the use of different host strains for the isolation of vesicles and/or the different compositions of the experimental buffers used in the fluorescence assays.

Considering Yp-NhaA as a prospective target for antibacterial treatments, we checked its susceptibility to the known NhaA-type antiporter inhibitor 2-aminoperimidine (2-AP). It is well known that amiloride and its analogues are competitive inhibitors of mammalian Na^+/H^+ antiporters that belong to the Na^+/H^+ exchanger (NHE) family (26). Amiloride also inhibits the bacterial Na^+/H^+ antiporter NhaB, but not NhaA (27). In a previous study, it was found that 2-AP, a guanidine-containing derivative fused to a naphthalene moiety and a distant analogue of amiloride, acted as a specific inhibitor of NhaA-type antiporters from *E. coli* and *V. cholerae* (28). We therefore tested the effect of this compound on Yp-NhaA (Fig. 8). As expected, we found that Yp-NhaA was sensitive to 2-AP, with 50% inhibitory concentrations (IC_{50}) at pH 8.0 of 3.1 μM for 10 mM Na^+ and 3.2 μM for 2 mM Li^+ (Fig. 8). Given the differences in the apparent K_m s of Yp-NhaA for Li^+ and Na^+ , as well as the fact that 2-AP acted as a competitive inhibitor, it is not surprising that the Li^+/H^+ antiport measured (with 2 mM LiCl added as a substrate) was inhibited by the same 2-AP concentrations as those inhibiting Na^+/H^+ antiport measured with 10 mM NaCl (Fig. 8A, compare left and right sides).

DISCUSSION

We have proposed previously that the essential role of the sodium cycle in the energy metabolism of many pathogenic organisms should provide a distinct set of targets for the development of novel intervention strategies (4). Some bacteria, including *Y. pestis*, can use sodium as a coupling ion, utilizing a number of primary and secondary Na^+ pumps that are important for overall bacterial physiology. In this study, we start to investigate the roles of sodium pumps in *Y. pestis* virulence. We found that while the primary sodium pump, NQR, did not play a major role in virulence, the combined loss of two major secondary sodium pumps, NhaA and NhaB, resulted in a strongly attenuated phenotype when strains were tested *in vivo*. To our knowledge, this is the first report that establishes a role for Na^+/H^+ antiporters in bacterial virulence.

The data presented here show that a *Y. pestis nhaA nhaB* double mutant has a survival defect in sheep blood and serum. However, the *nhaA nhaB* double mutant survived and replicated in RAW 264.7 macrophages similarly to the parent strain (data not shown). Furthermore, we found that the double mutant still showed decreased survival in heat-inactivated serum. Taken together, these data suggest that the antibacterial factors present in blood and serum are not responsible for the decreased survival of the *Y. pestis nhaA nhaB* mutant in blood or serum. Indeed, the double mutant also showed growth retardation when grown in an artificial serum medium, Opti-MEM, further confirming this idea. Blood plasma normally contains 135 to 155 mM Na^+ ; similar levels of sodium in bacterial cytoplasm are considered lethal (29). Thus, these observations led us to consider the possibility that the Na^+/H^+ antiporters NhaA and NhaB are essential for the survival of *Y. pestis* in blood, since they protect the bacterial cells from Na^+ toxicity. Indeed, the double mutant strain showed a growth defect in high sodium but not high potassium concentrations.

