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In Vitro and In Vivo Analyses of Constitutive and In Vivo-Induced Promoters in Attenuated Vaccine and Vector Strains of *Vibrio cholerae*

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The optimal promoter for in vivo expression of heterologous antigens by live, attenuated vaccine vector strains of *Vibrio cholerae* is unclear; in vitro analyses of promoter activity may not accurately predict expression of antigens in vivo. We therefore introduced plasmids expressing the B subunit of cholera toxin (CtxB) under the control of a number of promoters into *V. cholerae* vaccine strain Peru2. We evaluated the *tac* promoter, which is constitutively expressed in *V. cholerae*, as well as the in vivo-induced *V. cholerae* heat shock *htpG* promoter and the in vivo-induced *V. cholerae* iron-regulated *irgA* promoter. The functionality of all promoters was confirmed in vitro. In vitro antigenic expression was highest in vaccine strains expressing CtxB under the control of the *tac* promoter (2 to 5 μg/ml/unit of optical density at 600 nm [OD_{600}]) and, under low-iron conditions, in strains containing the *irgA* promoter (5 μg/ml/OD_{600}). We orally inoculated mice with the various vaccine strains and used anti-CtxB immune responses as a marker for in vivo expression of CtxB. The vaccine strain expressing CtxB under the control of the *tac* promoter elicited the most prominent specific anti-CtxB responses in vivo (serum immunoglobulin G [IgG], $P \leq 0.05$; serum IgA, $P \leq 0.05$; stool IgA, $P \leq 0.05$; bile IgA, $P \leq 0.05$), despite the finding that the *tac* and *irgA* promoters expressed equivalent amounts of CtxB in vitro. Vibriocidal antibody titers were equivalent in all groups of animals. Our results indicate that in vitro assessment of antigen expression by vaccine and vector strains of *V. cholerae* may correlate poorly with immune responses in vivo and that of the promoters examined, the *tac* promoter may be best suited for expression from plasmids of at least certain heterologous antigens in such strains.

Development of *Vibrio cholerae* as a vector organism capable of expressing heterologous antigens at mucosal surfaces is attractive. *V. cholerae* is a noninvasive organism that effectively colonizes the intestinal mucosa of humans, and infection with *V. cholerae* results in immune responses that are long-lasting (10, 15). Live, attenuated vaccine strains of *V. cholerae* can be administered orally, and such strains have been well characterized and shown to be both safe and immunogenic in humans (11, 12, 14, 22, 24). *V. cholerae* vaccines strains can secrete immunoadjuvants in vivo, such as the nontoxic immunoadjuvantive mutant of *Escherichia coli* heat-labile enterotoxin $LT_{(R192G)}$ (19), and *V. cholerae* vaccines strains can express large quantities of heterologous antigens in a balanced lethal plasmid expression system (20). *V. cholerae* vaccine strains can also efficiently express and secrete both large and small heterologous antigens (2, 3, 17, 18), and a mouse model of *V. cholerae* infection that permits rapid preliminary evaluation of *V. cholerae* vaccine and vector strains in vivo has been developed (4, 6). The optimal promoter for in vivo expression of heterologous antigens by vaccine and vector strains of *V. cholerae* is, however, unclear.

Constitutive promoters can drive high-level expression of certain antigens; however, such expression can be toxic to bacterial cells (5). In contrast, in vivo-induced promoters may have no or low-level activity when evaluated in vitro, but such promoters may be extremely active in vivo (5, 16). Strains expressing heterologous antigens from in vivo-induced promoters may be less compromised than those expressing antigens from constitutive promoters, and in vivo expression of heterologous antigens by in vivo-induced promoters may exceed that of constitutive promoters (5, 16).

To examine optimal promoter activity in *V. cholerae* vaccine and vector strains, we compared in vitro and in vivo activities of a number of promoters. We used derivatives of *V. cholerae* vaccine strain Peru2 (*V. cholerae* O1 El Tor C6709 Δ*attRS1*) expressing a nontoxic B subunit (CtxB) of cholera toxin from a plasmid; strains used in this study were identical except for the identity of the promoter driving CtxB expression. We evaluated the *tac* promoter, which is constitutively active in *V. cholerae* since *V. cholerae* lacks the *lacI*^{q} repressor (1). We also examined two in vivo-induced *V. cholerae* promoters: the heat shock *htpG* promoter (induced under conditions of environmental stress) (13) and the *V. cholerae* iron-regulated *irgA* promoter (induced under low-iron conditions) (7, 9). We confirmed appropriate in vitro regulation of these promoters, and we analyzed systemic and mucosal immune responses to CtxB in mice inoculated with the various vaccine strains of *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are described in Table 1. All strains were maintained at −70°C in Luria-Bertani (LB) broth (21) containing 15% glycerol. All cultures contained either streptomycin (100 μg/ml) or ampicillin (100 μg/ml). Cultures were grown at indicated temperatures with aeration.

