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Characterization and Protective Properties of Attenuated Mutants of *Salmonella choleraesuis*

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We have constructed *crp*::Tn*10* and *cya*::Tn*10* *Salmonella choleraesuis* mutants and their fusaric acid-resistant derivatives with deletions (Δ) of the Tn*10* and adjacent DNA sequences and found them to be avirulent and able to induce protection against a wild-type challenge in 8-week-old BALB/c mice. Mice survived infection with the *crp* and *cya* mutants at doses of more than 7×10^3 times the oral (p.o.) 50% lethal dose (LD_{50}) and more than 8×10^2 times the intraperitoneal LD_{50} of the wild-type *S. choleraesuis* parent. Mice vaccinated with attenuated strains were protected against challenge with more than 1.6×10^4 times the p.o. LD_{50} and more than 80 times the intraperitoneal LD_{50} of the wild-type virulent *S. choleraesuis* parent. One deletion mutation isolated in the *crp* region extends to an adjacent gene(s) that was shown to be associated with avirulence. This gene or operon has been designated *cdt* (colonization of deep tissues). A Δ(*crp-cdt*)*19* strain, when complemented with the wild-type *crp* gene and promoter on a pBR-derived plasmid, had p.o. LD_{50} values 10^3 times higher than those for the wild type. A Δ*cya* Δ(*crp-cdt*)*19* double mutant was less virulent than and afforded more complete protection against a challenge with the wild-type strain than a Δ*crp*-11 Δ*cya* double mutant or the individual *cya*, *crp*, or *crp*$^+$/*cdt* mutants. The deletion derivatives exhibited reduced invasion of CHO cells in vitro, and the numbers of the mutants recovered from mouse tissues were less than that of the parent strain. These studies suggest that one or more of the genes involved in cell attachment to and/or invasion of *S. choleraesuis* may be under catabolite repression. In addition, we describe a new deletion of a gene(s) located in the *crp* region between *cysG* and *argD* that is associated with virulence in *S. choleraesuis*.

Salmonella choleraesuis is host adapted to swine and is often the etiologic agent of a fatal septicemic disease with little involvement of the intestinal tract (33, 57). The resulting *S. choleraesuis* reservoir in swine is a concern, not only because of its disease-causing potential in young pigs but also because of its public health implications for humans (4). Although swine are the natural hosts, mice are frequently used as experimental animal models to study the pathogenesis of the disease (18, 37, 38, 51).

Currently, there is little information on vaccine use for the control of *S. choleraesuis* infections. A rough variant of *S. choleraesuis* used by Smith (51) in an attempt to demonstrate protection of pigs against a challenge with the virulent *S. choleraesuis* parent resulted in the development of fevers and sublethal diseases following an oral (p.o.) challenge.

Several approaches have been utilized in the construction of attenuated and immunogenic *Salmonella* vaccine strains. Germanier and Fürer found that a *galE* mutant of *Salmonella typhimurium* lacking UDP-galactose epimerase activity was avirulent and immunogenic in mice (17). Hohmann et al. found significant quantities of intestinal and serum immunoglobulin A antibody in mice immunized p.o. with the *galE S. typhimurium* mutant G30 (22). Hone also found that a genetically engineered Δ*galE* derivative of *S. typhimurium* was avirulent and immunogenic in mice (24). Hoiseth and Stocker initially isolated Δ*aroA* mutants of *S. typhimurium* which were avirulent and immunogenic in mice (23), cattle (44, 50), and sheep (35). Δ*asd*, Δ*thy* (9), and *pur* (32, 39) mutants of *S. typhimurium* were avirulent in mice but were not immunogenic when mice were challenged with the virulent parent strain. *S. typhimurium* strains with Δ*cya* and Δ*crp* mutations, which eliminate the ability to synthesize adenylate cyclase (ATP pyrophosphate lyase [cyclizing] [EC 4.6.1.1]) and the ability to synthesize the cyclic AMP (cAMP) receptor protein (CRP), respectively, are avirulent and immunogenic in BALB/c mice (10). Preliminary studies have shown the Δ*cya* Δ*crp* *Salmonella* strains to be avirulent by the p.o. route in chickens (11) and pigs (52, 53). In addition, *Salmonella typhi* Δ*cya* Δ*crp* mutants cause an occasional febrile response (54).

Attempts to attenuate *S. choleraesuis* by the methods discussed above for *S. typhimurium* have demonstrated the differences in virulence and immunogenicity in these two species. Nnalue and Stocker (37, 38) reported that galactose-sensitive, *galE* *S. choleraesuis* strains had reduced virulence, whereas galactose-resistant, *galE* derivatives remained as virulent as the wild-type Gal$^+$ parent in mice. When Δ*aroA*, Δ*thy*, and *pur* *S. choleraesuis* derivatives were tested for avirulence with mice, all of the mutants were reduced in virulence by the intraperitoneal (i.p.) route. However, only the *aroA* derivatives were sufficiently avirulent, and they were not effective as live vaccines (38).

Since attenuation strategies successfully demonstrated for *S. typhimurium* have had limited success for *S. choleraesuis*, we have studied *S. choleraesuis* mutants defective in the cAMP-CRP global regulatory system. We have constructed Δ*cya*, Δ*crp*, and Δ*cya* Δ*crp* derivatives of an *S. choleraesuis* bv. kunzendorf strain and determined their (i) virulence properties after p.o. and i.p. inoculation of BALB/c mice, (ii) colonization of and persistence in various mouse tissues, (iii) adherence to and invasive properties in mammalian cells, (iv) resistance to serum, and (v) efficacy in inducing protective immunity against p.o. and i.p. challenges with the virulent wild-type parent strain. In addition, we describe a mutation located between *argD* and *cysG* that encompasses not only *crp* but another gene(s) nearby that significantly attenuates *S. choleraesuis*.

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MATERIALS AND METHODS

Bacterial strains. The *S. choleraesuis* strains are listed in Table 1. The highly virulent strain χ3246, a swine-derived field isolate kindly provided by W. Fales (Veterinary Medical Diagnostic Laboratory, School of Veterinary Medicine, University of Missouri, Columbia), was chosen as the parent for all subsequent genetically modified strains. All strains were characterized for (i) type 1 pili in static broth cultures (27, 40) and motility in 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 chloride per ml, (ii) the appearance of lipopolysaccharide in sodium dodecyl sulfate-polyacrylamide gel electrophoresis when visualized by the silver-staining procedure as described previously (21, 56), (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 (7) supplemented with DL-methionine (20 μg/ml) when required and 0.5% (wt/vol) of the desired carbohydrate and in Luria broth (30) by methods described previously (10), and (v) group C_1 O antigen and H antigen (poly a-z) as confirmed by slide agglutination with antisera (Difco Laboratories).

Genetic manipulations. Transductions were performed with the bacteriophage P1L4 (8) or P22 HT*int* (2, 45) according to standard methods. Fusaric acid selection for deletion derivatives of strains harboring Tn*10* insertions was done as described by Maloy and Nunn (31). The plasmid pSD110 contains a 1.3-kb *Bam*HI-*Eco*RI fragment of *S. typhimurium* LT2 cloned into pBR322 (48) and was generously provided by C. Schroeder. This 1.3-kb fragment contains the *crp* promoter region and structural gene with approximately 300 bp of flanking DNA at the 5′ and 3′ ends.

Animal infections and protective immunity. Eight-week-old female BALB/c mice (Sasco, Omaha, Nebr.) were used for all infectivity and protection experiments. Methods for growth and preparation of mice and inoculation with *S. choleraesuis* were as described by Curtiss and Kelly (10).

Mice were inoculated p.o. or i.p. with various doses of the avirulent mutants. Thirty days later, mice that survived were challenged by the same route with 10^3 to 10^4 times the p.o. and 80 times the i.p. 50% lethal doses (LD_{50}) of the wild-type, virulent parent strain, χ3246. Morbidity and mortality were observed for an additional 45 days after the challenge with the wild-type strain. Deaths were recorded over the course of the experiment, and the LD_{50} were calculated by the method of Reed and Muench (43).

