Comparative Genomics and Immunoinformatics Approach for the Identification of Vaccine Candidates for Enterohemorrhagic *Escherichia coli* O157:H7

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 strains are major human food-borne pathogens, responsible for bloody diarrhea and hemolytic-uremic syndrome worldwide. Thus far, there is no vaccine for humans against EHEC infections. In this study, a comparative genomics analysis was performed to identify EHEC-specific antigens useful as potential vaccines. The genes present in both EHEC EDL933 and Sakai strains but absent in nonpathogenic *E. coli* K-12 and HS strains were subjected to an *in silico* analysis to identify secreted or surface-expressed proteins. We obtained a total of 65 gene-encoding protein candidates, which were subjected to immunoinformatics analysis. Our criteria of selection aided in categorizing the candidates as high, medium, and low priority. Three members of each group were randomly selected and cloned into pVAX-1. Candidates were pooled accordingly to their priority group and tested for immunogenicity against EHEC O157:H7 using a murine model of gastrointestinal infection. The high-priority (HP) pool, containing genes encoding a Lom-like protein (pVAX-31), a putative pilin subunit (pVAX-12), and a fragment of the type III secretion structural protein EscC (pVAX-56.2), was able to induce the production of EHEC IgG and sIgA in sera and feces. HP candidate-immunized mice displayed elevated levels of Th2 cytokines and diminished cecum colonization after wild-type challenge. Individually tested HP vaccine candidates showed that pVAX-12 and pVAX-56.2 significantly induced Th2 cytokines and production of fecal EHEC sIgA, with pVAX-56.2 reducing EHEC cecum colonization. We describe here a bioinformatics approach able to identify novel vaccine candidates potentially useful for preventing EHEC O157:H7 infections.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are zoonotic extracellular pathogens, members of the Shiga-toxin producing *E. coli* (STEC) pathogroup. EHEC causes sporadic outbreaks of diarrhea and hemorrhagic colitis, particularly in developed countries (reviewed in references 1, 2, and 3). In the United States, EHEC causes approximately 0.9 cases per 100,000 inhabitants, with a significant number of associated hospitalizations and death, particularly among children and the elderly (reviewed in references 4 and 5). *E. coli* O157:H7 is the serotype most commonly associated with outbreaks (6), and the expression of Shiga toxin (Stx), in addition to being linked to hemorrhagic colitis, is associated with the progression to the hemolytic-uremic syndrome (HUS), which causes renal failure and a high fatality rate (reviewed in reference 7). In addition, EHEC O157:H7 uses a type III secretion system (T3SS) to translocate effector proteins into the eukaryotic cell, causing changes in the host cytoskeleton, ultimately leading to improved bacterial adherence and colonization and, in some cases, host cell death (8). The EHEC T3SS is comprised of a basal ATP-dependent secretion apparatus, with an EscC polymer ring spanning bacterial outer membrane and a needle-like structure formed by polymers of the EscF protein and an extension structure comprised of polymerized EspA. Finally, the EspD and EspB proteins form a translocon structure in the host membrane (9–11).

Generally asymptomatic, ruminants are the principal EHEC reservoir. Contaminated meat or fresh produce resulting from animal shedding constitute an important route for human infection (12). Current prevention efforts are centered in the elimination of animal colonization, whether by vaccination or by improving sanitary and breeding practices (12, 13). Once the human

infection is acquired, supportive care is provided, since antibiotic treatment could induce Shiga toxin expression. To date, two vaccines able to reduce EHEC colonization in cattle are commercially available (13, 14). Nevertheless, development of other subunit-based vaccines has been focused in the T3SS and its associated proteins, as well as Stx (4, 12). For example, inactivated Stx derivatives are able to induce Stx-neutralizing antibodies in mice (15, 16), and hybrid A-B subunit-derived Stx toxins also induce antibody production and increase survival against toxemia and EHEC challenge *in vivo* (17–19). Fusion proteins comprising of Stx-derived peptides and T3SS-related proteins are promising vaccine candidates. St2B-Tir-Stx1B-Zot, Stx2B-Stx1B-Int281, EspA-Stx2A1, EspA-IntiminC300-Stx2B, and Stx2B-BLS fusions have been demonstrated to reduce EHEC colonization in animal models, such as mice and goats (20–27). Overall, cumulative information indicates that mucosal delivery routes seem to be an effective way to induce immune responses to block the adhesion of EHEC in the intestine, mainly through expression of secretory immunoglobulin A (sIgA) (4).

In addition to the worldwide outbreaks caused by STEC O157:

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FIG 1 Schematic summary representation of the comparative genomics and immunoinformatics approaches used in the present study to identify vaccine candidates from EHEC O157:H7.

