INFECTION AND IMMUNITY, Sept. 1999, p. 4628–4636 Vol. 67, No. 9
0019-9567/99/$04.00+0

Attenuation and Immunogenicity of Δ*cya* Δ*crp* Derivatives of *Salmonella choleraesuis* in Pigs

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Received 4 February 1999/Returned for modification 16 April 1999/Accepted 14 June 1999

Six different isogenic Δ*cya* Δ*crp* derivatives of a strain of *Salmonella choleraesuis* var. kunzendorf-χ3246 virulent for pigs were constructed by transposon-mediated deletion mutagenesis. These strains were evaluated for virulence and ability to elicit a protective immune response in young weaned pigs after oral administration and were compared to a commercially available vaccine which lacks the 50-kb virulence plasmid (vpl$^-$). These derivatives were Δ*cya* Δ*crp* vpl$^+$, Δ*cya* Δ*crp* vpl$^-$, Δ*cya* Δ(*crp-cdt*) vpl$^+$, Δ*cya* Δ(*crp-cdt*) vpl$^-$, Δ*cya* Δ*crp pmi-3834* vpl$^+$, and Δ*cya* Δ(*crp-cdt*) *pmi-3834*. In experiments to evaluate safety, no significant adverse effects of any of the vaccine constructs were observed, except that two of the strains which carried the virulence plasmid (vpl$^+$) caused a small, short-term elevation in maximum temperature compared to pretreatment temperature values. Orally immunized animals, except for those vaccinated with the Δ*cya* Δ*crp pmi-3834* vpl$^+$ strain or SC-54, developed significant serum antibody responses 21 days postvaccination as measured by enzyme-linked immunosorbent assay. No cell-mediated immune responses to heat-killed *S. choleraesuis* were noted at the same time point as measured with heat-killed bacteria as antigen in a lymphocyte proliferation assay. In an oral challenge exposure model with a highly virulent heterologous strain of *S. choleraesuis*, the Δ*cya* Δ*crp* strains with deletions in *pmi* were not protective. As measured by morbidity scores, the responses to challenge of the pigs vaccinated with the other four Δ*cya* Δ*crp* derivatives were significantly better than those of the nonvaccinated, challenged group. With the exception of temperature elevation and slight differences in diarrhea scores post-challenge, none of these strains differed significantly from each other in the other clinical parameters analyzed. While the commercial vaccine was protective by most of the parameters measured, it was not fully protective against challenge with virulent *S. choleraesuis* as judged by diarrhea scores and temperature elevation. Collectively, these data demonstrate that Δ*cya* Δ*crp* derivatives, with or without the virulence plasmid but not with deletions in the *pmi* gene, are candidates for vaccines for protection against salmonellosis in pigs.

Salmonella choleraesuis infections in swine cause a septicemic disease resulting in pneumonia and other systemic involvement, with some involvement of the intestinal tract (32, 47). In most outbreaks, mortality can be high, although morbidity is variable but usually less than 10% (47). The duration and severity of the disease in individual pigs are unpredictable, and recovered pigs have been found to be carriers and fecal shedders (47). The resulting *S. choleraesuis* reservoir in swine is of obvious concern due to its disease-causing potential for young pigs as well as its public health implications for humans (2).

Vaccination against *S. choleraesuis* is an appropriate strategy for control and prevention of this disease (47). This is particularly true because detection of carriers is difficult because of intermittent shedding of the organism (25) and because antimicrobial feed additives, which have helped to keep the disease in check, are being used with less frequency (47). The use of live-attenuated salmonellae as vaccines has been given a great deal of attention in recent years because avirulent strains of *Salmonella* are more effective than killed or subunit vaccines in inducing a protective immune response and attenuated strains colonize host tissues, stimulating secretory, humoral, and cellular immune responses (30).

Several attenuation strategies have been utilized to render *Salmonella* spp. avirulent (3, 4, 7, 10, 12). These include the use of temperature-sensitive mutants (e.g., see reference 10), auxotrophic mutants (e.g., Δ*aroA*, Δ*asd*, Δ*cys*, or Δ*thy* mutants [13, 19, 38, 43, 44]), mutants defective in purine or diaminopimelic acid biosynthesis (e.g., Δ*pur* and Δ*dap* mutants [5, 31, 35]), strains altered in the utilization or synthesis of carbohydrates (e.g., *galE* mutants [14, 20]), and mutants altered in global gene expression (e.g., Δ*cya* Δ*crp* or Δ*phoP* mutants [7, 10, 12]). As might be expected, attempts to attenuate salmonellae by these methods have led to varying degrees of success and demonstrated differences in virulence and immunogenicity (4, 5, 7, 10, 12). For instance, Δ*aroA* mutants and *galE* mutants of *Salmonella typhimurium* lacking UDP-galactose epimerase activity were avirulent and immunogenic in mice (14, 18–20). In contrast, Δ*asd*, Δ*thy*, and Δ*pur* mutants of *S. typhimurium* were avirulent in mice but also were not immunogenic when mice were challenged with the virulent parent strain (10, 34). When these same mutations were tested in *S. choleraesuis*, all mutants were reduced in virulence, but only Δ*aroA* mutants were sufficiently avirulent, and none were effective as live vaccines (33, 34).

Subsequently, Kelly et al. (23) constructed and characterized *S. choleraesuis* mutants defective in the cyclic AMP (cAMP)-cAMP receptor protein (CRP) global regulatory system. Preliminary studies have shown *S. choleraesuis* strains with Δ*cya*

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TABLE 1. Bacterial strains and vaccines

Strain	Relevant genotype	Vaccine or challenge inoculum (CFU/animal)	Source or reference
χ3246	Wild type, parent strain	1.9×10^9	23
χ3781	Δ*cya* Δ(*crp-cdt*) vpl$^+$	1.6×10^9	23
χ3923	Δ*cya* Δ(*crp-cdt*) vpl$^-$	2.2×10^9	23
χ4186	Δ*cya* Δ*crp* vpl$^+$	2.8×10^9	23
χ4497	Δ*cya* Δ*crp* vpl$^-$	4.7×10^9	This study
χ4522	Δ*cya* Δ(*crp-cdt*) *pmi-3834* vpl$^+$	2.0×10^9	This study
χ4814	Δ*cya* Δ*crp pmi-3834* vpl$^+$	3.6×10^9	This study
SC-54	vpl$^-$; unknown attenuation	Not determined[a]	40
P92-091	Amp^r	8.9×10^8	This study
UC6077			This study

[a] Vaccine administered as per manufacturer's recommendation.

and Δ*crp* mutations to be avirulent and immunogenic in BALB/c mice (23) and pigs (45). In the present report, we extend those observations by assessing the virulence and ability of a series of Δ*cya* Δ*crp* derivatives, with or without additional mutations and/or the 50-kb virulence plasmid, to induce a protective immune response in pigs. In addition, these strains were compared to a commercially available vaccine attenuated by passage five times through porcine neutrophils and found to have lost its 50-kb virulence plasmid (40).