In addition, we found, unexpectedly, that the $\Delta nhaA \Delta nhaB$

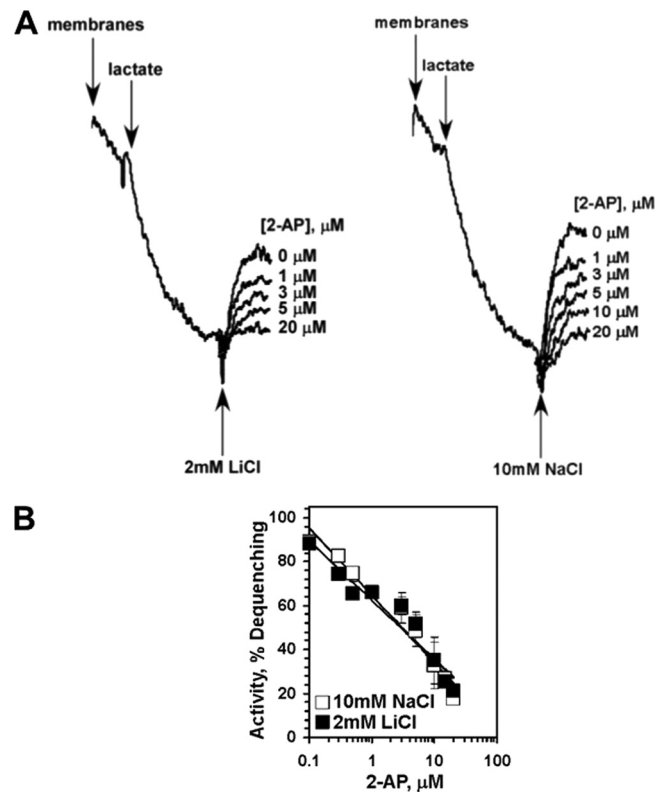


FIG 8 Effect of 2-AP on the antiport activity of Yp-NhaA in inside-out membrane vesicles isolated from EP432/pBAD-YpNhaA. Inside-out membrane vesicles were isolated from EP432/pBAD-YpNhaA cells as described in Materials and Methods. (A) Vesicles were assayed for ΔpH in a reaction mixture containing 200 μg of protein, 15 μM acridine orange, 200 mM sorbitol, 5 mM MgCl_2 , 50 mM BTP-HCl adjusted to pH 8.0, and 2-AP at the indicated concentrations. At the beginning of the experiment, vesicles were added to the reaction mixture (first arrows pointing down), followed by 10 mM Tris-D-lactate (second arrows pointing down), and acridine orange fluorescence quenching was recorded until a steady-state level of ΔpH was reached. LiCl (2 mM) (left) or NaCl (10 mM) (right) was then added (arrows pointing up), and acridine orange fluorescence quenching was recorded until a new steady state was obtained. All experiments were repeated four times, and the results were virtually the same. (B) For calculation of the IC_{50} of 2-AP, the activity of Yp-NhaA was plotted against the 2-AP concentration. One hundred percent dequenching corresponds to activity in the absence of 2-AP. The IC_{50} of 2-AP was 3.2 μM in the presence of 2 mM LiCl and 3.1 μM in the presence of 10 mM NaCl.

double mutant showed survival rates similar to those of the parent strain and the *nqr* mutant in human blood but was defective for growth in human serum (data not shown). Because human blood samples were treated by adding heparin, whereas the sheep blood samples were processed only to remove fibrin, with no additives or preservatives added, we hypothesized that heparin may affect bacterial survival in blood. Indeed, we observed that the $\Delta nhaA \Delta nhaB$ double mutant showed survival rates similar to those of the parent strain in sheep blood when sodium heparin was added to the blood (data not shown). However, our data indicated that the *Y. pestis* antiporters are not required for survival mechanisms related to immune factors, so likely heparin exerts its effect via an alternative mechanism, such as altering the sodium concentrations. It is noteworthy that heparin has the highest negative charge density of any known biological molecule (30), and clearly, future studies are required to explain this intriguing finding.

The lack of relevance of NQR to the survival of *Y. pestis* under these conditions might not be surprising, because this primary sodium pump creates a transmembrane electrical gradient ($\Delta\psi$) ("plus" outside) on the membrane as it expels Na^+ ions; therefore, its ability to build up a sizable chemical transmembrane gradient of Na^+ (which would lead to lower internal sodium levels) is very limited. On the other hand, the secondary sodium-proton exchangers, NhaA and NhaB, being electrogenic (they exchange 2 H^+ ions per Na^+ ion and 3 H^+ ions per 2 Na^+ ions, respectively), are able to consume $\Delta\psi$ for the very efficient removal of cytoplasmic Na^+ (see references 9 and 10 and references therein).