H7, this organism has come recently under renewed scientific investigation as a result of the emergence of a subpopulation of strains that have acquired critical virulence factors that contribute to more severe and lethal disease in humans (28, 29). Further, the discovery of cattle reservoirs shedding high levels of STEC O157:H7, which has been associated with the transmission between animals and across the human-animal interface (30, 31), strongly supports the idea that adoption of vaccination for livestock and/or susceptible individuals will have significant public health benefits, preventing substantial numbers of human STEC O157 cases (32). Therefore, further discovery for EHEC-specific antigens needs to be done to improve existing or to develop novel vaccines. Because the EHEC-associated disease is complex and many molecular and cellular processes affected during infection are not fully understood, it is plausible to propose that some EHEC-encoded virulence-associated proteins could have important, yet unveiled role in the immune/protective process. Therefore, in order to bypass the bias toward assaying a limited number of known virulence factors as components of a vaccine against O157:H7, we performed a genome-wide *in silico* search for proteins most likely to be effective as immunogenic/protective antigens. By comparative genomics, we identified EHEC-specific antigens with high probability to be exposed to the host during infection. Using an immunoinformatics approach, we further grouped our candidates into high-, medium-, and low-priority groups based on their putative antigenicity and screened a subset of them as vaccine candidates in a murine model of gastrointestinal infection. Our approach involved randomly selecting three candidates from each group which were evaluated as DNA vaccines for their capacity to induce an EHEC immune response and to reduce bacterial colonization in the murine intestine.

MATERIALS AND METHODS

Bacterial strains and media. The prototype enterohemorrhagic *E. coli* O157:H7 strains 86-24 (33) and EDL933 (34) were grown in Luria-Bertani (LB) broth at 37°C. Strains bearing pVAX1 derivatives were grown in

medium supplemented with kanamycin (Sigma, 25 µg/ml) as a requirement for recombinant plasmid selection.

Comparative genomics and immunoinformatics analysis. A comparative bioinformatic analysis was developed to obtain groups of orthologous proteins of *E. coli* O157:H7 strains that excluded orthologous proteins from other nonpathogenic (named external) strains. To achieve this, we included two *E. coli* O157:H7 strains (EDL933 and Sakai) and two external strains: *E. coli* HS and *E. coli* K-12 strain MG1655. The most probable set of orthologous proteins shared by the two *E. coli* O157:H7 strains were identified using a reciprocal best-hit criterion as follows. All of the predicted proteins of one genome were searched against the predicted proteins of the other genome and vice versa using BLAST-P with a cutoff E value of 10^{-12} . To be included in an orthologue group, the alignment region between the subject protein and the query protein had to be at least of 80%, and there had to be at least 40% similarity for both query and target sizes. Next, the orthologs groups that contained a protein of any of the two external strains were excluded (Fig. 1). This resulted in a set of 897 groups of orthologous proteins shared only by the *E. coli* O157:H7 strains. The orthologue sequences corresponding to the EDL933 strain were analyzed by the P-SORT software (<http://www.psort.org/psort/>) in order to predict their subcellular localization. Sixty-five proteins that were putatively secreted or outer membrane associated were selected as candidates. These proteins were extensively analyzed for critical features found in vaccine candidates, such as physicochemical properties, adhesiveness, and antigenicity and subsequently predicted for immunodominant epitopes. At least two different software programs were used for each property as described below. In most cases, the underlying principle of these programs for the same categorical property is different to ensure wide coverage and yet maintaining stringency by creating a consensus of predicted results (Table 1).

The transmembrane (TM) regions were predicted using TMHMM (35), HMMTOP (36), and Phobius (37). Phobius can discern TM topology and signaling peptide in a protein. It substantially reduces the errors in the predictions of these two characteristics compared to other algorithms, including TMHMM. After an initial analysis with PsortB (38), the signal or localization peptides were also predicted using SignalP (39) and NetChop (40). The presence of lipoprotein signal peptide was determined using the method described by Juncker et al. (41), which predicts both SpI and SpII signal peptidases. The Lipo program (42), which recognizes the

TABLE 1 Overview of prediction programs used

Program	Physicochemical properties ^a				Immunological properties		
	Transmembrane (TM)	Signal-peptide (SP)	Subcellular localization	Adhesion probability	B-cell epitopes	T-cell epitopes	Antigenicity
1	TMHMM	Signal-P (trained on Gram-positive bacteria)	Signal-P (trained on Gram-negative bacteria)	SPAAN	ABCPred (linear)	NetCTL (MHC-I)	VaxiJen
2	HMMTOP	PsortB	PsortB	LipoP		NetMHC-II (MHC-II)	
3	PHOBIUS	NetChop	NetChop	Lipo			

^a The consensus of results from all programs within a given category, wherever applied, is indicated.

lipo-box in protein sequences, was also used. SPAAN (43) predicts the probability of a protein being an adhesin, which often comprise important factors in bacterial virulence. VaxiJen (44), an alignment-free approach for antigen prediction, was used to score the overall antigenicity of the protein. A VaxiJen model used was “bacteria” with the threshold set to 0.5. B-cell linear epitopes were predicted using ABCPred (45), whereas NetCTL (46) and NetMHC-II (47) programs were used to predict major histocompatibility complex class I (MHC-I) and MHC-II binding peptides, respectively. The ABCPred provided an accuracy of 65.93% and equal sensitivity and specificity using a window length of 16-mer peptides with overlapping allowed. NetCTL predicts CTL epitopes restricted to 12 MHC-I supertypes, and a specificity of 97% was used. MHC-II was used with an epitope length of 15 residues against 14 HLA-DR alleles covering the nine HLA-DR, six HLA-DQ, and six HLA-DP supertypes. The overall strategy and its preferred outcome are outlined in Fig. 1.