MATERIALS AND METHODS

Bacterial strains and vaccines. The *S. choleraesuis* strains are listed in Table 1. The highly virulent strain χ3246, a swine-derived field isolate (23), was chosen as the parent strain for all subsequent genetically modified strains. They were suspended in Luria-Bertani (LB) broth (10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 10 g of NaCl per liter of deionized water) containing 20% glycerol and stored frozen at −70°C. These strains were characterized for (i) type 1 pili in static broth cultures (24, 36) and motility in motility medium composed of 1.0% casein enzyme hydrolysate (Sigma, St. Louis, Mo.), 0.5% NaCl, 0.5% agar (Difco Laboratories, Detroit, Mich.), and 50 μg of triphenyltetrazolium choride per ml; (ii) the appearance of lipopolysaccharide (LPS) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis when visualized by silver staining (17, 46); (iii) fermentation patterns on various carbohydrates and production of H_2S by using the API 20E system; (iv) growth rates both in minimal liquid medium (9) supplemented with DL-methionine (20 μg/ml) when required and 0.5% (wt/vol) of the desired carbohydrate and in Luria broth (27) by methods described previously (11); and (v) group C_1 O antigen and H antigen (poly a-z) as confirmed by slide agglutination with antisera (Difco Laboratories). These strains were all found to be *Salmonella* serotype C_1, and because the deletion in *cya* and *crp* alters their biochemical characteristics, they gave API 20E code number 4104100 (good identification as *Yersinia ruckeri* [1 of 5] and *Hafnia alvei* [1 of 213]). The reference vaccine was a commercially available vaccine (NOBL SC-54, U.S. veterinary license no. 319, serial no. 104) and was obtained from NOBL Laboratories, Inc. (Sioux Center, Iowa), through a distributor (Vetpo Distributors, Inc., Holland, Mich.). This vaccine was stored at 4°C, as per the manufacturer's recommendations. The virulent challenge strain, *S. choleraesuis* var. kunzendorf P92-091, was originally obtained from Lorraine J. Hoffman, Veterinary Diagnostic Laboratory, Ames, Iowa. This strain was stored at 4°C in lyophilized vials and was identified as *S. choleraesuis* by serotyping and API 20E.

Genetic manipulations. Transductions were performed with the bacteriophage P1L4 (8) as previously described (23). Fusaric acid selection for deletion derivatives of strains harboring Tn*10* insertions was done as described by Maloy and Nunn (29).

Animals, husbandry, and housing. Male and female (n = 72) purebred and crossbred pigs, 51.5 ± 1.5 days of age and weighing 12.7 ± 2.1 kg at vaccination, were used in the present study. These animals were obtained from the Pharmacia & Upjohn swine herd and were in overall good health, free of clinical signs of enteric diseases and negative for *Salmonella* species by microbiological culture and serology. Groups of eight pigs were housed in separate rooms (four pens/room, two pigs/pen) in a biosafety level 2 facility. Animals were acclimated to the diet and facilities for 7 days prior to initiation of the study. Pigs were fed a nonmedicated diet (S-850), and feed and water were provided ad libitum except where noted.

Bacterial inocula. Each of the Δ*cya* Δ*crp S. choleraesuis* vaccine derivatives was revived from frozen stocks by inoculating 0.1 ml of thawed culture into 10 ml of LB broth in a 16- by 125-mm polystyrene tissue culture tube (FALCON). After 14 h of static incubation at 37°C, each 10-ml culture was used to inoculate separate flasks containing 90 ml of fresh LB broth in 250-ml sterile polycarbonate Erlenmeyer flasks (Corning; Corning Glass Works, Corning, N.Y.). After a 5- to 6-h static incubation at 37°C, the resulting undiluted broth culture contained approximately 2×10^8 to 5×10^8 CFU/ml. An inoculum volume of 10 ml containing approximately 2×10^9 to 5×10^9 CFU was administered to each pig. The exact inoculum sizes are given in Table 1. Nonvaccinated control animals received 10 ml of sterile water as the vaccine inoculum. The SC-54 group was vaccinated intranasally with 2.0 ml (1.0 ml per nostril), as per the manufacturer's instructions. This inoculum was expected to contain approximately 2×10^9 CFU/pig.

The challenge strain, P92-091, was revived from lyophilization by streaking onto blood agar plates. After incubation of this culture for 18 to 24 h at 37°C, colonies from a heavy growth area were swept with a sterile loop and inoculated into several 10-ml tubes of LB broth. After 14 h of static incubation at 37°C, the 10-ml cultures were combined and 10 ml was used to inoculate each of several flasks containing 90 ml of fresh LB broth in 250-ml sterile polycarbonate Erlenmeyer flasks. After a 5- to 6-h static incubation at 37°C, the flask cultures were combined. The resulting undiluted broth culture contained 8.9×10^8 CFU/ml (Table 1). The stock broth culture was mixed with feed as described below and was used to inoculate all groups of pigs except the nonvaccinated, nonchallenged group. The nonvaccinated, nonchallenged group received LB as the inoculum. For the challenge inoculum, 10 ml of undiluted broth culture per pig was mixed with approximately 200 g of a 50:50 gruel mixture of feed and water, providing an inoculum size of 8.9×10^9 CFU/pig.

Animal vaccination and infections. Pigs were subjected to fasting for 24 h prior to inoculation and for a minimum of 30 min postinoculation with either Δ*cya* Δ*crp* derivatives, the vaccine strain, or the challenge organism. All eight pigs within a room received the same inoculum size (in bacterial numbers as well as volume) and the identical strain. For delivery of bicarbonate, Δ*cya* Δ*crp* derivatives, vaccine strains, or sterile broth (to nonvaccinated, control pigs), animals were lightly anesthetized with ketamine HCl (20 mg/kg of body weight) and restrained by hand, and the mouth was opened with a speculum to allow gastric intubation. Five to ten minutes prior to inoculation with the Δ*cya* Δ*crp S. choleraesuis* vaccine strains, stomach acidity was neutralized with 25 ml of a 10% (wt/vol) sodium bicarbonate solution by gastric intubation with a 16.5-in. SILASTIC tube (1/4-in. inside diameter and 3/8-in. outside diameter). The bacterial inoculum was then delivered by gastric intubation via the same-size tubing. The SC-54 vaccine was administered to animals intranasally as per the manufacturer's recommendations.