Enumeration of viable *S. choleraesuis* in mice. Necropsy procedures were as described by Curtiss and Kelly (10). Heart blood samples were collected with heparinized microhematocrit capillary tubes. The Peyer's patches and spleens were homogenized with a tissue homogenizer (Brinkmann Instruments, Inc., Westbury, N.Y.) in buffered saline with 1.0% gelatin added as a wetting agent (6). *S. choleraesuis* was recovered from these homogenates by plating on MacConkey agar (Difco Laboratories) supplemented with 1.0% (wt/vol) maltose. CFU are presented as geometric means with standard errors (n = four mice per time point). Antisera to *Salmonella* group C_1 O antigen (Difco Laboratories) were used to confirm that Mal^- colonies isolated from blood samples and tissues were serotype *S. choleraesuis*.

CHO cell adherence and invasion. Methods for the growth of bacteria and assays for the infection of CHO cells have been described previously (15, 19).

Bacterial transcytosis assay. Assays for bacterial transcytosis of polarized Madin-Darby canine kidney (MDCK) cell monolayers were performed by using the methods of Finlay et al. (14), with some modifications. Briefly, MDCK cells were grown on Costar Transwell filter units in Eagle's minimal essential medium (Sigma) containing 30 mM $NaHCO_3$–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) until they had reached confluent growth of approximately 3.5×10^5 cells per filter. A mixed inoculum of strains χ3246 (wild type) and χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] was added to the apical surface-polarized monolayers (in triplicate) at a ratio of 0.78 (wild type to mutant). Each hour, the basolateral medium was removed from each well, and χ3246 and χ3781 (which had transcytosed) were enumerated. The wild-type χ3246 and the Δ*cya-12* Δ(*crp-cdt*)*19* mutant χ3781 were distinguished on MacConkey agar supplemented with 1% maltose.

Serum resistance. Bacteria were grown at 37°C to log phase in L broth and then diluted in buffered saline with 1.0% gelatin to approximately 10^6 CFU/ml. A 10-μl sample containing approximately 10^4 CFU was added to 90 μl of normal rabbit serum (final serum concentration, 90%; unabsorbed and buffered with 20 mM HEPES, pH 7.2) which had been preequilibrated in a 2.5% CO_2 atmosphere to control the pH. The mixture was incubated at 37°C in a 2.5% CO_2 atmosphere. After 1 h, samples were diluted and plated on MacConkey agar containing 1% maltose for enumeration of CFU. *S. typhimurium* Δ(*galE-chl-uvrB*)*1005* χ3477 was used as a positive control for complement-mediated bacteriolysis.

RESULTS

Characterization of mutant strains of *S. choleraesuis*. The phenotypic characteristics of the wild-type *S. choleraesuis* strain, χ3246, and those of its deletion mutants are listed in Table 2. Type 1 pili could not be detected on the wild-type strain, χ3246, or its derivatives; *S. typhimurium* SL1344 χ3339 served as a positive control. The Δ*cya*, Δ*crp*, Δ*cya* Δ*crp*, and/or Δ(*crp-cdt*)*19* derivatives were nonmotile, unlike the wild-type parent. The phenotypes of the strains with *crp*::Tn*10*, Δ*crp-11*, and *cya*::Tn*10* mutations were the same as those of the mutants listed in Table 2. It has been suggested previously that *phs* (hydrogen sulfide production) is under control of the cAMP-CRP system when severe repression of H_2S is exerted by glucose in wild-type *S. typhimurium* strains (5). Crp^- Cya^- mutants of *S. choleraesuis* are unable to produce H_2S, unlike the wild-type parent. The mean generation times of Δ*cya-12* Δ(*crp-cdt*)*19* χ3781 and the wild-type strain, χ3246, in Luria broth were 33.4 and 24.3 min, respectively. Strain χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] failed to grow or give rise to mutant derivatives capable of growth when up to 10^9 CFU were plated on minimal agar medium supplemented with DL-methionine and various carbon sources that should not support its growth.

Virulence of mutant strains in mice. The p.o. and i.p. LD_{50} values of χ3246 (wild type) were determined to be approximately 8×10^4 and 36 CFU, respectively, as determined by the method of Reed and Muench (43). Although 1 to 5 mice died in the groups of 15 mice inoculated p.o. with the *crp* mutants, no mice died when given p.o. doses of approximately 5×10^8 CFU of the *cya* mutants (Table 3). The inclusion of pSD110 with the wild-type *crp* gene fully complemented the *crp-773*::Tn*10* mutation in χ4418 and the Δ*crp-11* mutation in χ4484 to wild-type virulence; however, the inclusion of the crp^+ clone in strain χ3755 containing the Δ(*crp-cdt*)*19* mutation did not restore full virulence by the

TABLE 1. Bacterial strains

Strain	Genotype or phenotype	Derivation, source, and/or reference
E. coli CA8445	*thi rpsL* Δ*crp-45* Δ*cya-06* (pSD110)	47
S. typhimurium LT2		
PP1002	*trpB223 cya*::Tn*10*	41
PP1037	*trpB223 crp-773*::Tn*10*	41
TT218	*metE862*::Tn*10*	46
TT2104	*argI539 proAB47 amtA1 trp-130 cya-961 zid-62*::Tn*10*	46
χ3000	Prototroph	19
χ3376	*fli-8007*::Tn*10*	28
χ3385	*hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	55
χ3477	pStST100$^-$ *hsdL6* Δ(*galE-chl-uvrB*)*1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	55
χ3485	*hsdL6 galE496 flaA66 trpB2 his-6165 rpsL120 xyl-404 cya*::Tn*10 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	P22 HT*int*(PP1002)→χ3385 with selection for Tc^r (Mal$^-$)
χ3486	*hsdL6 galE496 flaA66 trpB2 his-6165 rpsL120 xyl-404 cya*::Tn*10 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	P1 *clr clm* lysogen of χ3485 with selection for Cm^r at 30°C
χ3524	pStLT100$^-$ *hsdL6* Δ(*galE-chl-uvrB*)*1005 flaA66 rpsL120 crp-773*::Tn*10 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	P22 HT*int*(PP1037)→χ3477 with selection for Tc^r (Mal$^-$)
χ3670	*hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val* (pSD110)	P22 HT*int*(CA8445)→χ3385 with selection for Ap^r
χ3711	*hisG rpsL* Δ*cya-12 zid-62*::Tn*10*	P22 HT*int*(χ3738)→χ3615 with selection for Tc^r (Mal$^-$)
χ3738	*zid-62*::Tn*10*	P22 HT*int*(TT2104)→χ3000 with selection for Tc^r (Mal$^+$)
χ3757	*hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404* Δ*cya-12 zid-62*::Tn*10 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	P22 HT*int*(χ3711)→χ3385 with selection for Tc^r (Mal$^-$)
χ3856	*hsdL6* Δ(*galE-chl-uvrB*)*1005 flaA66 rpsL120 metE862*::Tn*10 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	P22 HT*int*(TT218)→χ3477 with selection for Tc^r (Met$^-$)
χ4157	*hsdL6 galE496 trpB2 fli-8007*::Tn*10 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	P22 HT*int*(χ3376)→χ3385 with selection for Tc^r (Fla$^-$)
SL1344		
χ3339	*rpsL hisG*	Mouse-passaged SL1344; 48
χ3604	*hisG46 rpsL cya*::Tn*10*	P22 HT*int*(PP1002)→χ3339 with selection for Tc^r (Mal$^-$)
χ3615	*hisG46 rpsL* Δ*cya-12*	Fusaric acid-resistant, tetracycline-sensitive, Mal$^-$ derivative of χ3604
S. choleraesuis		
χ3246	Wild type, prototroph	Swine isolate 5451-84 from William Fales, Veterinary Medical Diagnostic Laboratory, School of Veterinary Medicine, University of Missouri
χ3492	*cya*::Tn*10*	P1 *clr clm*(χ3486)→χ3246 with selection for Tc^r (Mal$^-$)
χ3751	*crp-773*::Tn*10*	P1L4(χ3524)→χ3246 with selection for Tc^r (Mal$^-$)
χ3752	Δ(*crp-cdt*)*19*	Fusaric acid-resistant, tetracycline-sensitive, Mal$^-$ derivative of χ3751
χ3755	(pSD110) Δ(*crp-cdt*)*19*	P1L4(χ3670)→χ3752 with selection for Ap^r (Mal$^+$)
χ3759	(pSD110) Δ(*crp-cdt*)*19* Δ*cya-12 zid-62*::Tn*10 metE551*	P1L4(χ3757)→χ3755 with selection for Ap^r Tc^r (Mal$^-$)
χ3781	Δ(*crp-cdt*)*19* Δ*cya-12 metE551* Δ(*zid-62*::Tn*10*)	Fusaric acid-resistant, tetracycline-sensitive, ampicillin-sensitive, Mal$^-$ derivative of χ3759
χ3820	Δ*crp-11 zhc-1431*::Tn*10*	P1L4(χ3819)→χ3246 with selection for Tc^r (Mal$^-$)
χ3858	Δ*cya-12 metE551 zid-62*::Tn*10*	P1L4(χ3757)→χ3246 with selection for Tc^r (Mal$^-$)
χ3860	Δ*crp-11* Δ(*zhc-1431*::Tn*10*)	Fusaric acid-resistant, tetracycline-sensitive, Mal$^-$ derivative of χ3820
χ4184	(pSD110) Δ*crp-11* Δ(*zhc-1431*::Tn*10*)	P1L4(χ3670)→χ3860 with selection for Ap^r (Mal$^+$)
χ4185	(pSD110) Δ*crp-11* Δ(*zhc-1431*::Tn*10*) Δ*cya-12*	P1L4(χ3757)→χ4184 with selection for Tc^r Ap^r (Mal$^-$)
χ4186	Δ*crp-11* Δ(*zhc-1431*::Tn*10*) Δ*cya-12* Δ(*zid-62*::Tn*10*) *zid-62*::Tn*10*	Fusaric acid-resistant, tetracycline-sensitive, ampicillin-sensitive, Mal$^-$ derivative of χ4185
χ4222	*metE862*::Tn*10*	P1L4(χ3856)→χ3246 with selection for Tc^r (Met$^-$)
χ4390	*fli-8007*::Tn*10*	P1L4(χ4157)→χ3246 with selection for Tc^r (Fla$^-$)