Plasmid construction. Plasmid pVAX1 was obtained from Invitrogen/Life Technologies (New York, NY). For our study, large candidates were subdivided in coding sequences (CDS) of a maximum 1,000 bp in length. For the high-priority pool, candidates 31, 56, and 12 were selected. Candidate 56 (EscC) was divided into two CDS. Since the amino-terminal portion of EscC is oriented toward the periplasm (10), we decided to test the fragment comprising the carboxy terminus (pVAX56.2). Candidates 43, 16, and 9 were selected for the medium-priority pool. Finally, candidate 51 and two CDS of candidate 49 were selected for the low-priority pool. The CDS for selected candidates were amplified by PCR from genomic DNA of *E. coli* EDL933 (O157:H7) using the corresponding forward (Fw) and reverse (Rv) primers containing HindIII and XhoI restriction sites, respectively (Table 2). The resulting fragments were digested

and cloned into the HindIII and XhoI sites of pVAX1. Fw primers were designed to generate a Kozak consensus sequence (ACCATGG) at the 5' end of each CDS. All of the clones were sequenced.

Immunization protocol. Six- to eight-week-old female BALB/c mice (Harlan Laboratories) were divided into six groups ($n = 10$). Mice were immunized intranasally with 20 μ l of the DNA vaccines in Tris-EDTA (TE) buffer (10 μ l on each nostril), arranged as follows: (i) TE buffer only, (ii) TE plus cholera toxin (CT; adjuvant), (iii) pVAX vector plus CT, (iv) pVAX-high priority (HP), (v) pVAX-medium priority (MP), and (vi) pVAX-low priority (LP) vaccine candidates. All of the vaccine candidates were administered in pools of three targets (20 μ g of each plasmid), along with the adjuvant CT (1 μ g/ μ l). For the immunization, the animals were anesthetized with isoflurane and primed with final dose of 60 μ g of DNA per mice, followed by boosts at 2 and 4 weeks using the same dose without CT. In the case of the individual candidate immunizations, the animals received a total of 60 μ g of individual plasmid. In the CT control group, priming was with CT, followed by TE boosts. One week after the last boost, blood and fecal samples were collected to monitor the mucosal antibody response.

Challenging the immunized mice. To determine the protective ability of the potential DNA vaccine candidates, all immunized mice were challenged with a dose of 5×10^9 CFU of the streptomycin resistant EHEC O157:H7 strain 86-24, via gavage, 2 weeks after the last boost. Two hours prior to the challenge, mice received an i.p. dose of cimetidine hydroxyzine (10 mg/ml) to reduce stomach acidity. Fecal samples were collected from each group at indicated days after infection. Stools were dissolved in 2 ml of phosphate-buffered saline (PBS), serially diluted, and plated. To recover bacteria from the intestine, the mice were euthanized at the indi-

TABLE 2 Primers used for plasmid construction

Construct	EHEC gene insert ^a	Primer	Sequence (5'–3')
pVAX-12	Z1538	PVAX12-Fw PVAX12-Rv	ACCAAGCTTACCATGGTTTCTACTTTCAAAAAAGCAG ACCCTCGAG TAGAGGTAGCTCAGGGTGTATTCT
pVAX-31	Z3075	PVAX31-Fw PVAX31-Rv	ATTAAGCTTACCATGGGTAACTTTATGCCGCCATTTTG ATTCTCGAGTCAATGATGATGATGATGGAACCTTATAACCGACACCCAC
pVAX-56.2	aa 253 to 512 from <i>escC</i>	PVAX56.2-Fw PVAX56.2-Rv	ACCAAGCTTACCATGGACCGCGAAATAACGATGGAT ACC CTCGAGTTATTCGCTAGATGCAGATTTTATC
pVAX-43	Z4321	PVAX43-Fw PVAX43-Rv	ATTAAGCTTACCATGGGTGGTTCAAGACTGGCTGATAATC ATTCTCGAGTTAAAAACGATAACCAACTCCAAC
pVAX-16	Z1908	PVAX16-Fw PVAX16-Rv	ATTAAGCTTACCATGGCTTTTTCTTTTTTTCTACAAAACCCATACC ATTCTCGAGTTATCCGCGCCGACCATTAACC
pVAX-9	Z0981	PVAX9-Fw PVAX9-Rv	ACCAAGCTTACCATGGGTAAAGTTTGTGCAGCAA ACCCTCGAGTCAAAATTTATAACCGACACCCAC
pVAX-51	<i>espB</i>	PVAX51-Fw PVAX51-Rv	ATTAAGCTTACCATGGATACTATTGATAATACTCAAG ATTCTCGAGTCAATGATGATGATGATGATGCCAGCTAAGCGACCCGATTG
pVAX-49.1	aa 641 to 960 from <i>ehaG</i>	PVAX49.1-Fw PVAX49.2-Rv	ACCAAGCTTACCATGGCCGATGCCGTTAACGGCTC TTATCTAGACTCGAGTTACTCGGCGTTCCGAATGGTG
pVAX-49.2	aa 1141 to 1380 from <i>ehaG</i>	PVAX49.2-Fw PVAX49.2-Rv	ACCAAGCTTACCATGGAACGTCTCGGTGCATTGTCT TTATCTAGACTCGAGTTAGCCGGAACCAATCGCGACG

^a aa, amino acids.

cated days, and ceca were excised and homogenized in 2 ml of PBS. Bacterial suspensions were serially diluted and plated. Both organ and fecal samples were plated on MacConkey agar plates containing streptomycin and then incubated at 37°C overnight prior to *E. coli* O157:H7 colony enumeration.