For challenge exposure, the mixture of feed and water served as the carrier for the inoculum containing strain P92-091. Approximately 400 g of the mixture containing the inoculum was mixed in a bowl (one per pen) and placed in the pen for consumption by the pigs (two pigs per pen). Preliminary studies showed that the counts of *S. choleraesuis* P92-091 in this feed mixture were unchanged for at least 1 h. The pigs consumed this entire amount of feed mixture in less than 15 min.

Experimental design and protective immunity. Animals were ranked from heaviest to lightest and divided into four groups of similar weight. Pigs were paired with another pig within their weight group, and one pair from each weight group was randomly assigned to each of the nine rooms. Within each room (treatment group), animal pairs were randomly assigned to one of the four pens. There were eight animals (four pens of two animals each) per treatment group.

Pigs were acclimated to the diet and housing for 7 days prior to initiation of the study. During a 4-day prevaccination period, baseline values for body temperature, fecal consistency, and physical condition were obtained for each pig. Pigs were vaccinated on day 4 of the trial, and the safety of the vaccines was assessed for 21 days by evaluation of clinical signs and shedding of the organism as noted below. At day 25, the pigs were challenged with the virulent strain, P92-091. At study day 52 or 53 (day 52/53) or at the death of the pig if earlier, necropsies were conducted on the animals and various samples were collected. Because of the large numbers of pigs involved, all postmortem necropsies could not be performed in a single day. Therefore, pigs were euthanized on day 52 or 53, the termination of the study.

Monitoring and sample collection. Rectal swabs were taken daily for the first week in both the postvaccination and the postchallenge periods. Thereafter, they were taken on Monday, Wednesday, and Friday for the remainder of the study and on day 52/53 or at necropsy for each pig for determination of the presence of *S. choleraesuis*. Two samples (1 to 2 g) of feed were collected from two different bags of feed from each production lot used in the study. These samples were cultured for *Salmonella* spp. as described below. Blood samples were collected from each pig on days 1 and 25 and at necropsy to obtain serum for antibody determinations. Blood (heparinized) for the cell-mediated immune (CMI) response assays was taken from three randomly selected pigs from each group (same pigs at each time period) on days 1 and 25 and at necropsy.

Fecal consistency, physical condition, and body temperature were evaluated daily throughout the study. Fecal consistency was scored as follows: 1 = normal, solid formed or soft with form; 2 = soft, unformed; 3 = watery with solid material; and 4 = profuse watery and/or projectile with little or no solid material. The mean of these values was converted to a diarrhea score (percent) by dividing the mean values by the maximum possible value (score of 4) and multiplying by 100%. The physical condition of the pigs was scored as follows: 1 = healthy, active, with a normal hair coat; 2 = intermediate, active, with a rough hair coat; 3 = inactive, lethargic, and/or gaunt irrespective of hair coat; or 4 = moribund.

The mean of these values was converted to a morbidity score (percent) by dividing the mean values by the maximum possible value (score of 4) and multiplying by 100%.

Body weights were recorded upon arrival (for randomization schedule), the day of vaccination, the day of challenge, and at death or necropsy. Mortality was recorded daily throughout the experiment, and moribund animals were euthanized. Necropsies were performed as soon as possible after death. Samples of tonsil, liver, lung, spleen, ileocecal mesenteric lymph node (MLN), ileocecal valve, and cecum were collected and cultured for *S. choleraesuis*, since these tissues were reported previously to yield *Salmonella* most consistently of the tissues tested from *S. choleraesuis*-infected pigs (21, 37). If present, ≥2 g of feces was collected from the descending colon and cultured for *S. choleraesuis*. All samples were stored at −70°C until culturing.

Macroscopic lesion scores typical of salmonellosis were evaluated at necropsy. The colon and cecum were scored together by a modification of the procedure of Jacks et al. (21) as follows: 0 = no lesions; 1 = mild inflammatory changes in the colon and/or cecum; 2 = cecal and/or colonic walls thickened and edematous and/or with focal or mild diffuse necrosis; and 3 = cecal and/or colonic walls thickened and edematous and/or with a fibrinonecrotic membrane. The lungs were removed intact and scored for lesions as follows: 0 = no lesions; 1 = mild lesions; 2 = moderate lesions; and 3 = severe lesions. Additional observations and comments were recorded for selected animals to characterize the disease process. The mean of these values also was converted to a percentage by dividing the mean values by the maximum possible value (score of 3) and multiplying by 100%.

Isolation of *Salmonella* species. Fecal samples (≥2 g), rectal swabs, and feed samples (≥2 g) were enriched in 10 ml of selenite cystine broth. Tissue samples were thawed at room temperature, a ≥2-g sample was minced with scissors, and the sample was homogenized for 2 min in 10 ml of selenite cystine broth with a Stomacher (Tekmar Co., Cincinnati, Ohio). The enrichments were incubated overnight (16 to 18 h) at 37°C. At the end of the incubation period, 0.1 ml of the enrichments was plated onto brilliant green agar or MacConkey agar supplemented with 100 μg of tetracycline and 100 μg of ampicillin/ml of medium (after challenge with strain P92-091 only). The agar plates were incubated at 37°C for 18 to 24 h and scored for *Salmonella* colonies.

Salmonella colonies were identified based on the phenotypic characteristics of the Δ*cya* Δ*crp* or virulent *Salmonella* strains on the medium and by serotyping. Confirmation of suspect colonies was made by using commercially available identification strips, API 20E.

Serology and ELISA. Blood samples (~10 ml) were taken from all pigs for serology on the day of, but prior to, vaccination and challenge and on the day of necropsy. An enzyme-linked immunosorbent assay (ELISA) was used to measure antibody titers. The antigen used to coat the plates for the ELISA was *S. choleraesuis* UC6077, which had been killed by heating in a boiling water bath for 10 min. The nonviable bacteria were washed in saline and suspended to approximately 1.4×10^{11} organisms per ml (as determined by viable count prior to boiling). A 1:40 dilution of this suspension was prepared in bicarbonate-carbonate buffer (pH 9.6), and 0.1 ml of this antigen was added to each well of a 96-well polystyrene ELISA microtiter plate (Corning Glass Works). The plates were incubated for 18 h at 4°C, and the wells were washed three times with 0.002 M imidazole-buffered saline containing 0.02% Tween 20 (wash buffer; Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The unreacted sites in the wells were blocked for 1 h at 37°C with 20% goat serum (Sigma Chemical Co.) in wash buffer. The plates were then washed three times. Serum samples were diluted 1:60 in wash buffer containing 10% goat serum, and 0.1 ml of this solution was dispensed to duplicate wells. One-tenth milliliter of affinity-purified, goat anti-swine immunoglobulin G conjugated to horseradish peroxidase (Kirkegaard & Perry; diluted 1:1,000 in 10% goat serum) was added to each well. The plate was incubated at room temperature for 45 min. The wells were washed three times, and 0.1 ml of 3,3′,5,5′-tetramethylbenzidine and peroxidase solution (TMB; Kirkegaard & Perry) was added to each well for 10 min. The reaction was stopped by the addition of 0.05 ml of a 1:400 dilution of hydrofluoride. The plate was read by use of an automated ELISA reader (3550 microplate reader; Bio-Rad Laboratories, Richmond, Calif.) set at 655 nm.

