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Characterization and Immunogenicity of *Salmonella typhimurium* SL1344 and UK-1 Δ*crp* and Δ*cdt* Deletion Mutants

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S. typhimurium **SL1344 and UK-1 mutants with deletions of the *crp* (cyclic AMP receptor protein) and *cdt* (colonization of deep tissues) genes have been constructed and characterized, and their levels of virulence and immunogenicity have been determined for BALB/c mice. All Crp$^-$ Cdt$^-$ and Crp$^+$ Cdt$^-$ mutants were avirulent, as mice survived oral doses of 10^9 cells without illness. All the mutants colonized the gut-associated lymphoid tissue efficiently, but capacities to colonize deeper tissues, such as those of the spleen and liver, and blood were significantly reduced for the Crp$^-$ Cdt$^-$ and Crp$^+$ Cdt$^-$ mutants compared with the Crp$^-$ Cdt$^+$ mutant and the wild-type parent strain. The Crp$^-$ Cdt$^-$ and Crp$^+$ Cdt$^-$ SL1344 strains induced complete protection, as all mice immunized with the mutants survived challenge with ~10^4 times the 50% lethal dose of the wild-type SL1344 strain. The Crp$^-$ UK-1 strain was least attenuated yet induced the highest level of protective immunity against challenge with the wild-type UK-1 strain. The Crp$^+$ Cdt$^-$ and Crp$^-$ Cdt$^-$ strains, although totally attenuated, differed in immunogenicity to challenge with the highly virulent UK-1 parent, with the apparently hyperattenuated Crp$^-$ Cdt$^-$ strain inducing a lower level of protective immunity than the Crp$^+$ Cdt$^-$ strain. Nevertheless, these UK-1 Crp$^-$ Cdt$^-$ and Crp$^+$ Cdt$^-$ strains induced complete protective immunity to challenge with the less-virulent SL1344 wild-type strain. Taken collectively, the results indicate that the attenuation of a highly virulent *S. typhimurium* strain can yield a vaccine that induces excellent protective immunity to challenge with less-virulent *S. typhimurium* strains.**

Salmonella infection in humans and some domestic animals has been a public concern for many years. The development of attenuated *Salmonella* vaccine strains to protect against diseases caused by *Salmonella* spp. has been a field extensively studied by many investigators. One reason for the use of live avirulent *Salmonella* organisms as an oral vaccine is that *Salmonella* spp. initially colonize the gut-associated lymphoid tissue (GALT) prior to colonizing deeper tissues, such as those of the spleen and liver (6), a characteristic which is particularly important since the delivery of antigens to the GALT leads to generalized secretory, humoral, and cellular immune responses (2, 3, 7, 10, 26, 28). The ideal attenuated vaccine strain should be safe, stably attenuated, and protective. To render *Salmonella* spp. avirulent without impairing immunogenicity, several means of attenuation have been used, including curing the virulence plasmid and the introduction of auxotrophic mutations, mutations altering utilization and synthesis of carbohydrates, and mutations altering gene expression (for reviews, see references 5, 8, and 14).

Curtiss and Kelly (13) described *Salmonella typhimurium* SR-11 mutants with deletions (Δ) of the genes encoding the adenylate cyclase (cya) and cyclic AMP receptor protein that are avirulent and protective in mice. *S. typhimurium* Δ*cya* Δ*crp* mutants have been shown to be avirulent and protective for swine (9) and chickens (19, 20). Kelly et al. (22) described a mutation in *Salmonella choleraesuis* that extended beyond the *crp* gene, that resulted in decreased virulence, and that could not be complemented to the wild-type state by a plasmid containing the *crp*$^+$ gene. Similar mutations were generated in *S. typhimurium* and also introduced into *Salmonella typhi* (16, 29, 38). This locus adjacent to *crp* was termed *cdt* (colonization of deep tissues), since strains with these extended *crp* deletion mutations, although colonizing the GALT with wild-type ability, were significantly impaired in their ability to colonize internal visceral organs (4).