Serum and feces collection for immunoglobulin determination.

Sera were obtained by retro-orbital bleeding, clotting whole blood for 30 min at room temperature, and centrifugation at $3,000 \times g$ for 15 min at 4°C. The resulting supernatant was collected and used for enzyme-linked immunosorbent assay (ELISA). For sIgA measurement, feces samples were weighted and diluted to 1 g/ml with PBS-phenylmethylsulfonyl fluoride. After vigorous homogenization by vortexing, feces samples were incubated for 1 h on ice and centrifuged at 4,000 rpm for 30 min at 4°C. The supernatant was collected and stored at -20°C prior to use.

Immune response. (i) **ELISA.** Total serum IgG antibody responses were determined by ELISA according to the manufacturer's instructions (eBioscience). Briefly, polystyrene 96-well high-binding ELISA plates (Nunc, Denmark) were coated overnight at 4°C with capture IgG antibody. The plates were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBS-T) and plated with blocking buffer. The diluted serum sample (1:1,000 and 1:10,000) and an IgG standard of known activity were incubated, followed by repeated washing with 0.05% PBS-T. Next, horseradish peroxidase-conjugated goat anti-mouse IgG in PBS-T (1:250) was added to the ELISA plates, followed by incubation at 37°C for 30 min, followed in turn by washing. A total of 100 µl of tetramethylbenzidine (TMB) was added to the cells, followed by incubation at room temperature for 15 min. The reaction was stopped with 100 µl of 2 M H₂SO₄, and the optical density at 450 nm was determined.

For the EHEC antibody response, wild-type EHEC O157:H7 was grown overnight in LB broth. Bacterial cells were pelleted (15 min at $5,000 \times g$) and resuspended in PBS. The bacterial suspension was pulse-sonicated on ice for 5 min. The sonicated sample was centrifuged ($10,000 \times g$ for 15 min at 4°C), and the total protein concentration in the supernatant was determined by using a bicinchoninic acid protein (BCA) assay. For ELISA, polystyrene 96-well Nunc plates were coated overnight at 4°C with 100 µl of EHEC extract (2 µg/well in coating buffer), and the assays were performed as described above.

(ii) **Bio-Plex assay.** The cytokine levels in serum and feces from immunized and nonimmunized mice were measured on a Bio-Plex 200 system powered by Luminex xMAP technology (Bio-Rad, USA) using specific 23-panel group mouse assay kits (catalog no. M60009RDPD) according to the manufacturer's instructions. The cytokines, chemokines, and growth factors include interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12p70, IL-13, IL-17, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, KC, eotaxin, macrophage chemoattractant protein 1 (MCP-1), granulocyte colony-stimulating factor (CSF), granulocyte-CSF (GM-CSF), and RANTES. A heat map of a normalized matrix was created that correlates cytokine response pattern to stimulation by high-priority vaccine candidates. For each cytokine, means and standard deviations (SD) were calculated from their induction values (pg/ml) across the three candidates and were normalized to controls (TE and pVAX-1). The Z-score transformation was calculated for each cytokine by subtracting each induction value from the row mean and dividing by the row SD (48). Overall, the Z-score gives an estimation of the deviation of the measurement from the row mean in SD units. Each block of red or green represents a high positive or negative correlation between the cytokine production and the vaccine candidate under investigation.

Statistical analysis. All of the statistical significance between control and vaccinated groups was assessed using SPSS software. One-way analysis of variance (ANOVA) and Student *t* test with threshold of $P < 0.05$ was used to analyze the data for colonization and antibody response. For the antibody responses the *P* value is indicated in the respective figures.

RESULTS

Subunit EHEC vaccine research has been mainly focused on known virulence factors. We sought to screen the genome of EHEC strains EDL933 and Sakai to identify sequences with high probability to encode protective antigens, independently of their previous assigned function. Mucosal immune responses are relatively difficult to induce by vaccine candidates due to the fact that mucosal surfaces are often in contact with nonpathogenic *E. coli*, which enhance the homeostatic state (reviewed in reference 49). Thus, our first criterion was to identify common EHEC antigens absent in nonpathogenic *E. coli* strains. For this, the set of common proteins between EDL933 and Sakai O157:H7 EHEC strains were determined by a reciprocal BLAST analysis as described in Materials and Methods. Next, DNA sequences encoding proteins present in the nonpathogenic *E. coli* K-12 MG1655 and commensal HS strains genomes were eliminated from the pool. This analysis rendered 897 protein sequences. It is proposed that the probabilities to provide protection increase for those antigens detected during natural infection, meaning that bacterium-exposed proteins comprise better vaccine candidates (50). In order to predict their subcellular localization, the 897 proteins selected in the previous analysis were evaluated by using the P-SORTb software. A total of 65 proteins putatively associated with the outer membrane and/or secreted were selected as vaccine candidates (Table 3). Further, we accelerated the process of identifying highly antigenic vaccine candidates by using immunoinformatics, which allowed us to assign priorities for the testing of these vaccine candidates in the mouse model of EHEC O157:H7 infection. As described in Fig. 1 and based on physicochemical properties, the proteins were prioritized on the basis of either possessing no transmembrane (TM) domains or containing one region only, having a signal peptide or whether its localization was predicted to be "secreted" and whether they display a high score for adhesiveness. Proteins that satisfy more of these characteristics were ranked higher than others. The VaxiJen server (44) predicted the protective bacterial antigens based on the overall immunogenicity score (higher was better) and also helped to rank the proteins. For B-cell epitope predictions, three parameters were calculated from the results: (i) the total number of epitopes per sequence, (ii) the total score of all epitopes combined per sequence, and (iii) the average score of an epitope. Similarly, for T-cell epitope predictions, parameters such as the total number of high-binding (HB) epitopes, the total score of HB epitopes, the percentage of HB epitopes among all epitopes predicted for a given sequence, and the number of human leukocyte antigens (HLA) alleles covered by the epitopes of a given sequence were also calculated. These parameters were also taken into account when ranking the proteins; however, a larger weight was assigned to B-cell epitopes since EHEC is an extracellular pathogen. The final ranking of all 65 proteins was conducted using a cumulative score from both physicochemical and immunological properties. Based on the combined informatics analysis, vaccine candidates were divided in three groups: high priority (HP; 17 candidates), medium priority (MP; 10 candidates), and low priority (LP; 38 candidates) (indicated in parentheses in Table 3).