Recombinant DNA methods. Isolation of plasmid DNA, restriction digestions, and agarose gel electrophoresis were performed using standard procedures (21). DNA sequencing was performed at the DNA Sequencing Core Facility, Department of Molecular Biology, Massachusetts General Hospital, using ABI Prism DiTerminator cycle sequencing with AmpliTaq DNA polymerase FS with an ABI 377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.) (17).

Plasmids were transformed into *E. coli* JM105 by using standard techniques or were electroporated into *V. cholerae* with a Gene Pulser (Bio-Rad Laboratories,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
V. cholerae		
C6709	O1, El Tor, Inaba, wild type; Sm^r	23
Peru2	C6709 Δ*attRS1*; Sm^r	23
O395	O1, classical strain; Sm^r	Lab collection
E. coli JM105	*thi rpsL endA sbcB15 hsdR4 supE* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]; Sm^r	Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.
Plasmids		
pVC100	pUC19-based plasmid, containing the *toxR-htpG* intergenic region of *V. cholerae* El Tor E7946 as an *Eco*RI-*Dra*I fragment cloned into *Hin*cII; Amp^r	13
pMBG126	pUC18-based plasmid containing a 510-bp *Hin*dIII-*Sma*I DNA fragment that includes the iron-regulated *irgBA* genes from *V. cholerae* O395, blunt ended into *Hin*dIII and *Sph*I sites of the polylinker; Amp^r	2
pKK223-3	pBR322-based plasmid containing a multiple cloning site between the *tac* promoter and the *rrnB* transcriptional terminator; Amp^r	Pharmacia P-L Biochemicals
pETR1	pKK223-3-based plasmid, with a 393-bp *Eco*RI-*Pst*I insert containing a modified *ctxB* derived from *V. cholerae* C6709 by recombinant mutagenic PCR. *ctxB* contains a unique *Nhe*I site 6 bp 3′ to the sequence of the carboxy terminus of the CtxB signal peptide. The *tac* promoter drives *ctxB* expression; Amp^r	This study
pMCSETR1B	pETR1 derived, with replacement of a *Bam*HI-*Eco*RI fragment containing the *tac* promoter with a polylinker (*Bam*HI-*Nsi*I-*Xba*I-*Eco*RV-*Bgl*II-*Spe*I-*Eco*RI-*Pst*I). The 3′ end of the polylinker contains stop codons in all three reading frames; Amp^r	18
pETR13	pMCSETR1B derived, with a 600-bp *Bam*HI-*Xba*I fragment containing the heat shock *htpG* promoter of *V. cholerae* C6709 controlling expression of *ctxB*; Amp^r	This study
pETR12	pMCSETR1B derived, with a 255-bp *Hin*dIII-*Nco*I blunt-ended insert containing the iron-regulated *irgA* promoter of *V. cholerae* O395 cloned into the *Eco*RV site of pMCSETR1B, such that the *irgA* promoter controls expression of *ctxB*; Amp^r	This study

[a] Sm^r, streptomycin resistant; Amp^r, ampicillin resistant.

Richmond, Calif.) as instructed by the manufacturer and modified for electroporation into *V. cholerae* as previously described (8). Electroporation conditions were 2,500 V at 25-mF capacitance, producing time constants of 4.8 to 4.9 ms.

DNA restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, and Klenow fragment of DNA polymerase I were used as specified by manufacturers. *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used for thermocyclic DNA amplification, using reaction mixtures and protocols as previously described (18). Restriction enzyme-digested plasmid DNA fragments were fractionated on 1% agarose gels; required DNA fragments were removed under UV illumination and recovered using GenElute agarose spin columns (Supelco Inc., Bellefonte, Pa.).