The present paper reports the characterization of *S. typhimurium* SL1344 and UK-1 Δ*crp* and Δ(*crp-cdt*) mutant strains and their avirulence, persistence, and abilities to induce protection against challenge with the highly virulent *S. typhimurium* SL1344 wild-type strain and the wild-type UK-1 strain.

The *Escherichia coli* and *S. typhimurium* LT-2, SL1344, and UK-1 strains used are listed in Table 1. Bacterial strains for most experiments, unless otherwise noted, were grown in Luria broth (LB) (24). Bacterial strains were maintained in 1% Bacto Peptone (Difco Laboratories, Detroit, Mich.) containing 5% glycerol and were fast frozen in dry ice-ethanol for storage in duplicate at −70°C. Plasmid pSD110 contains a 1.3-kb *Bam*HI-*Eco*RI fragment of *S. typhimurium* LT-2 DNA cloned into pBR322 and was generously provided by C. Schroeder (36). The 1.3-kb fragment carries the *crp* structural gene flanked by 370 bp of DNA which includes the promoter region at the 5′ end and 300 bp at the 3′ end. This plasmid was used to complement in *trans* the Δ*crp* mutation in the chromosome.

The Δ*crp* and Δ(*crp-cdt*) mutant strains were constructed by using bacteriophage P22HT*int* transduction following standard methods (1, 35). Fusaric acid selection for tetracycline-sensitive deletion mutants was performed as described by Maloy and Nunn (25). (Note that the *zhc-1431*::Tn*10* insertion used to introduce the linked *crp* and *crp-cdt* alleles into UK-1 strain χ3761 has not been removed by fusaric acid selection. This ensures that the only differences in the UK-1 strains are the specific *crp* allele and the presence or absence of pSD110.) Plasmid pSD110 was introduced into the Δ*crp* and Δ(*crp-cdt*) strains by electroporation or by P22HT*int* transduction.

All strains were tested for growth characteristics in LB at 37°C and for carbohydrate fermentation or utilization with MacConkey agar (Difco Laboratories) or minimal agar, re-