To screen the putative protective candidates as determined by their priority score, we randomly selected three candidates from each group (candidates 31, 56.2, and 12 from the HP group; candidates 43, 16, and 9 from the MP group; and candidates 51, 49.1, and 49.2 from the LP group) and cloned them into the widely used

TABLE 3 EHEC O157-specific secreted and outer-membrane-associated proteins identified by P-SORT analysis

No. (candidate type) ^a	GI	Annotation
1 (LP)*	12512859	Putative fimbrial protein
2 (LP)*	12512975	Hypothetical protein Z0266
3 (LP)*	12513130	Putative beta-barrel outer membrane protein
4 (LP)	12513211	Putative structural protein (partial)
5 (LP)*	12513363	Putative outer membrane export protein
6 (LP)	12513364	Hypothetical protein Z0609
7 (LP)	12513368	Putative RTX family exoprotein
8 (LP)	12513376	Hypothetical protein Z0639
9 (MP)*	12513752	Putative outer membrane protein of prophage CP-933K
10 (HP)	12514345	Putative outer membrane protein Lom precursor of bacteriophage BP-933W
11 (LP)	12514376	Hypothetical protein Z1516
12 (HP)	12514403	Putative pilin subunit
13 (LP)	12514410	Putative member of ShlA/HecA/FhaA exoprotein family
14 (HP)	12514411	Putative outer membrane transporter of ShlA/HecA/FhaA exoprotein family
15 (HP)	12514503	Putative receptor
16 (MP)	12514836	Putative tail component of prophage CP-933X
17 (LP)	12514898	Putative outer membrane receptor, probably <i>tonB</i> dependent
18 (LP)*	12515102	Putative tail component of prophage CP-933O
19 (LP)	12515159	ORF, hypothetical protein
20 (LP)*	12515160	Putative ATP-binding component of a transport system and adhesion protein
21 (LP)*	12515311	Putative outer membrane protein
22 (LP)*	12515315	Hypothetical protein Z2323
23 (MP)	12515551	Putative chaperone protein
24 (HP)*	13259573	Putative Lom-like outer membrane protein of cryptic prophage CP-933P
25 (LP)	13259574	Putative tail component of cryptic prophage CP-933P
26 (LP)	13259580	Putative tail component of cryptic prophage CP-933P
27 (LP)	12516024	Flagellar biosynthesis; flagellin, filament structural protein
28 (LP)	12516037	Putative secreted protein
29 (LP)	12516039	Putative secreted protein
30 (LP)	12516089	Unknown protein encoded within prophage CP-933U
31 (HP) ^a	12516092	Putative outer membrane protein of prophage CP-933U
32 (MP)	12516149	Putative integrase for prophage CP-933U
33 (LP)	12516174	Putative outer membrane receptor for iron compound or colicin
34 (HP)*	12516360	Putative Lom-like outer membrane protein of prophage CP-933V
35 (LP)*	12516361	Putative tail fiber protein of prophage CP-933V
36 (MP)	12516373	Putative major tail subunit encoded within prophage CP-933V
37 (LP)*	169822942	Putative fimbrial usher
38 (LP)	12517052	Hypothetical protein Z3920
39 (LP)*	12517087	Hypothetical protein Z3954
40 (MP)*	12517088	Putative enzyme

TABLE 3 (Continued)