We observed that the mice that survived infection with either the KIM5 $\Delta nhaA$ (*nhaB*) or the CO92 $\Delta nhaA \Delta nhaB$ strain never showed any signs of illness, and no CFU were recovered from any of their spleen and blood tissues (data not shown). Mice with bubonic plague with lymph node infection but prior to disseminated sepsis typically show no obvious signs of disease, and illness is detected only after disseminated spread. The concentrations of sodium ions in the majority of mammalian extracellular fluids, including those in liver and spleen, are indeed rather high and comparable to that in blood plasma, i.e., ~100 to 140 mM (reference 31 and references therein). It is reasonable to expect, therefore, that Na^+/H^+ antiporter-deficient *Y. pestis* would be unable to grow there, as in blood. Thus, we conclude that the $\Delta nhaA$ (*nhaB*) mutant did not spread to the internal organs; it died out at the intradermal injection site or in the draining lymph node. We also did not notice any swelling or pathology in these lymph nodes.

We found that the *Y. pestis nhaA nhaB* double mutant strain recovered the ability to grow to the wild-type (WT) level in Opti-MEM and LBB (pH 7.4) containing 140 mM NaCl after a longer incubation (48 h) (data not shown). Similar growth recovery has been observed in an *E. coli nhaA nhaB* double mutant in LB medium containing 300 mM NaCl, where it was attributed to the expression of the "silent" primary Na^+ pump in the respiratory chain (32). However, no such recovery was observed for the *Y. pestis* mutant in sheep blood and *in vivo* experiments. Thus, such a difference in growth abilities might be caused simply by the fact that LB medium (and Opti-MEM) is a richer medium than serum/blood.

Of note, when the *Y. pestis* KIM5 and KIM5 $\Delta nhaA$ strains were evaluated in growth and secretion experiments using standard TMH medium (38) with or without calcium, both strains showed typical calcium-dependent growth and Yop secretion, which were indistinguishable between them, eliminating the possibility that the low-calcium-response-mediated Na^+ -sensitive phenotype (33–35) is responsible for the Na^+ sensitivity of the KIM5 $\Delta nhaA$ (*nhaB*) strains.

Competitive inhibitors of mammalian Na^+/H^+ antiporters belonging to the NHE family, including the diuretic drug amiloride and its numerous derivatives, have been studied extensively (26). However, until recently, there were no inhibitors specific for the antiporters of the NhaA type (28). The data presented in this paper show that 2-AP acts efficiently on Yp-NhaA, similarly to its inhibition of the NhaA-type enzymes in *V. cholerae* or *E. coli* (29). This offers the potential for 2-AP to become a platform for the design of medically relevant inhibitors of the bacterial Na^+/H^+ antiporters of the NhaA type. This could be particularly important for the development of novel antimicrobials targeting blood-borne bacterial infections.

In summary, the data presented in this communication dem-

onstrate the essential role of Na^+/H^+ antiporter in *Y. pestis* virulence, possibly by protecting against sodium toxicity in blood. Because Na^+/H^+ antiporters are ubiquitous proteins, these findings might be applicable to other blood-borne pathogens.