Lymphoproliferation (CMI) assay. Five to 10 ml (day 25) or 25 ml (day 52/53) of heparinized blood was collected by venipuncture and was mixed with an equal volume of phosphate-buffered saline (PBS; pH 6.8). The suspensions were centrifuged at 1,000 × *g* for 20 min at 10°C. The buffy coat was removed, suspended in 2.0 ml of PBS, and then placed in 12.0 ml of sterile H_2O for 40 s to lyse the erythrocytes. Immediately thereafter, 6.0 ml of 2.7% NaCl-phosphate buffer was added to restore isotonicity. This suspension was then centrifuged at 10°C and 121 × *g* for 7 min, and the supernatant was discarded. The lysis procedure was repeated. The resulting pellet was resuspended in 5.0 ml of PBS, and 2.0 ml was layered onto 3 ml of lymphocyte separation medium (Organon Teknika, Durham, N.C.). The other 3.0 ml was used for the blood cultures (see below). The cells were centrifuged at 1,000 × *g* for 20 min at 10°C, and the cells at the interface were harvested and washed once in PBS. These cells were then resuspended at approximately 5×10^6 cells per ml in RPMI 1640 medium (Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. One-tenth milliliter of the cell suspension was dispensed into each well of a 96-well microtiter plate (Corning Glass Works). Into each column (12 columns containing eight wells labeled row A to H) were dispensed cells from a different pig. Cells in wells of rows A, B, and C were treated with 0.1 ml of *S. choleraesuis* UC6077 (heat inactivated as for the ELISA, resuspended to the same density, and diluted 1:1,600). Cells in wells from rows D and E (mitogen control) were treated with 0.1 ml of phytohemagglutinin (PHA) at 5 μg/ml. Cells in rows F, G, and H (negative control) were treated with 0.1 ml of medium. The plates were incubated at 37°C with 5% CO_2 for 72 h and then pulsed with 5-bromo-2′-deoxyuridine (BrdU) for 24 h (supplied as 5-bromo-2′-deoxyuridine labeling and detection kit III [Boehringer Mannheim Biochemica, Indianapolis, Ind.]).

The BrdU assay method for detecting lymphocyte proliferation is based on an ELISA (28). The cells were fixed to the 96-well plates by first drying at 60°C for 2 h followed by fixing at −20°C with 70% ethanol in 0.5 M HCl. Next, the cellular DNA was partially digested by nuclease treatment at 37°C for 30 min. Peroxidase-labeled antibody to BrdU was added, and peroxidase substrate [2,2′-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS)] was added. The peroxidase enzyme catalyzed the cleavage of the substrate, yielding a colored reaction product which was then read by use of the automated ELISA reader with a 405-nm–490-nm dual wavelength setting. Stimulation indices were calculated by dividing the absorbance reading for the antigen- or mitogen-treated wells by the absorbance for the medium-treated wells. A stimulation index of at least 3.0 generally is required to consider a well as positive (6). The means and standard deviations of the samples were calculated, and differences between treatments were assessed by Student's *t* test.

Blood cultures. The blood samples collected on days 25 and 52/53 for the CMI response assay were cultured for *Salmonella* as per the procedure of Roof et al. (39). Two separate components of the blood samples were cultured: the packed erythrocyte fraction and the pellet from the lymphocyte separation medium gradient. Three-milliliter suspensions of both components were added to 5.0 ml of peptone water (Difco) and incubated overnight at 37°C. From these broths, 0.1 ml was plated onto blood agar (Trypticase soy agar supplemented with 5% sheep blood) and brilliant green agar plates, streaked for isolation, and incubated at 37°C for 18 to 24 h.

Statistical analysis. The data were analyzed for the two phases of the experiment, the postvaccination (safety) period and the postchallenge (efficacy) period. The analysis of the safety period included the data from day 5 to day 11, while the analysis of the efficacy period included day 26 to day 32. The same statistical analyses were conducted for both periods. The primary response variables were fecal consistency (diarrhea score), physical condition (morbidity score), body temperature, and average daily gain (ADG). The other variables measured, including mortality, culturing, immune response, lesion severity scores at necropsy, and histologic lesions, were secondary and supportive data but were not analyzed statistically.

Differences in the maximum body temperature and the average preperiod body temperature (either prevaccination [days 1 to 4] or the 4 days prechallenge on which temperature measurements were taken [days 19, 22, 24, and 25]), fecal consistency and physical scores for 1 week following vaccination or challenge, respectively, and ADG for each entire period were analyzed on an experimental unit basis with type III sums of squares from the general linear models (GLM) procedure of SAS. The homogeneity of the elements of the test term was tested with Levene's test at $\alpha = 0.01$. The normality of the data was tested with the Shapiro-Wilks test at $\alpha = 0.05$.

RESULTS

Attenuation of Δ*cya* Δ*crp* mutants in pigs. The Δ*cya* Δ*crp* *S. choleraesuis* derivatives were administered by oral gavage at doses of approximately 10^9 CFU/pig, preceded by 25 ml of a 10% (wt/vol) sodium bicarbonate solution, to assess their virulence in swine. The SC-54 vaccine was administered to animals intranasally as per the manufacturer's recommendations. As shown in Table 2, all Δ*cya* Δ*crp* *S. choleraesuis* derivatives clearly were avirulent. Pigs survived oral challenge with a number of organisms that represented ≥10 times the oral 50% lethal dose of the wild-type parent strain (data not shown). Furthermore, these animals did not show any signs of disease and remained healthy for at least 21 days after challenge. No significant clinical signs were seen for any of the parameters measured except that the strains which carried the virulence plasmids (vpl^+; χ3781 and χ4186) caused a small, short-term elevation in the maximum rectal temperature ($\overline{\times}$ = 40.9 and 41.2°C, respectively) compared to pretreatment temperature values (Table 2). The increase in maximum temperature was not significantly different between vpl^+ strains but was significantly higher than that for all other groups including the other Δ*cya* Δ*crp* strains. The low-grade fevers for the pigs given the vpl^+ strains returned to normal after peaking at 3 days after