No. (candidate type) ^a	GI	Annotation
41 (HP)	12517355	Putative lipoprotein of type III secretion apparatus
42 (HP)	12517375	Type III secretion apparatus protein
43 (MP)	12517526	Putative PagC-like membrane protein
44 (HP)	12517607	Putative iron compound receptor
45 (HP)	12518206	Outer membrane heme/hemoglobin receptor
46 (HP)	12518273	Putative fimbrial subunit
47 (LP)	12518274	Putative fimbrial protein
48 (MP)	12518278	Putative major fimbrial subunit
49 (LP)	12518349	Putative adhesin
50 (LP)	12518435	<i>espF</i>
51 (LP)	12518439	Secreted protein EspB
52 (LP)	12518440	Secreted protein ExpD
53 (HP)	12518447	Intimin adherence protein
54 (LP)	12518449	Putative translocated intimin receptor protein
55 (LP)	12518464	<i>escJ</i>
56 (HP)	12518466	<i>escC</i>
57 (MP)	12518483	Hypothetical protein Z5142
58 (HP)	12518576	Putative fimbrial protein
59 (HP)	12518577	Putative fimbrial protein
60 (HP)*	12518578	Putative fimbrial usher
61 (LP)	12518581	Putative major fimbrial subunit
62 (MP)	12518689	Hypothetical protein Z5335
63 (LP)	3822134	Putative exoprotein precursor
64 (LP)	3822145	Hypothetical protein
65 (LP)	3822162	Hypothetical toxin protein

^a Low-priority (LP), medium-priority (MP), and high-priority (HP) candidates are indicated. *, Final candidates analyzed further using blastp for which paralogs of some of sequences were found in recently sequenced nonpathogenic *E. coli* strains.

pVAX1 DNA vaccine vector (51, 52). DNA vaccine construction comprises an approach that allows the rapid testing of several vaccine candidates instead of limiting the analysis to antigens requiring optimized expression and purification. Since EHEC is an extracellular intestinal pathogen, our approach sought to induce mucosal immune responses, and DNA vaccines have been shown to induce both mucosal and systemic immune responses against pathogenic bacteria antigens when delivered by the intranasal route (53–55). During the cloning process in pVAX-1, large DNA sequences were divided in coding sequences with a maximum length of 1 kb. The constructs were pooled in their respective priority group and delivered intranasally in groups of 10 mice. A schedule of priming and two boosts was followed, with 60 µg of the pooled plasmids (20 µg of each individual construct) and cholera toxin (CT) as an adjuvant, which is further detailed in the Materials and Methods. Next, we sought to evaluate the induction of immune responses in vaccinated mice. First, we monitored the production of EHEC IgG in immunized mice by ELISA using serum samples collected 1 week after the last boost. HP and MP candidate-immunized mice presented higher levels of EHEC IgG compared to animals receiving buffer (TE), adjuvant (CT) alone, or pVAX-1 (empty vector) (Fig. 2A). Next, EHEC IgA titers in feces were determined. HP candidate-vaccinated mice elicited higher titers of EHEC sIgA compared to MP and LP candidate-immunized animals and control groups (Fig. 2B), although the titers declined with increased serum dilution. These results

FIG 2 EHEC immune responses after priority groups' vaccination. (A) Anti-EHEC serum IgG following intranasal immunization. Groups of 10 mice were administered HP (pVAX-12, pVAX-31, and pVAX-56.2), MP (pVAX-43, pVAX-16, and pVAX-9), and LP (pVAX-51, pVAX49.1, and pVAX49.2) plasmids, along with CT as an adjuvant. As controls, nonimmunized mouse sera were obtained from mice receiving TE, pVAX-1, or CT. All sera were assessed using ELISA, and samples were diluted up to 1:1,000 for the assay. The results are expressed as means $\pm$ the standard deviations (SD) of duplicate values, and asterisks (*) are used to indicate statistical differences in the IgG response of HP, MP, and LP vaccine candidates compared to the TE group: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. (B) EHEC IgA measured in a fecal sample of immunized mice. Vaccinated mice were administered HP, MP, and LP vaccines, along with adjuvant (CT). Mice vaccinated with TE or pVAX-1 were used as controls. The samples were 2-fold diluted and assessed for EHEC sIgA antibody using ELISA. The results are expressed as means of duplicate values, and an asterisk (*) indicates a statistical difference ($P < 0.05$). HP, high priority; MP, medium priority; LP, low priority.

showed that although both HP and MP DNA vaccine pools induce EHEC antibody responses, only the HP group induced a larger amount of EHEC sIgA.

Two weeks after the last boost, animals were challenged with EHEC strain 86-24. The bacterial load in feces and cecum was determined from days 3 to 6 after infection. The results consistently showed a reduction pattern in the CFU recovered from feces in the HP candidate-vaccinated mice at days 4, 5, and 6 (Fig. 3A) compared to the TE buffer, CT, or pVAX-1 control groups. In addition, we observed a reduction in the LP candidate-vaccinated group at day 6, although it was less than that observed in the HP group. Cecum organ plating revealed a significant reduction of >1 log ($P < 0.05$) in the bacterial burden in HP candidate-vaccinated mice compared to the empty vector control group at day 3

(Fig. 3B). No reduction was observed in the ceca of MP or LP candidate-vaccinated mice. Overall, the data showed that HP plasmids were more efficient at reducing EHEC colonization in the murine model.