ACKNOWLEDGMENTS

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REFERENCES

- Perry RD, Fetherston JD. 2011. Yersiniabactin iron uptake: mechanisms and role in *Yersinia pestis* pathogenesis. *Microbes Infect.* 13:808–817.
- Skulachev VP (ed). 1988. Membrane bioenergetics. Springer-Verlag, Berlin, Germany.
- Lolkema JS, Speelmans G, Konings WN. 1994. Na^+ -coupled versus H^+ -coupled energy transduction in bacteria. *Biochim. Biophys. Acta* 1187:211–215.
- Häse CC, Fedorova ND, Galperin MY, Dibrov PA. 2001. Sodium ion cycle in bacterial pathogens: evidence from cross-genome comparisons. *Microbiol. Mol. Biol. Rev.* 65:353–370.
- Mulkiidjanian AY, Dibrov P, Galperin MY. 2008. The past and present of sodium energetics: may the sodium-motive force be with you. *Biochim. Biophys. Acta* 1777:985–992.
- Resch CT, Winogrodzki JL, Patterson CT, Lind EJ, Quinn MJ, Dibrov P, Häse CC. 2010. The putative Na^+/H^+ antiporter of *Vibrio cholerae*, Vc-NhaP2, mediates the specific K^+/H^+ exchange *in vivo*. *Biochemistry* 49:2520–2528.
- Quinn MJ, Resch CT, Sun J, Lind EJ, Dibrov P, Häse CC. 2012. NhaP1 is a $\text{K}^+(\text{Na}^+)/\text{H}^+$ antiporter required for growth and internal pH homeostasis of *Vibrio cholerae* at low extracellular pH. *Microbiology* 158:1094–1105.
- Herz K, Vimont S, Padan E, Berche P. 2003. Roles of NhaA, NhaB, and NhaD Na^+/H^+ antiporters in survival of *Vibrio cholerae* in a saline environment. *J. Bacteriol.* 185:1236–1244.
- Padan E, Venturi M, Gerchman Y, Dover N. 2001. Na^+/H^+ antiporters. *Biochim. Biophys. Acta* 1505:144–157.
- Padan E, Bibi E, Ito M, Krulwich TA. 2005. Alkaline pH homeostasis in bacteria: new insights. *Biochim. Biophys. Acta* 1717:67–88.
- Ganoth A, Alhadeff R, Kohan D, Arkin IT. 2011. Characterization of the Na^+/H^+ antiporter from *Yersinia pestis*. *PLoS One* 6:e26115. doi:10.1371/journal.pone.0026115.
- Pinner E, Kotler Y, Padan E, Schuldiner S. 1993. Physiological role of *nhaB*, a specific Na^+/H^+ antiporter in *Escherichia coli*. *J. Biol. Chem.* 268:1729–1734.
- Pinner E, Padan E, Schuldiner S. 1994. Kinetic properties of NhaB, a Na^+/H^+ antiporter from *Escherichia coli*. *J. Biol. Chem.* 269:26274–26279.
- Nakamura T, Fujisaki Y, Enomoto H, Nakayama Y, Takabe T, Yamaguchi N, Uozumi N. 2001. Residue aspartate-147 from the third transmembrane region of Na^+/H^+ antiporter NhaB of *Vibrio alginolyticus* plays a role in its activity. *J. Bacteriol.* 183:5762–5767.
- Bartra SS, Styer KL, O'Bryant DM, Nilles ML, Hinnebusch BJ, Aballay A, Plano GV. 2008. Resistance of *Yersinia pestis* to complement-dependent killing is mediated by the Ail outer membrane protein. *Infect. Immun.* 76:612–622.
- Kolodziejek AM, Sinclair DJ, Seo KS, Schneider DR, Deobald CF, Rohde HN, Viall AK, Minnich SS, Hovde CJ, Minnich SA, Bohach GA. 2007. Phenotypic characterization of OmpX, an Ail homologue of *Yersinia pestis* KIM. *Microbiology* 153:2941–2951.
- Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97:6640–6645.
- Conchas RF, Carniel E. 1990. A highly efficient electroporation system for transformation of *Yersinia*. *Gene* 87:133–137.
- Derbise A, Chenal-Francois V, Huon C, Fayolle C, Demeure CE, Chane Woon-Ming B, Médigue C, Hinnebusch BJ, Carniel E. 2010. Delineation and analysis of chromosomal regions specifying *Yersinia pestis*. *Infect. Immun.* 78:3930–3941.