Plasmid constructions. Plasmid pETR1 was constructed by recombinant mutagenic PCR using two oligonucleotide primer pairs (primers 1 plus 3 and primers 2 plus 4) to amplify *ctxB* from the genome of *V. cholerae* C6709 and to introduce a unique *Nhe*I site 6 bp 3′ to the coding sequence of the CtxB leader peptide. Primer 1 (5′-ACTGACAGCTGGGAATTCTAAGGATGAATTATG ATTAAATTAAAA-3′) and primer 3 (5′-AGTAATATTTTGGCTAGCAGGT GTTCCATGTGCATATGC-3′), including *Eco*RI and *Nhe*I restriction enzyme sites, respectively (underlined), were initially used to amplify a 110-bp PCR product extending from the Shine-Dalgarno sequence to that of the leader peptide of CtxB, inclusively. Primer 2 (5′-TCAGACTTCAGACTGCAGGGGC AAAACGGTTGCTTCTCATCATCG-3′) and primer 4 (5′-CATGGAACACC TGCTAGCCAAAATAATACTGATTTGTGT-3′), including *Pst*I and *Nhe*I restriction enzyme sites, respectively (underlined), were used to amplify a 406-bp PCR product that extended from the leader sequence to the stop codon of *ctxB*, inclusively. Overlap extension PCR, using primers 1 and 2, was performed with equimolar concentrations of gel-purified 110- and 406-bp PCR products for 10 cycles (94°C, 1 min; 50°C, 1 min; 72°C, 30 s). The final 393-bp fragment was cloned into the *Eco*RI and *Pst*I sites of pKK223-3 immediately downstream of the *tac* promoter.

To construct pMCSETR1B, a *Bam*HI-*Eco*RI DNA fragment containing the *tac* promoter of plasmid pETR1 was replaced with a polylinker that included *Bam*HI-*Nsi*I-*Xba*I-*Eco*RV-*Bgl*II-*Spe*I-*Eco*RI-*Pst*-I restriction enzyme sites (18). To prohibit translational read-through into *ctxB*, the 3′ end of the multiple cloning site included stop codons in all three reading frames. Plasmid pMCSETR1B, therefore, contained a multiple cloning site immediately upstream of a 393-bp promoterless *ctxB* gene from *V. cholerae* C6709.

Plasmid pVC100 contains the *toxR-htpG* intergenic region of *V. cholerae* El Tor E7946 as an *Eco*RI-*Dra*I DNA fragment inserted into the *Hin*cII restriction site of the polylinker of pUC19 (13). Plasmid pETR13 was constructed by cloning an approximately 600-bp *Bam*HI-*Xba*I DNA fragment containing the *toxR-htpG* intergenic region from pVC100 into pMCSETR1B, such that the modified *ctxB* gene was placed under the transcriptional control of the *htpG* promoter.

Plasmid pMBG126 contains a 510-bp *Hin*dIII-*Sma*I DNA fragment that includes the iron-regulated *irgBA* genes from *V. cholerae* O395; this fragment had previously been blunt-end ligated into the *Hin*dIII-*Sph*I sites of the polylinker of pUC18 (2). To construct plasmid pETR12, a 438-bp *Hin*dIII-*Eco*RV DNA fragment from pMBG126 containing the *irgA* promoter was blunt-end ligated into the *Eco*RV site of pMCSETR1B. The 438-bp insert was then truncated with *Nco*I and *Eco*RV, treated with the Klenow fragment of DNA polymerase I, and religated; this truncation removed a 183-bp nonpromoter fragment of DNA and placed *ctxB* under the transcriptional control of a 255-bp insert containing the *irgA* promoter.

Plasmids pMCSETR1B, pETR1, pETR13, and pETR12 were confirmed by restriction enzyme digestion and sequence analysis; plasmids were electroporated into *V. cholerae* vaccine strain Peru2.