TABLE 2. Prechallenge safety—clinical signs following vaccination with NOBL SC-54 or various vaccine candidate strains

Strain or group	Time[a]	Mortality (%)[b]	Morbidity score (%)[b]	Diarrhea score (%)[b]	% of pigs culture positive (vaccine strains)	ADG (kg)	Mean temp (°C)[c]
Nonvaccinated, challenged	Pre	0	0	1.0			39.4
	Post	0	0	1.2	0	0.73	40.2*
Nonvaccinated, nonchallenged	Pre	0	0	0			39.6
	Post	0	0	0	0	0.69	40.3*
NOBL SC-54	Pre	0	0	0			39.8
	Post	0	0	4.2	0	0.64	40.4*
χ3246	Pre	0	0	0			NT
	Post	20.0	23.3	46.2	80.0	−0.03	NT
χ3781	Pre	0	0	8.3			39.4
	Post	0	0	1.8	0	0.65	40.9**
χ3923	Pre	0	0	3.1			39.7
	Post	0	0	1.2	0	0.65	40.6*
χ4186	Pre	0	0	1.0			39.5
	Post	0	0	2.4	0	0.66	41.2**
χ4497	Pre	0	0	1.0			39.4
	Post	0	0	0	0	0.70	40.0*
χ4522	Pre	0	4.1	5.2			39.8
	Post	0	0	1.2	0	0.69	40.4*
χ4814	Pre	0	0	4.2			39.7
	Post	0	0	3.0	0	0.70	40.2*

[a] Pre, mean for the 4 days prior to vaccination; post, mean for the 1-week period postvaccination except for temperatures.
[b] Maximum possible severity is 100%.
[c] "Pre" values are the mean temperatures for the 4 days before vaccination. "Post" values are the mean maximum temperatures for the post vaccination period. NT, not tested. * and **, values in the same column with different superscripts are significantly different ($P < 0.05$).

vaccination. No significant differences were noted in the ADG between naive animals and animals given Δ*cya* Δ*crp* strains or SC-54. None of the Δ*cya* Δ*crp* derivatives nor SC-54 was recovered by rectal swab from the pigs at any day postvaccination (Table 2).

Immunogenicity of Δ*cya* Δ*crp* mutants in pigs. Twenty-one days after vaccination with the Δ*cya* Δ*crp* derivatives or SC-54, pigs were challenged with a heterologous, virulent strain of *S. choleraesuis*, P92-091. Pigs challenged with P92-091 without previous exposure to the Δ*cya* Δ*crp* derivatives or SC-54 had a moderate to severe fever, which peaked 2 days after infection ($\overline{\times} = 41.3$°C) and persisted for at least 7 days after infection (Table 3). These pigs were depressed and had diminished appetites for 2 to 3 days. Approximately 90% of the pigs experienced watery diarrhea, which persisted for half of the monitored period (21 days). Fifty percent of these pigs also shed the challenge organism for 3 to 4 days postchallenge. The ADG was significantly lower than that of the nonvaccinated, nonchallenged pigs.

It should be noted that the morbidity and diarrhea scores postchallenge reported in Table 3 are the means for the first week postchallenge, the time during which the symptoms are the worst, and not for the entire period. The statistical analysis for morbidity scores and diarrhea scores also encompasses only the first week postchallenge.

The response to challenge of pigs vaccinated with four of the Δ*cya* Δ*crp* strains (χ3781, χ3923, χ4186, and χ4497) was significantly better than that of the nonvaccinated, challenged group and was not significantly different from that of the nonchallenged, nonvaccinated group in physical condition (morbidity scores) and ADG. Similarly, these pigs also had significantly less diarrhea, lower temperature responses, and less shedding of the challenge organism than did nonvaccinated, challenged animals. Animals vaccinated with either χ3781 or χ3923 did not shed the challenge organism at all. Except for temperature elevation and diarrhea scores for the first week postchallenge (Table 3), none of the four Δ*cya* Δ*crp* strains differed significantly from each other in the categories analyzed.

Pigs challenged with P92-091 after prior exposure to Δ*cya* Δ*crp* strains with an additional deletion in the gene encoding phosphomannose isomerase (*pmi*; χ4522 and χ4814) were not protected. Morbidity scores, diarrhea scores, ADGs, and mean maximum temperatures of animals in these groups were not significantly different from those of the nonvaccinated, challenged group or of each other (Table 3). In addition, the number of pigs with positive rectal swab cultures, the average number of culture-positive days, and the percent days of shedding were slightly greater for pigs vaccinated with these strains than for the nonvaccinated, challenged group (Table 3).

Pathology and bacteriologic examinations. In this study, only one animal died prior to termination. This animal had lesions consistent with septicemia caused by *S. choleraesuis*. Lesion scores for all animals at necropsy are summarized in Table 4. The challenge strain, *S. choleraesuis* P92-091, did not induce macroscopic lung lesions in these pigs. This was somewhat unexpected, since in preliminary studies with animals of similar age and size we had seen lung lesions in pigs suffering

TABLE 3. Postchallenge efficacy—clinical signs after challenge with virulent *S. choleraesuis* P92-091[e]

Strain or group	Time[a]	Mortality (%)[b]	Morbidity score (%)[b]	Diarrhea score (%)[b]	% of pigs culture positive (92-091)	ADG (kg)[c]	Mean temp (°C)[d]
Nonvaccinated, challenged	Pre	0	0	0		0.73	39.5
	Post	0	16.1*	50.0*	50.0	0.69*†	41.3*
Nonvaccinated, nonchallenged	Pre	0	0	0		0.69	39.8
	Post	0	0†	0†	0	0.99‡	40.2†
NOBL SC-54	Pre	0	0	0		0.64	39.6
	Post	0	3.6†	14.9‡	62.5	0.89‡	41.3*
χ3781	Pre	0	0	0		0.65	39.5
	Post	0	0†	11.9‡§	0	0.94‡	40.1†‡
χ3923	Pre	0	0	0		0.65	39.7
	Post	0	0†	7.7†§	0	0.88*‡	40.8§
χ4186	Pre	0	0	0		0.66	39.6
	Post	0	1.2†	3.6†	25.0	1.00‡	40.4‡§
χ4497	Pre	0	0	0		0.70	39.3
	Post	0	0.6†	10.7‡§	12.5	0.89‡	40.3§
χ4522	Pre	0	0	0		0.69	39.7
	Post	0	17.3*	43.5*	62.5	0.54†	41.7*
χ4814	Pre	0	0	0		0.70	39.6
	Post	12.5	19.1*	44.4*	87.5	0.63†	41.3*