To determine the extent of the immunogenic/protective effect of each candidate in the overall HP vaccine group, an individual plasmid vaccination experiment was performed. A total of 60 μ g of pVAX-12, pVAX-31, pVAX-56.2, or TE buffer was administered to groups of mice with the same vaccination schedule as the previous experiment. To assess the immunogenicity of individual DNA vaccines, mouse sera were collected and assayed for EHEC IgG. The results showed increased EHEC IgG levels in pVAX-12. The IgG levels were also elevated with the pVAX-56.2-vaccinated group compared to the TE control group (Fig. 4A). The pVAX-31

FIG 3 Bacterial counts in feces and cecum of EHEC-infected mice. (A) Fecal shedding of vaccinated mice with HP, MP, and LP vaccines with CT adjuvant and challenged with 10^9 EHEC O157:H7 for days 3 through 6 postimmunization. Feces were homogenized and plated onto MacConkey agar plates, and the numbers of CFU per gram were determined. Values are represented as means $\pm$ the SD from feces obtained from five mice, and asterisks (*) indicate statistical differences ($P < 0.05$). (B) Cecum segments were obtained from each mouse receiving HP, MP, and LP vaccines with CT adjuvant, infected with EHEC O157:H7. Cecum segments obtained at day 3 postinfection were homogenized and plated onto MacConkey agar plates. The ceca from mice receiving TE, pVAX-1, and CT were used as controls, and results are depicted as the log CFU/g of cecum. Means $\pm$ the SD reflect values of five mice per group, and an asterisk (*) denotes a statistically significant difference ($P < 0.05$).

FIG 4 EHEC immune responses after pVAX candidate vaccination. (A) EHEC IgG antibody titers were detected in serum by ELISA. Sera were obtained from mice immunized with HP group and individual vaccine candidates: pVAX-12, pVAX-31, and pVAX-56.2 with CT adjuvant. A nonimmunized mice serum from TE group was used as control. The samples were diluted up to 1:1,000 for the assay. The results are expressed as means $\pm$ the SD of duplicate values obtained from five mice in each group, and an asterisk (*) indicates a significant difference ($P < 0.05$). (B) Titers of EHEC IgA measured from fecal samples of immunized mice with HP group and individual vaccine candidates: pVAX-12, pVAX-31, and pVAX-56.2 with CT adjuvant. Mice inoculated with TE were used as a control. The feces samples were serially diluted up to 1:8 for detection of sIgA antibody by ELISA. The results are expressed as means $\pm$ the SD of duplicate values from five mice in each group, and asterisks (***) indicate statistically significant differences ($P < 0.05$).

plasmid failed to induce the production of EHEC IgG. In parallel, we measured EHEC sIgA in the feces of all vaccinated animals to evaluate mucosal immune induction. The mice immunized with pVAX-56.2 showed increases in sIgA up to the 1:8 dilution (Fig. 4B) compared to control animals. The pVAX-31 and, to a minor extent, pVAX-12 also increased the levels of sIgA in feces compared to the TE control group. Overall, pVAX-12 produced the higher induction of serum IgG, whereas pVAX-56.2 was able to stimulate high titers of both serum IgG and sIgA.

Next, mice vaccinated with individual candidates were challenged with EHEC 86-24 two weeks after the last boost. Daily bacterial loads in feces and cecum colonization from days 3 through 6 postinfection were assessed. The results show a clear reduction of EHEC in feces in all vaccinated groups at day 6 (Fig. 5A). No differences in cecum colonization were observed at day 3 (data not shown). However, a small reduction in EHEC CFU on the cecum from the pVAX-56.2-immunized group was detected at day 6 (Fig. 5B). Overall, pVAX-56.2 consistently induced EHEC serum IgG and fecal sIgA and reduced EHEC shedding and colonization, suggesting that candidate 56.2, which encodes the C-ter-

минаl fragment of EscC, the T3SS secretin, was the most effective candidate tested.

Because the cellular immune response plays a leading role in protection and long-term immune response, we also evaluated the expression of cytokines in sera of HP, MP, and LP candidate-immunized and nonimmunized mice using a Bio-Plex assay. Eleven of 23 inflammatory mediators were increased in HP candidate-immunized mice compared to nonimmunized mice, including the Th1 cytokines IL-1 β and TNF- α , the Th2 cytokines IL-3, IL-5, IL-6, and IL-10, the chemokines MIP-1 α , MIP-1 β , eotaxin, KC, and MCP-1, and the growth factor GM-CSF (the data are summarized using heterogeneous colors between columns in the heat map in Fig. 6A). Proinflammatory cytokines such as IL-1 β , TNF- α , and IL-6 are increased at an early stage of the immune response and play a central role in the host defense mechanism. However, increased levels of Th2 cytokines, including IL-3, IL-5, IL-6, and IL-10 but not IL-4, in HP candidate-immunized mice were also detected. We propose that these Th2-type cytokine responses may account for the generation of the humoral antibody response. Sera obtained from mice vaccinated

FIG 5 Bacterial counts in feces and cecum of EHEC-infected mice. (A) Bacterial fecal shedding was determined in mice vaccinated with pVAX-12, pVAX-31, pVAX-56.2, and HP group candidates (10 mice per group). Excretion of EHEC O157:H7 was determined as CFU/g of feces for the first 6 days postimmunization. The results are expressed as means $\pm$ the SD from five mice in each group, and asterisks (***) indicates a statistical difference ($P < 0.05$). (B) Cecum segments were obtained from each vaccinated mice (pVAX-12, pVAX-31, pVAX-56.2, and HP groups) challenged with 10^9 EHEC O157:H7. Cecum segments obtained at day 6 postinfection were homogenized and plated onto MacConkey agar. Means $\pm$ the SD of the CFU/g from 10 mice are presented.