In vitro analysis of various promoters controlling CtxB expression. In vitro expression of CtxB by the various *V. cholerae* vaccine strains was analyzed by measuring CtxB concentrations in culture supernatants as previously described (6, 18, 19). Overnight cultures of *V. cholerae* Peru2(pMCSETR1B), Peru2 (pETR1), and Peru2(pETR13) were grown at 25, 30, 37, and 42°C. Overnight cultures of Peru2(pMCSETR1B) and Peru2(pETR12) were grown at 37°C in LB containing ampicillin (normal iron conditions) and in LB containing ampicillin and 0.150 mM 2,2′-dipyridyl, an iron chelator producing low-iron conditions (7). Cell-free supernatants of overnight cultures were serially diluted in phosphate-buffered saline (PBS)–0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) (PBS-T; undiluted to 1:128), applied to 96-well microtiter plates previously

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TABLE 2. In vitro CtxB expression under the control of various promoters[a]

Promoter/plasmid	Mean CtxB expression (μg/ml/OD_{600}) ± SE				
	Temp (°C)				With 0.150 mM 2-2′-dipyridyl
	25	30	37	42	
*tac*p/pETR1	0.67 ± 0.67	4.64 ± 2.82	1.93 ± 0.8	0	—
*htpG*p/pETR13	0	0	0.6 ± 0.04	0	—
*irgA*p/pETR12	—	—	0.120 ± 0.007	—	4.50 ± 1.10
Control (pMCSETR1B[b])	0	0	0	0	0

[a] All experiments were performed in *V. cholerae* Peru2. Peru2(pETR1) and Peru2(pETR13) were grown at indicated temperatures. Peru2(pETR12) was grown at 37°C without or with 0.150 mM 2,2′-dipyridyl (an iron chelator). —, not determined.

[b] Contains a promoterless *ctxB* gene.

coated with 100 ng of type III ganglioside (Sigma) per well in 50 mM carbonate buffer (pH 9.6), and subsequently blocked with PBS–1% bovine serum albumin (Sigma). Plates were incubated at 37°C for 1 h and washed with PBS-T, and a 1:2,000 dilution of goat anti-CtxB (List Biological Laboratories, Inc., Campbell, Calif.) in PBS-T was applied to each well. Following incubation at 37°C for 1 h, plates were washed with PBS-T, and a 1:2,000 dilution of anti-goat immunoglobulin G (IgG)-horseradish peroxidase conjugate (Southern Biotechnology Associates Inc., Birmingham, Ala.) was added to each well. Plates were then incubated at 37°C for 1 h, washed with PBS-T, and developed with a 1-mg/ml solution of 2,2′-azinobis(ethylbenzthiazolinesulfonic acid) (ABTS; Sigma) with 0.1% H_2O_2 (Sigma). The optical density at 405 nm (OD_{405}) was read in a Vmax microplate reader (Molecular Devices Corp., Sunnyvale, Calif.) and compared with that of a standard curve generated using dilutions of purified CtxB (List) in PBS-T.

Inoculation of germfree mice. Immediately upon removal from their shipping container, four groups of 5 to 19 germfree female Swiss mice, 3 to 4 weeks old (Taconic Farms, Inc., Germantown, N.Y.), were orally inoculated by gastric intubation with 250 μl of inoculum containing approximately 10^9 CFU of Peru2(pMCSETR1B), Peru2(pETR1), Peru2(PETR13), or Peru2(pETR12) resuspended in 0.5 M $NaHCO_3$ (pH 8.0) (6). Mice were subsequently housed in non-germfree conditions. All groups of mice received a primary oral vaccination series administered on days 0, 2, 4, and 6; oral booster inoculations of 10^9 CFU in 125 μl were administered on days 28, 42, 56, and 70.

Immunological sampling. Mice were sacrificed on day 84, at which time blood, bile, and stool samples were collected, processed, aliquoted, and stored as previously described (19).

Detection of immune responses. Serum vibriocidal antibody titers were measured in a microassay as previously described (18). Anti-CtxB antibody responses were detected using microtiter plates previously coated with ganglioside and CtxB (18, 19). To detect anti-CtxB IgG and IgA antibodies in sera, duplicate samples of 1:200 dilutions of sera in PBS-T were added to wells previously coated with ganglioside-CtxB. Following overnight incubation, goat anti-mouse IgG antibody conjugated to biotin or goat anti-mouse IgA antibody conjugated to biotin (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was applied to each well. After the addition of a 1:4,000 dilution of streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, Calif.) in PBS-T, plates were developed for peroxidase activity as described above. The OD_{405} was determined kinetically with the Vmax microplate reader. Plates were read for 5 min at 19-s intervals, and the maximum slope for an OD change of 0.2 U was reported as milli-OD units per minute (6, 17).