[a] Pre, mean values 1 week prior to challenge; post, mean values 1 week postchallenge.
[b] Maximum possible severity is 100%.
[c] "Pre" values are for vaccination period; "post" values are for period from challenge to necropsy (or death).
[d] "Pre" values are the mean temperatures for the 4 days prior to challenge; "post" values are the mean maximum temperatures for the postchallenge period.
[e] Values in the same column with different superscripts (*, †, ‡, and §) are significantly different ($P < 0.05$).

lethal infection with this strain (data not shown). However, two animals in the SC-54 group did have a mild interstitial pneumonia, reported to be consistent with a systemic lesion of *S. choleraesuis* infection. Lesion scores from the cecum and/or colon were more severe than those from the lungs. Lesion scores for pigs vaccinated with the Δ*cya* Δ*crp* Δ*pmi* strains were more severe than scores for the nonvaccinated, challenged group. Interestingly, lesion scores for χ3923-vaccinated pigs also were higher than those for the nonvaccinated group, even though pigs vaccinated with this strain were never positive by rectal swab or fecal culture for virulent *S. choleraesuis*. Lesions in the cecum and/or colon were consistent with *S. choleraesuis* pathogenesis (37).

Lung, liver, spleen, MLN, ileocecal junction, and feces have been reported to yield *Salmonella* isolates from *S. choleraesuis*-infected pigs (37). Necropsy of most of the animals was conducted on day 28 or 29 postchallenge (day 52/53). Recovery of the challenge strain, *S. choleraesuis* P92-091, from tissues at necropsy was limited to the MLN in one pig from the χ4497-vaccinated group and the liver and MLN of the pig that died in the χ4814-vaccinated group. No Δ*cya* Δ*crp* strains were recovered from the vaccinated pigs at necropsy. No *S. choleraesuis* P92-091 organisms were recovered from the fecal samples at necropsy. In contrast to the success reported by Roof et al. (39) in recovery of *S. choleraesuis* from the blood of infected pigs, no *S. choleraesuis*, wild type or vaccine strain, was cultured from the buffy coat or the packed erythrocyte fractions of the blood which was taken at day 25 or at necropsy.

Serum antibody responses. The humoral immune response measured by ELISA (Table 5) revealed that groups vaccinated with all of the Δ*cya* Δ*crp* strains except χ4814 responded with a significant increase in antibody titer by 21 days postvaccination. No significant increase in antibody response was seen with nonvaccinated pigs or pigs vaccinated with χ4814 or SC-54. Four weeks after challenge (study day 52/53) with virulent *S. choleraesuis*, all groups except the nonvaccinated, nonchallenged group showed a significantly increased antibody response to *S. choleraesuis* antigen.

CMI responses. The CMI response data are summarized in Table 6. For a stimulation index (absorbance of test sample/background absorbance) to be considered positive, it generally should exceed 3.0 (6). As measured by antigen-reactive cells in the peripheral blood, there was no significant CMI response to any of the vaccine strains at 21 days postvaccination. This was

TABLE 4. Gross lung and large intestinal lesion severity scores postchallenge and tissue colonization at necropsy

Strain or group	Lesions (%[a]) at necropsy	
	Lung	Cecum and/or colon
Nonvaccinated, challenged	0	12.7
Nonvaccinated, nonchallenged	0	0
NOBL SC-54	4.3	8.3
χ3781	0	4.3
χ3923	0	21.0
χ4186	0	0
χ4497	0	0
χ4522	0	46.0
χ4814	0	21.0

[a] Mean percentage of maximal score (score of 3).

TABLE 5. Antibody responses to *S. choleraesuis* as measured by ELISA[a]

Strain or group	ELISA absorbance at 655 nm for sera collected at:		
	Day 1 (prevaccination)	Day 25 (prechallenge)	Day 52/53 (necropsy)
Nonvaccinated, challenged	0.094 ± 0.013*	0.149 ± 0.039*	0.770 ± 0.133**
Nonvaccinated, nonchallenged	0.128 ± 0.019*	0.305 ± 0.218*	0.340 ± 0.240*
NOBL SC-54	0.137 ± 0.030*	0.294 ± 0.159*	0.521 ± 0.215**
χ3781	0.144 ± 0.006*	0.349 ± 0.105**	0.452 ± 0.094***
χ3923	0.103 ± 0.013*	0.228 ± 0.084**	0.549 ± 0.189***
χ4186	0.097 ± 0.014*	0.328 ± 0.151**	0.489 ± 0.239**
χ4497	0.102 ± 0.033*	0.253 ± 0.103**	0.657 ± 0.253***
χ4522	0.085 ± 0.002*	0.217 ± 0.111**	0.623 ± 0.159***
χ4814	0.126 ± 0.023*	0.227 ± 0.094*	0.696 ± 0.220**
Positive control (59-9)	ND	1.114	1.113
Negative control sera	0.106	0.134	0.130

[a] ND, not determined. * to ***, values with different superscripts within rows differed significantly ($P < 0.05$).

unexpected, since others have reported that oral administration of a recombinant derivative of χ3781 resulted in a positive delayed-type hypersensitivity (DTH) response in four of four vaccinated pigs at 28 days postvaccination (45). That these lymphocytes may not be detected unless the timing of their sampling is precise was shown by the results obtained with the positive-control pig. This pig had been hyperimmunized with acetone-killed *S. choleraesuis* 7 days prior to bleeding and had a high-titer antibody response as measured by ELISA (Table 5) on study day 25 and day 52/53. At day 25, when this animal had not been revaccinated for more than 2 months, a positive stimulation index was not obtained even though its ELISA titer was very high (Table 5). In anticipation of the day 52/53 bleed, the positive-control pig was immunized 7 days prior to the bleeding with acetone-killed *S. choleraesuis*. The stimulation index of these cells for the *S. choleraesuis* antigen was positive. These results suggest that the lymphocyte proliferation assay may not be as useful as a DTH assay for measuring CMI.

After challenge with *S. choleraesuis* P92-091 at day 52/53 (the day of necropsy and 28/29 days postchallenge), several of the groups had positive stimulation indices (>3.0). Perhaps not unexpectedly, the group of pigs which was most severely affected by the challenge with *S. choleraesuis* P92-091, the group vaccinated with strain χ4814, had the lowest stimulation index at day 52/53. The nonspecific response of the lymphocytes to the mitogen PHA was positive at all times for all groups.