20. Dzioba-Winogrodzki J, Winogrodzki O, Krulwich TA, Boin MA, Häse CC, Dibrov P. 2009. The *Vibrio cholerae* Mrp system: cation/proton antiport properties and enhancement of bile salt resistance in a heterologous host. *J. Mol. Microbiol. Biotechnol.* 16:176–186.
21. Dzioba J, Ostroumov E, Winogrodzki A, Dibrov P. 2002. Cloning, functional expression in *Escherichia coli* and primary characterization of a new Na⁺/H⁺ antiporter, NhaD, of *Vibrio cholerae*. *Mol. Cell. Biochem.* 229:119–124.
22. Ostroumov E, Dzioba J, Loewen PC, Dibrov P. 2002. Asp(344) and Thr(345) are critical for cation exchange mediated by NhaD, Na⁺/H⁺ antiporter of *Vibrio cholerae*. *Biochim. Biophys. Acta* 1564:99–106.
23. Habibian R, Dzioba J, Barrett J, Galperin MY, Loewen PC, Dibrov P. 2005. Functional analysis of conserved polar residues in Vc-NhaD, Na⁺/H⁺ antiporter of *Vibrio cholerae*. *J. Biol. Chem.* 280:39637–39643.
24. Leung KY, Reisner BS, Straley SC. 1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. *Infect. Immun.* 58:3262–3271.
25. Sebbane F, Jarrett C, Gardner D, Long D, Hinnebusch BJ. 2009. The *Yersinia pestis* caf1M1A1 fimbrial capsule operon promotes transmission by flea bite in a mouse model of bubonic plague. *Infect. Immun.* 77:1222–1229.
26. Masereel B, Pochet L, Laeckmann D. 2003. An overview of inhibitors of Na⁺/H⁺ exchanger. *Eur. J. Med. Chem.* 38:547–554.
27. Pinner E, Padan E, Schuldiner S. 1995. Amiloride and harmaline are potent inhibitors of NhaB, a Na⁺/H⁺ antiporter from *Escherichia coli*. *FEBS Lett.* 365:18–22.
28. Dibrov P, Rimon A, Dzioba J, Winogrodzki A, Shalitin Y, Padan E. 2005. 2-Aminoperimidine, a specific inhibitor of bacterial NhaA Na⁺/H⁺ antiporters. *FEBS Lett.* 579:373–378.
29. Padan E, Tzuberly T, Herz K, Kozachkov L, Rimon A, Galili L. 2004. NhaA of *Escherichia coli*, as a model of a pH-regulated Na⁺/H⁺ antiporter. *Biochim. Biophys. Acta* 1658:2–13.
30. Cox M, Nelson D. 2004. Lehninger, principles of biochemistry, 4th ed, p. 1100. WH Freeman, New York, NY.
31. Lang F. 1996. The body compartments and dynamics of water and electrolytes, p. 1545–1556. In Greger R, Windhorst U (ed), *Comprehensive human physiology*. Springer-Verlag, Berlin, Germany.
32. Harel-Bronstein M, Dibrov P, Olami Y, Pinner E, Schuldiner S, Padan E. 1995. MH1, a second-site revertant of an *Escherichia coli* mutant lacking Na⁺/H⁺ antiporters (Δ nhaA Δ nhaB), regains Na⁺ resistance and a capacity to excrete Na⁺ in a delta microH⁺-independent fashion. *J. Biol. Chem.* 270:3816–3822.
33. Fowler JM, Brubaker RR. 1994. Physiological basis of the low-calcium response in *Yersinia pestis*. *Infect. Immun.* 62:5234–5241.
34. Brubaker RR. 2005. Influence of Na⁺, dicarboxylic amino acids, and pH in modulating the low-calcium response of *Yersinia pestis*. *Infect. Immun.* 73:4743–4752.
35. Fowler JM, Wulff CR, Straley SC, Brubaker RR. 2009. Growth of calcium-blind mutants of *Yersinia pestis* at 37°C in permissive Ca²⁺ deficient environments. *Microbiology* 155:2509–2521.
36. Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, Prentice MB, Sebahia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeño-Tarraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrell BG. 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* 413:523–527.
37. Wang RF, Kushner SR. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene-expression in *Escherichia coli*. *Gene* 100:195–199.
38. Straley SC, Bowmer WS. 1986. Virulence genes regulated at the transcriptional level by Ca²⁺ in *Yersinia pestis* include structural genes for outer membrane proteins. *Infect. Immun.* 51:445–454.