To detect specific IgA antibody responses in stool and bile, concentrations of total IgA in stool and bile were first measured. Duplicate samples of serial twofold dilutions of processed stool (1:100 to 1:800) or bile (1:800 to 1:6,400) in PBS-T were added to wells previously coated with 100 ng of rat monoclonal anti-mouse IgA antibody R5-140 (Pharmingen, San Diego, Calif.) (19). After the addition of a 1:2,000 dilution of goat anti-mouse IgA antibody conjugated to biotin and streptavidin-horseradish peroxidase conjugate, plates were developed for peroxidase activity as described above. Comparison was made to a mouse IgA standard (Kappa TEPC 15; Sigma). To detect specific anti-CtxB antibodies in stool and bile, 200 ng of total biliary IgA (single) or 200 ng of total stool IgA (duplicate) in PBS-T was added to wells previously coated with ganglioside-CtxB (19, 20). Plates were incubated overnight at room temperature and washed with PBS-T, following which goat anti-mouse IgA antibody conjugated to biotin and streptavidin-horseradish peroxidase conjugate were added. Plates were developed for peroxidase activity, and the OD_{405} was determined kinetically.

Statistics and graphs. Statistical analysis for the comparison of geometric means was performed with the Mann-Whitney *U* test for nonparametric data using SPSS for Windows 8.0. Data were plotted using GraphPad PRISM, version 3 (GraphPad Software Inc., San Diego, Calif.).

RESULTS AND DISCUSSION

In vitro analysis of CtxB expression. Cultures of *V. cholerae* Peru2 containing plasmid pETR1, pETR13, pETR12, or control plasmid pMCSETR1B were grown under a number of in vitro conditions, and supernatants were analyzed for CtxB (Table 2). Peru2(pETR1), with the *tac* promoter controlling CtxB expression, secreted CtxB to the supernatant at a range of temperatures (25, 30, and 37°C). Peru2(pETR1) expressed approximately 2 to 5 μg/ml/OD_{600} at 30 and 37°C. Lower expression (0.5 to 1 μg/ml/OD_{600}) occurred at 25°C. CtxB was not detected in culture supernatants of Peru2(pETR1) grown at 42°C. Strain Peru2(pETR13), with the *V. cholerae* heat shock *htpG* promoter controlling CtxB expression, did not secrete detectable amounts of CtxB when grown at 25 or 30°C but did express CtxB (approximately 60 ng/ml/OD_{600}) when grown at 37°C. Peru2(pETR12), with the *irgA* promoter controlling CtxB expression, secreted approximately 40- to 50-fold more CtxB into supernatants when grown under low-iron conditions (approximately 4 to 5 μg/ml/OD_{600}) compared to expression during normal iron conditions (approximately 100 ng/ml/OD_{600}). Peru2(pMCSETR1B), containing a promoterless *ctxB* gene, produced no CtxB under any in vitro condition.

These results demonstrate that the *tac*, heat shock *htpG*, and iron-regulated *irgA* promoters were all active in vitro and that they responded to environmental conditions as expected: the *tac* promoter drove constitutive expression of CtxB under a number of in vitro conditions, the *irgA* promoter was most active in low-iron conditions, and the *htpG* promoter was most active at higher temperatures. Under the in vitro conditions tested, the constitutively active *tac* promoter and the *V. cholerae* iron-regulated *irgA* promoter (in low-iron conditions) both drove high-level and equivalent expression of CtxB.

Measurement of vibriocidal antibody responses. Vibriocidal antibodies were measured in serum samples collected on day 84 (Fig. 1). Vibriocidal antibodies are directed against *V. cholerae* organisms themselves and reflect the ability of *V. cholerae* strains to both survive in vivo and colonize the intestinal surface (20). Vibriocidal antibody levels were equivalent in all groups of animals, suggesting that in vivo survival patterns of the various *V. cholerae* vaccine strains, including Peru2 (pETR1), in which the *tac* promoter drives high-level constitutive expression of CtxB, were equivalent, as well.