DISCUSSION

Live vaccines in general are more effective against salmonellosis than are killed vaccines (3, 11). This is thought to be because live vaccines better stimulate cellular immune responses (26), perhaps because of greater persistence (22) and/or because different antigens are expressed in vivo (42). *Salmonella* strains that lack the ability to synthesize adenylate cyclase and CRP have been found to be nonvirulent for mice, chickens, and swine and effective as live vaccines when given by injection or by feeding (16). In order to better characterize this attenuation strategy, we constructed a series of isogenic Δ*cya* Δ*crp* mutants of *S. choleraesuis* χ3246, a strain virulent for pigs, with or without additional deletions in genes associated with virulence (*cdt*) or synthesis of LPS (*pmi*) and/or cured of the virulence plasmid (vpl). The aim was to assess the effect of additional deletions on strains that were proven to be effective live-vaccine constructs but which showed different degrees of attenuation in a rodent model (23).

The results of the present study demonstrated that all of the Δ*cya* Δ*crp* *S. choleraesuis* derivatives tested are safe for swine in that they showed essentially complete loss of virulence. This is not entirely unexpected, since many genes and operons are under the control of cAMP and CRP (11). For instance, Δ*cya* and Δ*crp* mutants are impaired in their abilities to transport and break down carbohydrate and amino acid catabolites (1) and are unable to synthesize functional fimbriae (41). Strains harboring a deletion in the *cdt* gene are unable to penetrate and persist in deeper tissues (23). Nonetheless, it was somewhat surprising to note that there was no apparent difference in virulence of, for example, χ3781 and χ4186. A comparison of these two strains in mice showed that χ3781 was less virulent than χ4186 (23). These strains are identical except that χ3781 carries an additional deletion in a gene(s) adjacent to *crp* that is also associated with *S. choleraesuis* virulence and may be involved in the colonization of deep tissues (23). It may be that *cya* and *crp* derivatives of *S. choleraesuis* are sufficiently attenuated such that deletion of additional genes found on the virulence plasmid that are necessary for, or contribute to, virulence would result in a phenotype that is unmeasurable when the strain is given orally to swine. This can be noted from the finding that curing of the virulence plasmid from χ3781 and

TABLE 6. CMI response in vaccinated pigs[a]

Strain or group	Pigs tested	Stimulation index at day of experiment					
		Day 1 (prevaccination)		Day 25 (prechallenge)		Day 52/53 (necropsy)	
		SC	PHA	SC	PHA	SC	PHA
Nonvaccinated, challenged	2,3,7	0.94 ± 0.66	4.32 ± 2.06	0.97 ± 0.06	3.72 ± 0.71	3.22 ± 2.63	5.99 ± 2.24
Nonvaccinated, nonchallenged	10,13,14	1.34 ± 0.81	5.68 ± 2.84	0.93 ± 0.06	4.42 ± 0.33	2.36 ± 1.01	5.47 ± 1.93
NOBL SC-54	65,70,71	1.61 ± 1.00	6.38 ± 2.71	0.67 ± 0.49	4.65 ± 2.0	2.65 ± 1.25	4.31 ± 1.83
χ3781	34,35,36	1.49 ± 0.88	5.86 ± 2.60	0.96 ± 0.23	3.47 ± 0.47	3.32 ± 1.26	5.08 ± 1.45
χ3923	41,42,46	2.14 ± 0.42	7.78 ± 1.76	0.66 ± 0.08	3.67 ± 0.70	3.70 ± 0.96	4.12 ± 0.99
χ4186	17,22,24	0.67 ± 0.24	4.08 ± 1.19	1.06 ± 0.33	4.33 ± 1.59	3.55 ± 2.02	6.0 ± 0.44
χ4497	28,30,32	1.08 ± 1.13	3.91 ± 2.93	0.82 ± 0.14	3.67 ± 1.01	4.43 ± 1.19	5.05 ± 0.94
χ4522	57,60,62	1.41 ± 0.51	7.13 ± 4.66	1.06 ± 0.25	5.22 ± 2.80	2.55 ± 0.63	6.02 ± 1.95
χ4814	54,55,56	1.59 ± 0.35	6.70 ± 1.26	1.03 ± 0.53	4.08 ± 2.58	1.93 ± 0.72	3.47 ± 1.09
Positive control	59-9	ND	ND	1.18	5.34	4.11[b]	7.23

[a] SC, response to *S. choleraesuis* antigen; PHA, response to PHA; ND, not determined.
[b] Vaccinated 7 days before bleeding.

χ4186 (resulting in strains χ3923 and χ4497, respectively) did not further attenuate these strains (Table 2). The same was true for strains χ4522 and χ4814, which contain the virulence plasmid but carry an additional deletion in a gene (*pmi*) involved in the synthesis of LPS. In contrast, Gulig and Curtiss (15) showed that vpl$^-$ strains of *S. typhimurium* were less invasive, and therefore more attenuated, than vpl$^+$ strains.

Nonetheless, even though all of the Δ*cya* Δ*crp* derivatives studied were sufficiently attenuated to be of interest as vaccine candidates, they were not all protective to the same degree. It should be noted that the challenge model used did not cause excessive mortality but induced disease of sufficient severity to make possible a number of important conclusions concerning the protective efficacy of the various vaccine strains used in this study.

Pigs vaccinated once with Δ*cya* Δ*crp* derivatives, with or without the virulence plasmid, were well protected against challenge with ca. 10 50% lethal doses of *S. choleraesuis* P92-091. In contrast, similar vaccination of pigs with Δ*cya* Δ*crp* strains carrying an additional deletion in a gene involved in LPS biosynthesis did not protect against challenge with *S. choleraesuis* P92-091. This result is compatible with the hypothesis that the O antigen is the main protective immunogen in *Salmonella* species (34). However, we have not ruled out the possibility that the strains carrying a deletion in the *pmi* gene are less able to persist in pigs or are unable to induce a protective immune response for some other reason.

Maximum temperature increases in animals vaccinated with one of the four effective Δ*cya* Δ*crp* derivatives were significantly lower than those in animals in the nonvaccinated, challenged group and the Δ*cya* Δ*crp* Δ*pmi* strain-vaccinated group. However, only pigs vaccinated with χ3781 had mean maximum temperatures which were not significantly different from those of the nonvaccinated, nonchallenged group. The maximum temperature of pigs vaccinated with the other vpl$^+$ strain, χ4186, while not significantly different from that of χ3781-vaccinated animals, was different from the maximum temperature of the nonvaccinated, nonchallenged group. Further statistical analysis of the area under the curve (AUC) for the temperatures after challenge with *S. choleraesuis* P92-091 generally supported the conclusions from analysis of the mean maximum temperature. Pigs vaccinated with strain χ3781 and χ4186 had temperature AUCs which were significantly lower than those of animals in the nonvaccinated, challenged group and the Δ*cya* Δ*crp* Δ*pmi* strain-vaccinated groups but which were not significantly different from those of animals in the nonvaccinated, nonchallenged group. The apparently poorer protective activity of χ4497, based solely on temperature maximum and AUC, is something of a statistical aberration (and probably not of biological significance), since pigs in this group had a lower baseline temperature before challenge (Table 3).