TABLE 2. Phenotypic characterization of *S. choleraesuis* strains

Strain (genotype)	Result for the following characteristic[a]:												MGT (min)[f]
	P1L4[b]	Type 1 pili[c]	Mot[d]	Carbohydrate fermentation and use of[e]:								Auxotrophy for Met	H_2S	
				Mal	Mtl	Ino	Stl	Rha	Mel	Gal	Glc			
χ3246 (wild type)	I	−	+	+	+	−	+	+	+	+	+	+	+	24.3
χ3752 [Δ(*crp-cdt*)*19*]	I	−	−	−	−	−	−	−	−	+/−	+	+	−	ND
χ3858 (Δ*cya-12*)	I	−	−	−	−	−	−	−	−	+/−	+	−	−	ND
χ3781 [Δ(*crp-cdt*)*19* Δ*cya-12*]	I	−	−	−	−	−	−	−	−	+/−	+	−	−	33.4

[a] −, negative; +, positive; +/−, incomplete.
[b] I, immune (bacteriophage P1L4 adsorbs and injects DNA into *S. choleraesuis* but cannot replicate).
[c] *S. typhimurium* χ3339 was used as a positive control for the presence of type 1 pili.
[d] Mot, motility.
[e] Fermentation on MacConkey base agar medium and API 20E and growth on minimal agar medium plus 0.5% of the carbon sources indicated.
[f] MGT, mean generation time; assay was conducted at 37°C with L broth. ND, not determined.

p.o. route of inoculation. Mice survived p.o. doses of more than 10^3 times the wild-type LD_{50} of the Δ(*crp-cdt*)*19* mutant χ3755 (Table 3).

Data on survival following i.p. infections with wild-type and mutant *S. choleraesuis* strains are included in Table 4. As with the results of the p.o. challenge, strains with *crp* mutations were somewhat more virulent than strains with *cya* mutations. Again, the inclusion of pSD110 with the wild-type *crp* gene fully complemented the *crp-773*::Tn*10* mutation in χ4418 and the Δ*crp-11* mutation in χ4484 (Table 4). Surprisingly, i.p. infections with strain χ3755 carrying the pSD110 plasmid resulted in LD_{50} levels comparable to those of the wild-type parent, χ3246, which implies that the *cdt* mutation, although attenuating *S. choleraesuis* when delivered by the p.o. route of inoculation, did not attenuate when delivered by the i.p. route. The mean number of days before death for mice inoculated i.p. with the wild-type parent, χ3246, was 5. Of the mice that died following i.p. challenges with the *S. choleraesuis* mutant strain χ3751, χ3752, or χ3860, most of these did so 15 days after being inoculated.

All 15 mice survived infections with p.o. doses of 10^9 CFU, and 14 of 15 mice survived infections with i.p. doses of 10^4 CFU of the Δ*cya-12* Δ(*crp-cdt*)*19* mutant χ3781. Strain χ4186 (Δ*cya-12* Δ*crp-11*) was not as attenuated as χ3781. Mice that received doses greater than 6×10^8 CFU became ill, and some died (Table 5).

χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*], χ3858 (Δ*cya-12*), and χ4186 (Δ*cya-12* Δ*crp-11*) require methionine for growth (Table 2), which provides an additional identification marker for recovery from tissues. Bacon et al. (3) tested several *S. typhi* strains containing independent *met* mutations and found all to be virulent by the i.p. route. We have tested Tn*10* insertion mutations in *met* by the p.o. and i.p. routes and observed similar results. A p.o. dose of 10^6 CFU and an i.p. dose of 85 CFU of χ4222 (*S. choleraesuis metE*) resulted in the deaths of all infected mice. These results suggest that a *metE* mutation does not attenuate *Salmonella* species.

Flagellar synthesis has been reported to be under the control of cAMP in *Escherichia coli* and *S. typhimurium* (58). Flagellation and motility are not required for virulence in *S. typhimurium* (20, 28); however, a Fim^- (fimbriae) Fla^- (flagella) derivative of *S. typhimurium* was shown to have increased LD_{50} values in BALB/c mice (29). The wild-type parent, χ3246, is type 1 fimbriae negative. To determine whether type 1 fimbriae and flagella are necessary virulence

TABLE 3. Mortality of BALB/c mice 30 days after p.o. inoculation with wild-type and mutant *S. choleraesuis* strains

Strain	Genotype	Inoculating dose (CFU)	No. of surviving mice/total[a]
χ3246	Wild type	8.0×10^4	3/6
χ3751	*crp-773*::Tn*10*	6.4×10^8	14/15
χ4418	pSD110$^+$ *crp-773*::Tn*10*	4.8×10^4	3/6
χ3860	Δ*crp-11*	2.6×10^8	10/15
χ4184	pSD110$^+$ Δ*crp-11*	5.1×10^5	3/6
χ3752	Δ(*crp-cdt*)*19*	7.6×10^8	14/15
χ3755	pSD110$^+$ Δ(*crp-cdt*)*19*	9.4×10^7	15/30
χ3492	*cya*::Tn*10*	4.0×10^8	15/15
χ3858	Δ*cya-12*	6.2×10^8	15/15

[a] Defined by the number of mice that survived the designated inoculating dose of the total number of mice inoculated with that dose range.

TABLE 4. Mortality of BALB/c mice 30 days after i.p. inoculation with wild-type and mutant *S. choleraesuis* strains

Strain	Genotype	Inoculating dose (CFU)	No. of surviving mice/total[a]
χ3246	Wild type	36	3/6
χ3751	*crp-773*::Tn*10*	2.4×10^2	5/5
		2.4×10^3	4/5
		2.4×10^4	4/5
χ4418	pSD110$^+$ *crp-773*::Tn*10*	18	3/6
χ3860	Δ*crp-11*	6.5×10^2	4/5
		6.5×10^3	3/5
		6.5×10^4	1/5
χ4184	pSD110$^+$ Δ*crp-11*	<19	3/6
χ3752	Δ(*crp-cdt*)*19*	3.0×10^2	4/5
		3.0×10^3	5/5
		3.0×10^4	5/5
χ3755	pSD110$^+$ Δ(*crp-cdt*)*19*	<37	15/30
χ3492	*cya*::Tn*10*	2.1×10^2	5/5
		2.1×10^3	5/5
		2.1×10^4	5/5
χ3858	Δ*cya-12*	2.5×10^2	4/5
		2.5×10^3	5/5
		2.5×10^4	5/5

[a] Defined by the number of mice that survived the designated inoculating dose of the total number of mice inoculated with that dose range.