Measurement of systemic and mucosal anti-CtxB antibodies. Systemic (serum) and mucosal (stool and bile) anti-CtxB antibodies were measured in samples collected on day 84 (Fig. 2). Compared to responses in mice that received control strain Peru2(pMCSETR1B), mice that received vaccine strains expressing CtxB under the control of the *tac* or *irgA* promoter had significant serum anti-CtxB IgG responses ($P \leq 0.05$),

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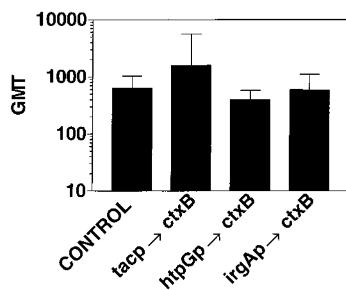

FIG. 1. Geometric mean titers (GMT) of vibriocidal antibody responses on day 84 samples. Groups of mice were inoculated with Peru2(pMCSETR1B) (CONTROL), Peru2(pETR1) (tacp → ctxB), Peru2(pETR13) (htpGp → ctxB), or Peru2(pETR12) (irgAp → ctxB). See text for details. Error bars represent standard errors of the mean for each group. Vibriocidal responses were comparable in all groups of mice.

confirming previous results (3). Compared to responses in mice that received Peru2(pETR13) expressing CtxB under the control of the heat shock *htpG* promoter, and compared to responses in mice that received Peru2(pETR13) expressing CtxB under the control of the iron-regulated *irgA* promoter, anti-CtxB responses were highest in mice that received Peru2(pETR1), the strain expressing CtxB under the control of the *tac* promoter. Mice that received *V. cholerae* Peru2 (pETR1) had the highest level of anti-CtxB responses in all samples: serum IgG ($P \leq 0.05$), serum IgA ($P \leq 0.05$), stool IgA ($P \leq 0.05$), and bile IgA ($P \leq 0.05$).

In summary, we used anti-CtxB antibody responses in mice as a surrogate marker for in vivo activity of various promoters in attenuated vaccine and vector strains of *V. cholerae*. Despite the fact that optimal activities of the *irgA* and *tac* promoters were equivalent in vitro, we found that strains expressing CtxB from the constitutively active *tac* promoter resulted in the most prominent immune responses in animals. Changing the identity of the expressed antigen may alter the relative efficiencies of the promoters that we examined, as may changing from a plasmid to a chromosomally based antigen expression system; however, for plasmid-based systems in vaccine and vector strains of *V. cholerae*, the *tac* promoter appears to be well suited for high-level in vivo expression of at least certain heterologous antigens. Since the level of expression of an antigen correlates with the prominence of induced immune responses, our data should facilitate development of effective vaccine and vector strains of *V. cholerae*.

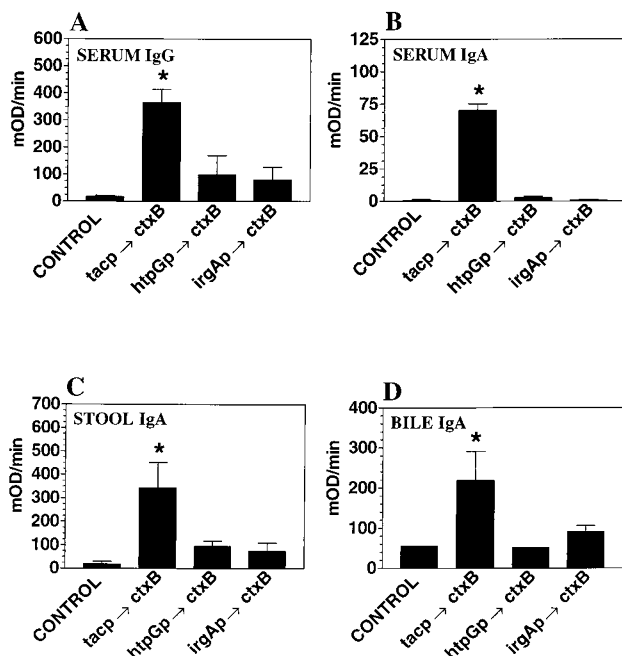

FIG. 2. Anti-CtxB enzyme-linked immunosorbent assay results on day 84 samples. Mice were inoculated orally with Peru2(pMCSETR1B) (CONTROL), Peru2(pETR1) (tacp → ctxB), Peru2(pETR13) (htpGp → ctxB), or Peru2(pETR12) (irgAp → ctxB). The geometric mean and standard error of the mean are reported for each group. *, $P \leq 0.05$ compared to mice that received Peru2(pETR13) and compared to mice that received Peru2(pETR12).

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