FIG 6 Schematic heat map representation of cytokines, chemokines, and growth factors in response to vaccinated and/or infected mice. (A and B) Bio-Plex assays were performed using the sera of immunized mice administered with HP, MP, and LP candidates (A) and the individual vaccine candidates pVAX-12, pVAX-31, and pVAX-56.2 (B). Nonimmunized sera from TE and CT mice were included as controls. The data are Z-score transformed and relative to controls and are expressed as SD units from the row mean. The samples were diluted up to 1:1 for the assay. The results are expressed as means $\pm$ the SD of duplicates. Statistical significance was calculated in both the A and B groups by using one-way ANOVA. All of the cytokines presented in the heat map were significantly different ($P < 0.001$) compared to the TE group.

with the individual candidates pVAX-12 and pVAX-56.2 showed an increase in Th1 cytokines (IL-1 β , IL-12p70, and TNF- α), Th2 cytokines (IL-3, IL-5, IL-6, and IL-10), chemokines (MIP-1 β , eotaxin, MCP-1, and RANTES), and the GM-CSF growth factor compared to control mice (Fig. 6B).

DISCUSSION

Most EHEC subunit vaccine candidates tested to date are comprised of known virulence factors, such as Stx and the T3SS-related proteins. These virulence factors are well characterized and are essential for the onset of EHEC colonization and/or host damage. Further, it is well documented that the main protection mechanism for these vaccine candidates is the induction of neutralizing antibodies (4). However, the DNA sequences encoded in the genome of EHEC strains may contain unveiled gene-encoding antigenic proteins, which have not yet been investigated as vaccine candidates. Computational vaccinology tools have been proposed as a potentially powerful aid in vaccine development, particularly for new or emerging pathogens for which critical antigenic determinants and/or virulence factors knowledge is limited (56). This work combines comparative genomics and immunoinformatics analysis of available EHEC genomes in the search for vaccine candidates. This approach represents an unbiased screening method, since it examined encoded sequences irrespective of their putative or experimental function, which allowed us to discover potential candidates overlooked by other EHEC vaccine studies. Subsequently, this method led us to de-

velop a list of 65 vaccine candidates, three of which were proven to be able to induce immune responses, and one of these also reduced EHEC colonization when delivered as a DNA vaccine. It is noteworthy that none of these three experimentally assayed candidates has been used as an EHEC vaccine before. We propose that similar comparative genomics plus immunoinformatics analysis approaches may be useful for the unbiased search of vaccine candidates in other bacterial pathogens.

Since intestinal mucosal surfaces are composed of exposed tissue in permanent contact with harmless environmental bacteria, which likely participate in the maintenance of a homeostasis state (49), immune responses are relatively difficult to induce by vaccine candidates delivered in the gastrointestinal mucosa. The HP candidates were able to induce EHEC humoral responses both in serum and in intestinal mucosa. Although some candidates assayed in mice have been shown to be protective by inducing exclusively serum responses (20, 57), most involved the production of sIgA (17, 58–61). In our case, the induction of sIgA by the HP pool correlated with a reduction in colonization, while the MP pool was also able to increase serum IgG levels but failed to reduce EHEC colonization after challenge. Assays performed with the three HP candidates individually also seem to support the role of a humoral response by the intestinal mucosa in protection, since pVAX-12 failed to protect against EHEC colonization, despite inducing a strong serum humoral response. On the other hand, pVAX-56.2, the candidate inducing the highest sIgA titers in feces,

was able to cause a reduction in the bacterial load in the cecum. Nevertheless, it seems that the combined immune response induced by the three candidates is required to achieve the colonization reduction levels observed with the HP pool. Arguably, the murine model of EHEC infection possesses inherent limitations. Although Stx delivery into mice resembles human HUS damage, such as thrombocytopenia, hemolysis, and renal failure (62), EHEC lethality in mice requires large amounts of the initial inoculum and does not promote the development of attaching and effacing lesions and diarrhea, in sharp contrast to the human infection (63, 64). However, due to their affordability and ease in handling, mice have largely been used for the initial screening of vaccine candidates (4). In addition, results obtained using EHEC-vaccinated mice have resembled those obtained for more relevant animal models (57).

Although much less investigated, cellular immune responses may play a role in protection against EHEC. Our data suggest that HP candidates induced an inflammatory response, with a mixed Th1/Th2 response. As part of an effective immune response strategy, the induction of a T helper immune response is required for a robust and long-lasting humoral immune response (65). In light of this, we examined Th1 and Th2 cytokine production, which could contribute to the generation of an efficient humoral immune response. Our data suggest that HP candidates induced an inflammatory response, with mixed Th1 and Th2 cytokine production. Previously, a similar mixed Th1-Th2 response was obtained when assaying EHEC bacterial ghosts as vaccine candidates (66). However, our findings showing a high production of IL-3, IL-5, IL-16, IL-10, and TNF- α by HP candidates may indicate a Th2-dominated response for subsequent stimulation of the observed humoral immune response. A further in-depth analysis is required for the cellular mechanisms triggered by the HP pool.

Overall, our approach allowed us to identify a group of EHEC-specific vaccine candidates, some of them displaying a high potential to encode protective immunogens. This bioinformatics and initial experimental assay confirms the immunogenic and relative protective potential of a subset of the candidates and further prompts the experimental characterization of the remaining ones in order to increase the stockpile of immunogens that can become components of an efficient multisubunit vaccine for humans or a new generation of vaccines for cattle.

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