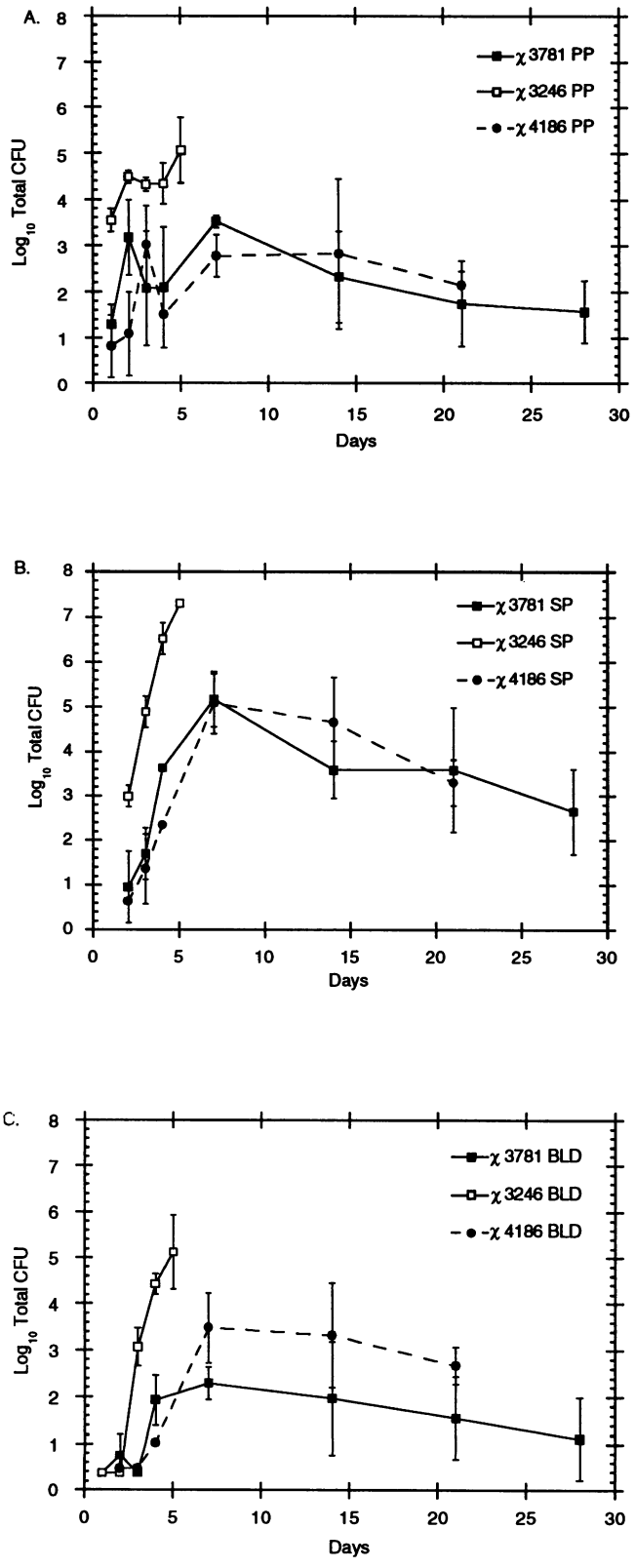

FIG. 1. Recovery of *S. choleraesuis* Δ*cya-12* Δ(*crp-cdt*)*19* χ3781, Δ*cya-12* Δ*crp-11* χ4186, and wild-type χ3246 from Peyer's patches (A), spleens (B), and blood samples from mice hearts (C) at specified times after p.o. inoculations with 7.8×10^8 CFU of χ3781, 1.5×10^9 CFU of χ4186, and 1.3×10^9 CFU of χ3246. Four mice were sacrificed at each time point. The results are given as geometric means ± standard deviations. PP, Peyer's patches; SP, spleens; BLD, blood samples.

TABLE 5. Effectiveness of p.o. and i.p. immunizations with *S. choleraesuis* Δ*cya-12* Δ(*crp-cdt*)*19* χ3781 and Δ*cya-12* Δ*crp-11* χ4186 in protecting against p.o. or i.p. challenge with wild-type χ3246[a]

Strain and route of inoculation	Inoculating dose (no. of surviving mice/total)	Challenge dose (no. of surviving mice/total)
χ3246		
p.o.	8.0×10^4 (3/5)	
i.p.	36 (2/5)	
χ3781		
p.o.	3.6×10^9 (15/15)	1.3×10^9 (15/15)
i.p.	8.0×10^4 (14/15)	3.0×10^3 (14/14)
χ4186		
p.o.	6.8×10^8 (4/5)	6.0×10^8 (4/4)
i.p.	3.3×10^3 (3/5)	3.0×10^3 (3/3)

[a] Thirty days after 8-week-old BALB/c mice were immunized p.o. with the indicated attenuated strains, they were challenged with wild-type virulent χ3246. Morbidity and mortality observations were recorded daily for an additional 45 days postchallenge. Both inoculating and challenge doses were measured in CFU.

attributes of *S. choleraesuis*, a Fla$^-$ derivative of the wild-type Fim$^-$ *S. choleraesuis* parent strain was constructed. The p.o. LD_{50} values of the wild-type (Fim$^-$) χ3246 and the Fla$^-$ (Fim$^-$) derivative χ4390 for BALB/c mice were identical.

Tissue tropism and persistence of avirulent mutants in mice. The Δ*cya-12* Δ(*crp-cdt*)*19* mutations in χ3781 and the Δ*cya-12* Δ*crp-11* mutations in χ4186 do not prevent *S. choleraesuis* cells from attaching to, invading, and/or persisting in Peyer's patches or the spleen (Fig. 1A and B) and reaching significant titers in the blood (Fig. 1C), but these abilities are significantly impaired compared with those of the wild-type strain, χ3246. All mice infected with 10^9 CFU of the wild-type strain, χ3246, died by day 5. χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] was detected up to 28 days postinoculation, with peak titers appearing 7 days after oral inoculation. The data recorded for χ4186 (Δ*cya-12* Δ*crp-11*) paralleled those for Peyer's patch and spleen colonization for χ3781. However, some differences (95% confidence level) in levels of bacteremia were observed (Fig. 1C). From days 7 to 28 postinoculation, spleens were enlarged approximately 10 times their normal size and weight in all mice inoculated with χ3781 and χ4186; however, mice inoculated with χ4186 (Δ*cya-12* Δ*crp-11*) had spleens and livers that appeared significantly more diseased than those of mice inoculated with χ3781 [Δ(*crp-cdt*)*19* Δ*cya-12*].

To further investigate differences in in vivo invasiveness, a mixed p.o. infection experiment with χ3246 (wild type) and χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] was done. Titers of CFU recovered from Peyer's patches, intestinal walls, and spleens revealed that χ3246 (wild type) initially colonized these tissues 4 to 20 times more efficiently than χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] (Table 6). By 4 days postinoculation, the χ3246/χ3781 ratios in the intestinal tract were 8.6. The greatest differences in CFU were observed in spleens, in

TABLE 6. Mixed p.o. infections of mice with wild-type *S. choleraesuis* χ3246 and Δ(*crp-cdt*)*19* Δ*cya-12* χ3781 and ratios of CFU in various organs[a]

Time after infection	Ratio of χ3246 to χ3781 in[b]:		
	Peyer's patches	Ilea[c]	Spleens
24 h	8.0 ± 3.6	4.7 ± 1.9	ND
48 h	8.6 ± 6.9	4.3 ± 4.9	4.6 ± 3.4
3 days	2.5 ± 0.5	2.1 ± 0.4	6.1 ± 5.1
4 days	9.5 ± 7.6	7.8 ± 5.1	22.9 ± 0
5 days	8.2 ± 5.1	8.9 ± 4.2	22.6 ± 1.3

[a] Eight-week-old BALB/c female mice received a p.o. mixture of 5.0×10^8 CFU of χ3246 and 9.6×10^8 CFU of χ3781 for an initial ratio of 0.52.