Of the effective Δ*cya* Δ*crp* strains, no shedding of virulent *S. choleraesuis* was noted from pigs vaccinated with χ3781 or χ3923, while only a few pigs which were vaccinated with χ4186 and χ4497 shed virulent *S. choleraesuis*. The former two vaccine constructs contained an additional deletion in the *cdt* gene. It may be that these two strains, which are less invasive (data not shown), remained more associated with the gut mucosa and caused a more localized immune response, which resulted in increased protection against gut colonization by the challenge strain. The average number of days of shedding of virulent *S. choleraesuis* was slightly higher for the pigs vaccinated with the *pmi*-deletion strains than for the nonvaccinated, challenged group (Table 3).

While the NOBL SC-54 vaccine was protective by most of the parameters measured, it was not fully protective as judged by the diarrhea scores, temperature elevation, and shedding of virulent *S. choleraesuis* postchallenge. The diarrhea scores, although significantly improved over those of the nonvaccinated, challenged group, were significantly higher than those for pigs given χ3923 and χ4186. The maximum temperature elevation for pigs given SC-54 was significantly higher than the maximum temperature of those given the effective Δ*cya* Δ*crp* strains and of pigs in the nonvaccinated, nonchallenged group. In fact, the maximum temperature of the SC-54 group was identical to that of the nonvaccinated, challenged group (Table 3). Moreover, the percentage of animals shedding the virulent challenge strain and the duration of shedding for pigs given SC-54 were significantly greater than those for pigs given the four effective Δ*cya* Δ*crp* strains. The percentage of pigs shedding after challenge was even higher in the group vaccinated with SC-54 than in the nonvaccinated, challenged group.

The lack of recovery of the vaccine strains or of *S. choleraesuis* P92-091 at the time of necropsy implied that these strains either do not invade tissues, do not persist in the tissues if they do invade, or are present only in low numbers and were not efficiently isolated by our culture techniques. In mice, both χ3781 and χ4186 were invasive and persisted in the blood, Peyer's patches, and spleen for at least 28 days postinoculation (23). Similarly, Stabel and coworkers found that a recombinant derivative of χ3781 could invade and persist in the spleens and livers of pigs for 14 to 21 days (45), but they used an inoculum that was at least 10-fold larger than the vaccine inoculum used in the present study. They did not report whether they could recover the isolate by culturing rectal swabs. Since, in order to evaluate immunogenicity, we did not sample tissues until 28 days after challenge with virulent *S. choleraesuis* (49 days after vaccination), it is possible that we missed the window of persistence of these strains. We were able to culture *S. choleraesuis* P92-091 from the liver and MLN of the sole pig that died due to salmonellosis at 5 to 6 days postchallenge. Also, an incubation temperature of 37°C for cultural isolation was used in the present study. Subsequent experiments performed in order to enhance our culturing efficiency showed that a higher temperature, 39°C, provided better growth of *S. choleraesuis* and reduced the growth of contaminants in enrichment broths (data not shown). Thus, it may be that our culturing technique was not optimized for recovering *S. choleraesuis* in this study.

In an attempt to evaluate the induction of specific immune responses, anti-*S. choleraesuis* antibodies and antigen-reactive cells in the peripheral blood were measured. Although serum antibody titers rose significantly following vaccination with the Δ*cya* Δ*crp* strains (except χ4814), it is unclear what role vaccination played in eliciting anti-*Salmonella* antibody responses, as the nonvaccinated, nonchallenged pigs had the highest ELISA value (0.305). All but two vaccine strains (χ3781 and χ4186) induced lower responses at this time, and the two strains that induced higher responses induced only slightly higher responses. Since the nonvaccinated animals showed increases in ELISA values, this result may be nonspecific. Similarly, the highest response of all groups at day 52/53 was observed in the naive but challenged animals (ELISA value = 0.77). Thus, this suggests that challenge and not vaccination was primarily responsible for the increase in anti-*Salmonella* titers noted at the time of challenge.

No CMI response to any of the vaccine strains at 21 days postvaccination was noted by the lymphocyte proliferation assay. This was unexpected, since Stabel and coworkers found that oral administration of a recombinant derivative of χ3781 resulted in a positive DTH response in four of four vaccinated pigs at 28 days postvaccination (45). However, as mentioned above, their vaccination dose was at least 10-fold higher than

that used here, the antigen preparations used to test antigen reactivity were different, and these assays, although having certain of the cell types in common, measure very different types of responses. In the DTH reaction, macrophages activated by antigen-reactive T cells are primarily responsible for the reaction measured. In the lymphoproliferation assay used here, only antigen-specific T cells circulating in the blood are measured and the time of their presence in the blood is related to the timing of antigen stimulation (6). That these lymphocytes may not be detected unless the timing of their sampling is precise was shown by the results obtained with the positive-control pig, which had been hyperimmunized with acetone-killed *S. choleraesuis* and had a high-titer antibody response as measured by ELISA on day 25 and day 52/53 (Table 5). At day 25, when this animal had not been revaccinated for more than 2 months, a positive stimulation index was not obtained even though its ELISA titer was very high. However, when this pig was immunized with acetone-killed *S. choleraesuis* 7 days prior to the bleeding, the stimulation index then became positive. These results suggest that the lymphocyte proliferation assay may not be as useful as a DTH assay for measuring CMI response. After challenge with *S. choleraesuis* P92-091 at day 52/53 (the day of necropsy and 28/29 days postchallenge), several of the groups had positive stimulation indices (>3.0). The group of pigs which was most adversely affected by the challenge with *S. choleraesuis* P92-091, the group vaccinated with strain χ4814, also had the lowest stimulation index at necropsy (day 52/53). The nonspecific response of the lymphocytes to the mitogen PHA was positive at all times for all groups.

In summary, we evaluated the safety and protective efficacy in pigs of six Δ*cya* Δ*crp* isogenic constructs of *S. choleraesuis* χ3246 compared to those of a commercial vaccine which was attenuated by accidental deletion of the virulence plasmid upon serial passage through neutrophils in vitro (25, 40). Four of these strains, χ3781, χ3923, χ4186, and χ4497, were found to be as protective as or more protective than the commercially available vaccine. Moreover, the percentage of animals shedding the virulent challenge strain and the duration of shedding were significantly lower in pigs given the four effective Δ*cya* Δ*crp* strains than in pigs given the commercial vaccine.

ACKNOWLEDGMENTS

We thank Sarah A. Salmon, Gates M. Baird, Gerald R. Bos, and Mary Jane Duke for excellent technical assistance.

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Editor: J. R. McGhee