[b] Results are given as geometric means ± standard deviations of ratios of wild-type χ3246 CFU to Δ(*crp-cdt*)*19* Δ*cya-12* χ3781 CFU recovered from Peyer's patches, ilea, and spleens of four mice at each time of assay. Data are corrected for input ratio. ND, not done.

[c] Ten-centimeter sections of the ilea were extensively washed to remove the contents, and the Peyer's patches were removed before homogenization.

which ratios were greater than those observed in either Peyer's patches or intestinal walls (Table 6).

Invasion of CHO cells. Comparative studies of the wild-type and Crp$^-$ mutant strains showed differences in their adherences to CHO cells; however, the Cdt$^-$ mutant maintained a wild-type ability to adhere to mammalian cells in culture. All of the mutants' invasive properties were significantly reduced by as much as 100-fold (Table 7).

After 24 h of growth following infection, noticeable differences in the appearances of the CHO cells which had been infected with the wild-type strain, χ3246, and those which had been infected with Δ*cya-12* Δ(*crp-cdt*)*19* χ3781 were seen (Fig. 2B and C). The CHO cells infected with the χ3781 mutant (Fig. 2C) appeared less rounded, and fewer cells had detached than seen with the monolayer infected with wild-type χ3246 (Fig. 2B). The nonconfluent growth of the wild-type χ3246-infected monolayer was most likely due to bacterial cytotoxicity.

Bacterial transcytosis. Bacterial transcytosis of polarized MDCK monolayers was investigated to determine the rate at which χ3781 could penetrate such a monolayer compared with the rate for the wild-type strain (Fig. 3). At 2 to 3 h postinfection, neither the wild-type strain, χ3246, nor the mutant strain, χ3781, passed through the monolayer, thus verifying that the monolayers were intact. After 3 to 4 h, low numbers of χ3246 (wild type) could be detected in the basolateral medium, while strain χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] began to penetrate the monolayer after 5 h. At 8 h postinfection, 8.5×10^5 CFU of χ3246 (wild type) per ml had transcytosed compared with 8.0×10^4 CFU of χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] per ml. This revealed a nearly 10-fold-decreased penetration efficiency by the mutant strain. It should be noted that the mean generation time for both the wild-type strain and the Δ*cya* Δ*crp* mutant was 1.3 h in the basolateral medium. Therefore, the actual number of bacteria passing through the monolayer would be slightly less than the number titered in the basolateral medium.

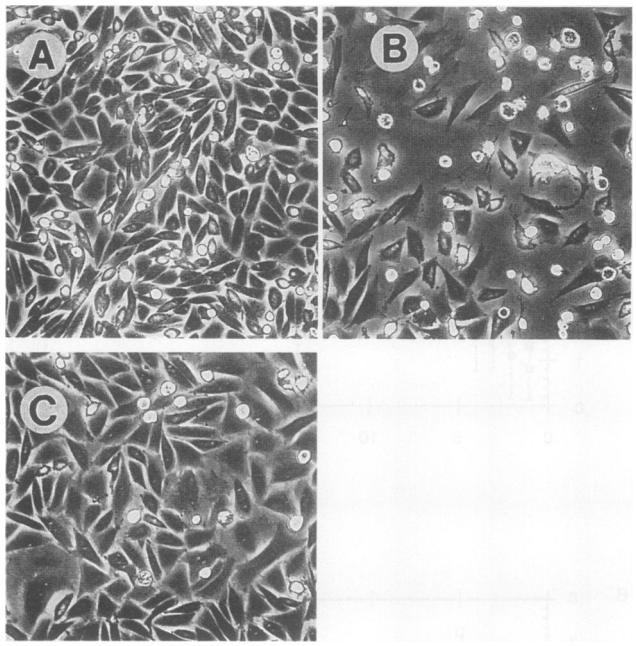

FIG. 2. CHO cell monolayers, incubated for 24 h, which were uninfected (A), infected with wild-type *S. choleraesuis* χ3246 (B), and infected with Δ*cya-12* Δ(*crp-cdt*)*19 S. choleraesuis* χ3781 (C), as seen with a Zeiss Axiomat microscope. Magnification, ×270.

Immunogenicity of mutant strains in mice. The *S. choleraesuis* mutants protect vaccinated BALB/c mice against a subsequent p.o. (Table 8) or i.p. (Table 9) challenge with the wild-type, virulent parent, χ3246. Differences in degrees of

TABLE 7. Infection of CHO cells with wild-type and mutant *S. choleraesuis*

Expt no. and strain	Genotype	Relevant phenotype	% Adherence[a]	% Invasion
Expt 1				
χ3246	Wild type	Wild type	9.2 ± 1.6	8.8 ± 0.9
χ3751	*crp-773*::Tn*10*	Crp$^-$	2.7 ± 0.2*[b]	0.05 ± 0.01*
χ3752	Δ(*crp-cdt*)*19*	Crp$^-$ Cdt$^-$	1.5 ± 0.1*	0.2 ± 0.004*
χ3755	pSD110$^+$ Δ(*crp-cdt*)*19*	Cdt$^-$	7.6 ± 1.0	3.1 ± 0.3*
χ4418	pSD110$^+$ *crp-773*::Tn*10*	Wild type	10.9 ± 0.5	5.8 ± 0.2
Expt 2				
χ3246	Wild type	Wild type	6.6 ± 0.6	11.7 ± 3.1
χ3781	Δ(*crp-cdt*)*19* Δ*cya-12*	Crp$^-$ Cdt$^-$ Cya$^-$	5.1 ± 0.6	1.1 ± 0.4*

[a] Values are mean percentages ± standard errors of the mean ($n = 3$) of CFU recovered after 2 h of adherence or 2 h of incubation in 100 μg of gentamicin per ml.

[b] All values marked with an asterisk are significantly different by Student's t test ($P < 0.005$) when compared with χ3246 (wild type) or χ4418 (*crp-773*::Tn*10*/pSD110$^+$). Similar results were observed when experiments were repeated several times.

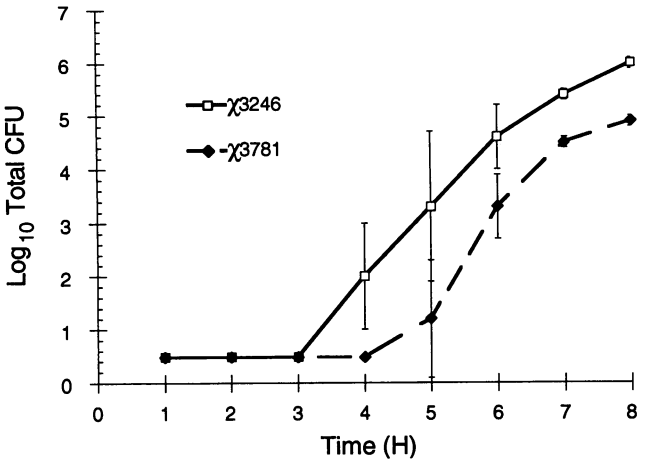

FIG. 3. Bacterial transcytosis of polarized MDCK monolayers as determined by recovery of *S. choleraesuis* wild-type χ3246 and Δ*cya-12* Δ(*crp-cdt*)*19* χ3781 from basolateral medium below the MDCK monolayers at specified times after infection of the apical surface with a mixed inoculum of the strains described above at a ratio of 0.78 (wild type to mutant).

protection were apparent, as mice vaccinated either i.p. or p.o. with the slightly more virulent *crp*::Tn*10*, Δ*crp-11*, and Δ(*crp-cdt*)*19* strains displayed better health and higher frequencies of survival after a challenge with virulent χ3246 than mice vaccinated i.p. or p.o. with the less virulent *cya*::Tn*10* and Δ*cya* constructs. All of the animals vaccinated p.o. with 10^8 CFU of χ3751 (*crp-773*::Tn*10*), χ3860 (Δ*crp-11*), or χ3752 [Δ(*crp-cdt*)*19*] were protected against a p.o. challenge with 5×10^8 to 7×10^8 CFU of virulent χ3246 (~8×10^3 times the wild-type LD_{50}), whereas those vaccinated p.o. with 10^8 CFU of χ3492 (*cya*::Tn*10*) or χ3858 (Δ*cya*-12) did not survive a challenge with 7×10^8 CFU of the wild-type strain and yielded survivors only when challenged with lower doses of χ3246 (Table 8). Interestingly, mice immunized p.o. with χ3755, which contains the pSD110 crp^+ plasmid to complement the Δ*crp* mutation, were fully protected (15 of 15 mice) against a p.o. challenge with 6.0×10^8 CFU of the wild-type χ3246 parent (Table 8). Thus, strains with Δ*cdt* are both avirulent and immunogenic. Mice vaccinated i.p. with 1×10^3 to 6×10^4 CFU of χ3751 (*crp*::Tn*10*), χ3860 (Δ*crp-11*), χ3752 [Δ(*crp-cdt*)*19*], or χ3858 (Δ*cya-12*) were completely protected against a wild-type challenge with 80 times the LD_{50}, while those vaccinated i.p. with 10^2, 10^3, or 10^4 CFU of χ3492 (*cya*::Tn*10*) developed significant illnesses, with some deaths, after an i.p. challenge (Table 9).

All 15 mice vaccinated p.o. with 3.6×10^9 CFU of the double mutant χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] were protected against a p.o. challenge with 1×10^9 CFU of virulent χ3246 (more than 1.6×10^4 times the wild-type LD_{50}) (Table 5). Mice surviving i.p. doses of χ3781 (2×10^3 times the wild-type LD_{50}) were fully protected against 3×10^3 CFU of the wild-type strain, χ3246 (80 times the wild-type i.p. LD_{50}). No ill effects were noted during the 45 days postchallenge with the wild-type χ3246. Although the Δ*cya-12* Δ*crp-11* double mutant χ4186 was not as attenuated in virulence as χ3781, all of the surviving mice were protected against a challenge with the wild-type strain, χ3246.

TABLE 8. Effectiveness of p.o. immunization with attenuated *S. choleraesuis* mutant strains in protecting against p.o. challenge with wild-type χ3246[a]

Strain (genotype)	Immunizing dose (CFU)	Challenge dose (CFU)	No. of surviving mice/total
χ3492 (*cya*::Tn*10*)	4.0×10^8	7.2×10^6	3/5
		7.2×10^7	1/5
		7.2×10^8	0/5
χ3858 (Δ*cya-12*)	6.2×10^8	7.2×10^6	2/5
		7.2×10^7	2/5
		7.2×10^8	0/5
χ3751 (*crp*-773::Tn*10*)	6.4×10^8	7.2×10^6	5/5
		7.2×10^7	5/5
		7.2×10^8	4/4
χ3860 (Δ*crp-11*)	2.6×10^8	5.0×10^8	10/10
χ3752 [Δ(*crp-cdt*)*19*]	2.4×10^8	7.2×10^6	4/4
		7.2×10^7	5/5
		7.2×10^8	5/5
χ3755 [pSD110$^+$ Δ(*crp-cdt*)*19*]	9.4×10^7	6.0×10^8	15/15

[a] Thirty days after 8-week-old BALB/c mice were immunized p.o. with the indicated attenuated strains, they were challenged with wild-type virulent χ3246. Morbidity and mortality observations were recorded daily for an additional 45 days postchallenge.

DISCUSSION

We have constructed mutants of mouse- and pig-virulent *S. choleraesuis* bv. kunzendorf χ3246 that lack the ability to synthesize adenylate cyclase and/or CRP. We have also discovered a gene(s) adjacent to *crp* that is also associated

TABLE 9. Effectiveness of i.p. immunization with attenuated *S. choleraesuis* mutant strains in protecting against i.p. challenge with wild-type χ3246[a]

Strain (genotype)	Immunizing dose (CFU)	No. of surviving mice/total
χ3492 (*cya*::Tn*10*)	2.1×10^2	2/5
	2.1×10^3	1/5
	2.1×10^4	3/5
χ3858 (Δ*cya-12*)	2.5×10^2	4/4
	2.5×10^3	5/5
	2.5×10^4	5/5
χ3751 (*crp*-773::Tn*10*)	2.4×10^2	3/5
	2.4×10^3	4/4
	2.4×10^4	4/4
χ3860 (Δ*crp-11*)	6.5×10^2	4/4
	6.5×10^3	3/3
	6.5×10^4	1/1
χ3752 [Δ(*crp-cdt*)*19*]	3.0×10^2	4/4
	3.0×10^3	5/5
	3.0×10^4	5/5

[a] Thirty days after 8-week-old BALB/c mice were immunized i.p. with the indicated attenuated strains, they were challenged with 3.0×10^3 CFU of wild-type virulent χ3246. Morbidity and mortality observations were recorded daily for an additional 45 days postchallenge.

with *S. choleraesuis* virulence. The mutants were avirulent and immunogenic and easy to grow and store. The mutant phenotype is not subject to alteration either by diet or by the animal host. Since many genes and operons are under the control of cAMP and CRP, *cya* and *crp* mutants are impaired in their abilities to transport and break down carbohydrate and amino acid catabolites (1). The cAMP concentration within the cell in conjunction with CRP also regulates the synthesis of pili (fimbriae), OmpA, glycogen (12, 34, 45), and hydrogen sulfide (5) and influences phage lysogeny (25, 42). cAMP is required for flagella formation in *E. coli* and *S. typhimurium* (58). However, *cfs* (constitutive flagellar synthesis) mutants have been isolated in Cya^- and Crp^- strains that obviate the need for cAMP (49). Since the H antigen is potentially an important immunogen, motile variants of *cya* or *crp* mutants can be selected to elicit the antiflagellar immune response. Although both χ3781 [Δ(*crp-cdt*)*19* Δ*cya-12*] and χ4186 (Δ*crp-11* Δ*cya-12*) agglutinated antisera to H antigen (poly a-z), motility agar assays were negative. *cfs* mutants arose more frequently in *S. typhimurium cya crp* strains than in *S. choleraesuis cya crp* strains (data not shown). These results imply that the *cya crp* strains may still synthesize flagella or flagellar hook protein but that they may be paralyzed and therefore nonmotile. Electron microscopy studies are under way to determine whether flagella are located on the bacterial cell surfaces of *cya crp* and/or *cdt* mutants.

The *cdt* locus was originally discovered in *S. typhimurium* after several independent deletion mutants derived after fusaric acid resistance selection of a strain carrying *crp-773*::Tn*10* were screened. One strain containing the Δ*crp-10* deletion could not be complemented with pSD110 and remained 10,000 times less virulent than the wild-type parent or the other Δ*crp* deletion mutants constructed. This Δ*crp-10* mutation was subsequently crossed into several different *S. typhimurium* serotypes as well as other *Salmonella* species, and the avirulence phenotype in mice was repeatedly demonstrated by complementation with pSD110. In vivo experiments with *S. typhimurium* Δ*crp-10* with or without pSD110 revealed that colonization of the gut-associated lymphoid tissue and deep organs was consistent with that by other previously described *Salmonella* mutants that were categorically defective in deep organ colonization, i.e., strains cured of the virulence plasmid and strains with Δ*phoP*, *ompR*, Δ*aroA*, Δ*cya*, and *galE*, etc. Thus, we generically named the allele *cdt*, as *Salmonella* strains mutated in this locus significantly reduced the abilities of the mutants to reach and colonize deep tissues compared with those of their wild-type parents.

To demonstrate that the imprecise excision of *crp-773*::Tn*10* deleted a neighboring gene(s) that is associated with virulence, the pSD110 plasmid was used to evaluate the complementation of each *crp* deletion mutation. pSD110 restored wild-type virulence to strains with the *crp-773*::Tn*10* and Δ*crp-11* mutations but not to strains carrying the Δ(*crp-cdt*)*19* mutation, which thus revealed the presence of an additional attenuating mutation. χ3781 appears to display an additive effect of both attenuation strategies, namely, the debilitated cAMP-CRP system and the *cdt* locus. The exact role of the *cdt* locus has not been conclusively determined. It is clear that the gene product(s) is involved in invasion past the gut after p.o. inoculation of mice; however, Crp^+ Cdt^- strains retain wild-type virulence when administered i.p. At this time, no other phenotype besides reduced p.o. virulence, deep tissue colonization after p.o. inoculation, and invasion of mammalian cells in culture has been identified for this deletion mutant.

Although all strains with *cya*::Tn*10*, *crp*::Tn*10*, or single-deletion mutations were attenuated, the double-deletion mutant χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] was less virulent, since mice survived infection with doses exceeding 4.5×10^4 and 2×10^3 times the p.o. and i.p. wild-type LD_{50} levels, respectively. The strain χ4186 (Δ*cya-12* Δ*crp-11*) was not as attenuated as χ3781, since mice that received doses higher than 6×10^8 CFU became ill, and some died.

S. choleraesuis Δ*cya* Δ(*crp-cdt*)*19* χ3781 retained the abilities to attach to, invade, and/or persist in Peyer's patches and to reach deep tissues, but its capacities for these were significantly reduced compared with those of the wild-type parent strain. This mutant persisted at levels in the spleen, Peyer's patches, and blood lower than those for Δ*cya-12* Δ*crp-11* χ4186 or the virulent parent strain. Although signs of disease on the spleens and livers of mice infected with χ3781 [Δ*cya-12* Δ(*crp-cdt*)*19*] were noted, the strain χ4186 (Δ*cya-12* Δ*crp-11*) induced more symptoms and greater virulence and persisted in blood at higher levels. An additional attenuating mutation or curing of the virulence plasmid may reduce the invasiveness of χ3781 and its persistence in blood without significantly impairing its immunogenicity.

The animal infection studies discussed above demonstrate the reduced invasiveness of the mutant strains to deep tissues of mice infected p.o., suggesting that *crp* and/or *cdt* might be involved in the ability of *S. choleraesuis* to resist phagocytosis or killing by murine macrophages or the ability to multiply within macrophages. Experiments to evaluate these hypotheses are in progress.

Mammalian cell lines have been useful in evaluating the virulence properties of attenuated bacterial strains (13–15, 26). The Crp^- strains adhered to CHO cells at levels slightly lower than but at a significant level compared with those for the wild-type parent, χ3246. The Cdt^- strain maintained a wild-type ability to adhere to mammalian cells in culture. However, distinct differences were seen in the abilities of all of the mutants to invade CHO cells. Similar results were obtained by using the bacterial transcytosis assay in which Δ*cya-12* Δ(*crp-cdt*)*19* χ3781 transcytosed the MDCK monolayer more slowly and at a lower level than the wild type. Therefore, it appeared that the Δ*cya-12* Δ(*crp-cdt*)*19* mutations in *S. choleraesuis* χ3781 slightly impaired the ability to adhere to CHO cells and significantly decreased the ability to invade CHO and MDCK cells compared with those of the wild-type parent.

Data from mixed infections of the wild-type strain, χ3246, and the Δ*cya-12* Δ(*crp-cdt*)*19* mutant χ3781 in BALB/c mice indicate that the Δ*cya-12* Δ(*crp-cdt*)*19* mutations moderately impair the abilities of *S. choleraesuis* to attach to, invade, and/or persist in Peyer's patches and the intestinal wall but impair more its ability to reach or to survive in deep tissues. These results are therefore very much in accord with the results from the cell attachment and invasion assays and transcytosis measurements discussed above. It therefore follows that attachment to and, to a greater extent, invasion of *S. choleraesuis* to mammalian cells may be partially dependent on cAMP and CRP. This might imply that the synthesis of *S. choleraesuis* invasins is subject to catabolite repression. These questions are currently being investigated.

Resistance to complement-mediated bacteriolysis by rabbit serum was not affected by introducing the Δ*cya-12* Δ(*crp-cdt*)*19* mutations into wild-type *S. choleraesuis*. The reduced ability of χ3781 to appear in blood (Fig. 1C) was

probably not due to increased sensitivity to serum bactericidal killing.

Although the single *crp* mutants appeared more protective than the *cya* mutants, the double Δ*cya-12* Δ(*crp-cdt*)*19* mutant χ3781 induced a higher level of protection, as was demonstrated when mice survived inoculation with the wild-type strain, χ3246, at more than 1.6×10^4 times and 80 times the p.o. and i.p. LD_{50} levels, respectively. Although χ4186 (Δ*cya-12* Δ*crp-11*) was more virulent than χ3781, it also induced a high level of protection against the wild-type challenge.

We have shown that *S. choleraesuis* can be attenuated by deletion of the *cya* and *crp* genes, which synthesize cAMP and CRP, respectively. We have also discovered a locus linked to *crp* that is associated with virulence in *S. choleraesuis*. Significant differences between the mutant and parent strains were seen in both invasion assays with mammalian cells and in vivo tissue tropism and persistence studies. Definitive molecular genetic studies are under way, and the region encompassing the Δ(*crp-cdt*)*19* deletion has been cloned.

Collaborative studies to investigate the potential use of Δ*cya-12* Δ(*crp-cdt*)*19 S. choleraesuis* as an immunizing agent for swine against colonization and persistence of naturally occurring *S. choleraesuis* and fatal swine salmonellosis are in progress. Attenuated *Salmonella* strains can be used as carriers to deliver cloned foreign colonization or virulence antigens from other pathogens (52). A vector-host system that constitutes a "balanced-lethal" system was developed to eliminate the need for antibiotic-resistant plasmid selective maintenance. A mutant with a deletion in the chromosome-blocking synthesis of an essential metabolite that is not readily available in nature or in the animal host was constructed for use with a plasmid vector containing a nonhomologous gene sequence complementing the chromosomal gene defect (36). We have made use of *E. coli* and *Salmonella* strains with deletions for the gene for aspartate β-semialdehyde dehydrogenase (*asd*) and plasmid vectors that contain the asd^+ gene cloned from *S. typhimurium* and genes for colonization or virulence antigens from a number of pathogens (16, 36). An Asd^- derivative of χ3781 has been constructed for use with the asd^+ balanced-lethal system for the expression and delivery of foreign gene products (16, 36).

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REFERENCES

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133**:149–157.
2. **Anderson, R. P., and J. R. Roth.** 1978. Tandem chromosomal duplications in *Salmonella typhimurium*: fusion of histidine genes to novel promoters. J. Mol. Biol. **119**:147–166.
3. **Bacon, G. A., T. W. Burrows, and M. Yates.** 1950. The effects of biochemical mutation on the virulence of *Bacterium typhosum*: the virulence of mutants. Br. J. Exp. Pathol. **31**:714–724.
4. **Blaser, M. J., and R. A. Feldman.** 1981. Salmonella bacteremia: reports to the Center for Disease Control, 1968–1979. J. Infect. Dis. **143**:743–746.
5. **Clark, M. A., and E. L. Barrett.** 1987. The *phs* gene and hydrogen sulfide production by *Salmonella typhimurium*. J. Bacteriol. **169**:2391–2397.
6. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
7. **Curtiss, R., III.** 1968. Ultraviolet-induced genetic recombination in a partially diploid strain of *Escherichia coli* K-12. Genetics **58**:9–54.
8. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
9. **Curtiss, R., III, R. M. Goldschmidt, S. M. Kelly, M. Lyons, S. M. Michalek, R. Pastian, and S. Stein.** 1987. Recombinant avirulent *Salmonella* for oral immunization to induce mucosal immunity to bacterial pathogens, p. 261–271. *In* H. Kohler and P. T. LoVerde (ed.), Vaccines: new concepts and developments. Proceedings of the Tenth International Convocation on Immunology. Longman Scientific and Technical, Harlow, Essex, United Kingdom.
10. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55**:3035–3043.
11. **Curtiss, R., III, S. B. Porter, M. Munson, S. A. Tinge, J. O. Hassan, C. Gentry-Weeks, and S. M. Kelly.** 1991. Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry, p. 169–198. *In* L. C. Blankenship, J. S. Bailey, N. A. Cox, N. J. Stern, and R. J. Meinersmann (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, New York.
12. **Dietzler, D. N., M. P. Leckie, W. L. Sternheim, T. L. Taxman, J. M. Unger, and S. E. Porter.** 1977. Evidence for the regulation of bacterial glycogen synthesis by cyclic AMP. Biochem. Biophys. Res. Commun. **77**:1468–1477.
13. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243**:1059–1062.
14. **Finlay, B. B., B. Gumbiner, and S. Falkow.** 1988. Penetration of *Salmonella* through a polarized Madin-Darby canine kidney epithelial cell monolayer. J. Cell Biol. **107**:221–230.
15. **Galan, J. E., and R. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86**:6383–6387.
16. **Galan, J. E., K. Nakayama, and R. Curtiss III.** 1990. Cloning and characterization of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene **94**:29–35.
17. **Germanier, R., and E. Fürer.** 1971. Immunity in experimental salmonellosis. II. Basis for the avirulence and protective capacity of *galE* mutants of *Salmonella typhimurium*. Infect. Immun. **4**:663–673.
18. **Griffith, R. W., T. T. Kramer, and J. F. Pohlenz.** 1984. Relationship between the antibody-complement susceptibility of smooth *Salmonella choleraesuis* var *kunzendorf* strains and their virulence for mice and pigs. Am. J. Vet. Res. **45**:1342–1348.
19. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55**:2891–2901.
20. **Hackett, J., S. Attridge, and D. Rowley.** 1988. Oral immunization with live avirulent fla^+ strains of *Salmonella* protects mice against subsequent oral challenge with *Salmonella typhimurium*. J. Infect. Dis. **157**:78–84.
21. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269–277.
22. **Hohmann, A., G. Schmidt, and D. Rowley.** 1979. Intestinal and

serum antibody responses in mice after oral immunization with *Salmonella*, *Escherichia coli*, and *Salmonella-Escherichia coli* hybrid strains. Infect. Immun. **25:**27–33.

23. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
24. **Hone, D. M.** 1987. Construction of defined *galE* mutants of *Salmonella* for use as vaccines. J. Infect. Dis. **156:**167–174.
25. **Hong, J., G. R. Smith, and B. N. Ames.** 1971. Adenosine 3′:5′-cyclic monophosphate concentrations in the bacterial host regulates the viral decision between lysogeny and lysis. Proc. Natl. Acad. Sci. USA **68:**2258–2262.
26. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
27. **Korhonen, T. K.** 1979. Yeast cell agglutination by purified enterobacterial pili. FEMS Microbiol. Lett. **6:**421–425.
28. **Lockman, H. A., and R. Curtiss III.** 1990. *Salmonella typhimurium* mutants lacking flagella or motility remain virulent in BALB/c mice. Infect. Immun. **58:**137–143.
29. **Lockman, H. A., and R. Curtiss III.** 1992. Virulence of non-type 1-fimbriated and nonfimbriated nonflagellated *Salmonella typhimurium* mutants in murine typhoid fever. Infect. Immun. **60:** 491–496.
30. **Luria, S. E., and J. W. Burrous.** 1957. Hybridization between *Escherichia coli* and Shigella. J. Bacteriol. **74:**461–476.
31. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:** 1110–1112.
32. **McFarland, W. C., and B. A. D. Stocker.** 1987. Effect of different purine auxotrophic mutations on mouse-virulence of a Vi-positive strain of *Salmonella dublin* and of two strains of *Salmonella typhimurium*. Microb. Pathog. **3:**129–141.
33. **Morehouse, L. G.** 1972. Salmonellosis in swine and its control. J. Am. Vet. Med. Assoc. **160:**593–601.
34. **Movva, R. N., P. Green, K. Nakamura, and M. Inouye.** 1981. Interaction of cAMP receptor protein with the *ompA* gene, a gene for a major outer membrane protein of *Escherichia coli*. FEBS Lett. **128:**186–190.
35. **Mukkur, K. S., G. H. McDowell, B. A. D. Stocker, and A. K. Lascelles.** 1987. Protection against experimental salmonellosis in mice and sheep by immunisation with aromatic-dependent *Salmonella typhimurium*. J. Med. Microbiol. **24:**11–19.
36. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an Asd^+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6:**693–697.
37. **Nnalue, N. A., and B. A. D. Stocker.** 1986. Some *galE* mutants of *Salmonella choleraesuis* retain virulence. Infect. Immun. **54:**635–640.
38. **Nnalue, N. A., and B. A. D. Stocker.** 1987. Tests of the virulence and live-vaccine efficacy of auxotrophic and *galE* derivatives of *Salmonella choleraesuis*. Infect. Immun. **55:**955–962.
39. **O'Callaghan, D., D. Maskell, R. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–423.
40. **Old, D. C., I. Corneil, L. F. Gibson, A. D. Thomson, and J. P. Duguid.** 1968. Fimbriation, pellicle formation and the amount of growth of salmonellas in broth. J. Gen. Microbiol. **51:**1–16.
41. **Postma, P. W., H. G. Keizer, and P. Kookwijk.** 1986. Transport of trehalose in *Salmonella typhimurium*. J. Bacteriol. **168:**1107–1111.
42. **Rao, N. R., and C. V. S. Raj.** 1973. *Salmonella typhimurium* mutants affecting establishment of lysogeny. Mol. Gen. Genet. **125:**119–123.
43. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27:**493–497.
44. **Robertsson, J. A., A. A. Lindberg, S. K. Hoiseth, and B. A. D. Stocker.** 1983. *Salmonella typhimurium* infection in calves: protection and survival of virulent challenge bacteria after immunization with live or inactivated vaccines. Infect. Immun. **41:**742–750.
45. **Saier, M. H., Jr., M. R. Schmidt, and M. Leibowitz.** 1978. Cyclic AMP-dependent synthesis of fimbriae in *Salmonella typhimurium*: effects of *cya* and *pts* mutations. J. Bacteriol. **134:**356–358.
46. **Sanderson, K. E., and J. R. Roth.** 1988. Linkage map of *Salmonella typhimurium*, edition VII. Microbiol. Rev. **52:**485–532.
47. **Schmeiger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
48. **Schroeder, C. J., and W. J. Dobrogosz.** 1986. Cloning and DNA sequence analysis of the wild-type and mutant cyclic AMP receptor protein genes from *Salmonella typhimurium*. J. Bacteriol. **167:**616–622.
49. **Silverman, M., and M. Simon.** 1974. Characterization of *Escherichia coli* flagellar mutants that are insensitive to catabolite repression. J. Bacteriol. **120:**1196–1203.
50. **Smith, B. P., M. Reina-Guerra, S. K. Hoiseth, B. A. D. Stocker, F. Habasha, E. Johnson, and F. Merritt.** 1984. Aromatic-dependent *Salmonella typhimurium* as modified live vaccines for calves. Am. J. Vet. Res. **45:**59–66.
51. **Smith, H. W.** 1965. The immunization of mice, calves, and pigs against *Salmonella dublin* and *Salmonella choleraesuis* infections. J. Hyg. **63:**117–135.
52. **Stabel, T. J., J. E. Mayfield, D. C. Morfitt, and M. J. Wannemuehler.** Oral immunization of mice and swine with an attenuated *Salmonella choleraesuis* Δ*cya* Δ*crp* containing a recombinant plasmid. Submitted for publication.
53. **Stabel, T. J., J. E. Mayfield, L. B. Tabatabai, and M. J. Wannemuehler.** 1991. Swine immunity to an attenuated *Salmonella typhimurium* mutant containing a recombinant plasmid which codes for production of a 31-kilodalton protein of *Brucella abortus*. Infect. Immun. **59:**2941–2947.
54. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δ*cya* Δ*crp* *Salmonella typhi* strains in adult volunteers. Infect. Immun. **60:**536–541.
55. **Tinge, S. A., and R. Curtiss III.** 1990. Isolation of the replication and partitioning regions of the *Salmonella typhimurium* virulence plasmid and stabilization of heterologous replicons. J. Bacteriol. **58:**3084–3092.
56. **Tsai, C.-M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
57. **Wilcock, B. P.** 1986. Salmonellosis, p. 508–519. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine, 6th ed. Iowa State University Press, Ames.
58. **Yokota, R., and J. S. Gots.** 1970. Requirement of adenosine 3′-5′-cyclic phosphate for flagella formation in *Escherichia coli* and *Salmonella typhimurium*. J. Bacteriol. **103:**513–516.