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TABLE 1. Bacterial strains

Strain	Genotype	Derivation or reference
E. coli		
CA8445	*thi rpsL* Δ*crp-45* Δ*cya-06* (pSD110)	36
S. typhimurium		
LT-2		
PP1037	*trpB223 crp-773*::Tn*10*	31
DU8802	*zhc-1431*::Tn*10*	33
TT172	*cysG*::Tn*10*	33
χ3000	Wild type	18
χ3385	*hsdL6 galE496 trpB2 flaA66 rpsL120 xyl-404 metE551 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *val his-6165 hsdSA29 metA22*	39
χ3477	F$^-$ *hsdL6* Δ(*galE-chl-uvrB*)*1005 flaA66 rpsL120 xyl-404 lamB*$^+$	39
χ3670	*hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val* (pSD110)	P22HT*int* (CA8445) →χ3385 with selection for Ap^r
χ3741	*zhc-1431*::Tn*10*	P22HT*int* (DU8802)→χ3000 with selection for Tc^r
χ4430	F$^-$ *hsdL6* Δ(*gal-chl-uvrB*)*1005 flaA66 rpsL120 zhc-1431*::Tn*10 xyl-404 lamB*$^+$ (*E. coli*) *hsdSA29* Δ(*zja*::Tn*10*)	P22HT*int* (DU8802)→χ3477
SL1344		
χ3339	Wild type *rpsL hisG*	18
χ3605	*hisG rpsL crp-773*::Tn*10*	P22HT*int* (PP1037)→χ3339 with selection for Tc^r, Mal$^-$
χ3622	*hisG rpsL* Δ(*crp-cdt*)*10*	FA^r Tc^s Mal$^-$ Arg$^-$ Cys$^-$ derivative of χ3605
χ3623	*hisG rpsL* Δ*crp-11*	FA^r Tc^s Mal$^-$ derivative of χ3605
χ3706	*hisG rpsL* Δ(*crp-cdt*)*10* (pSD110)	χ3622 transformed by pSD110
χ3712	*hisG rpsL* Δ(*crp-cdt*)*10* zhc-1431::Tn*10*	P22HT*int* (χ3741)→χ3622 with selection for Tc^r, Mal$^-$ Cys$^-$ Arg$^-$
χ3737	*hisG rpsL* Δ(*crp-cdt*)*10* (pSD110)	Mouse-passaged χ3706
χ3773	*hisG rpsL* Δ*crp-11 zhc-1431*::Tn*10*	P22HT*int* (χ3741)→χ3623 with selection for Tc^r, Mal$^-$
χ3774	*hisG rpsL* Δ*crp-11* (pSD110)	χ3623 transformed by pSD110
χ3910	*hisG rpsL cysG*::Tn*10*	P22*int* (TT172)→χ3339 with selection for Tc^r, Cys$^-$
χ3931	*hisG rpsL* Δ(*crp-cdt*)*14*	FA^r Tc^s derivative of χ3910; Cys$^-$ Crp$^-$
χ4452	*hisG rpsL* Δ(*crp-cdt*)*14 zhc-1431*::Tn*10*	P22HT*int* (χ4430)→χ3931 with selection for Tc^r, Mal$^-$ Cys$^-$
UK-1		
χ3761	Wild type	15
χ3779	Δ(*crp-cdt*)*10 zhc-1431*::Tn*10*	P22HT*int* (χ3712)→χ3761 with selection for Tc^r, Mal$^-$ Cys$^-$ Arg$^-$
χ3828	Δ*crp-11 zhc-1431*::Tn*10*	P22HT*int* (χ3773)→χ3761 with selection for Tc^r, Mal$^-$
χ4464	Δ(*crp-cdt*)*14 zhc-1431*::Tn*10*	P22HT*int* (χ4452)→χ3761 with selection for Tc^r, Mal$^-$ Cys$^-$
χ4635	Δ(*crp-cdt*)*10 zhc-1431*::Tn*10* (pSD110)	P22HT*int* (χ3670)→χ3779 with selection for Ap^r, Mal$^+$
χ4636	Δ*crp-11 zhc-1431*::Tn*10* (pSD110)	P22HT*int* (χ3670)→χ3828 with selection for Ap^r, Mal$^+$

spectively, with maltose, mannitol, sorbitol, melibiose, xylose, rhamnose, mannose, or glucose as the sole carbon source. The motilities of the mutants were determined by using medium composed of 1% casein enzyme hydrolysate (Sigma, St. Louis, Mo.), 0.5% NaCl, 0.5% agar (Difco Laboratories), and 50 μg of triphenyltetrazolium chloride per ml. The presence of group B O antigen (factors 1, 4, 5, and 12) and H antigen (poly a-z) was confirmed by slide agglutination with antisera (Difco Laboratories). The lipopolysaccharide profiles of the mutant and parent strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the profiles were visualized by the silver staining procedure (21, 40). Normal human serum (90%) was used to test the resistance of the mutants to complement-mediated bacteriolysis. Bacteria were grown at 37°C to log phase in LB and then diluted in buffered saline with gelatin (BSG) (11), which was added as a wetting agent to approximately 10^6 CFU/ml. Each strain was added at 10^4 CFU to serum buffered with HEPES (Sigma) (1 mM, pH 7.0) and allowed to incubate for 1 h at 37°C in a 5% CO_2 atmosphere. Samples were then diluted and plated on MacConkey agar containing 1% maltose for enumeration of CFU. *S. typhimurium* χ3477 [Δ(*gal-chl-uvrB*)*1005*], a rough strain, was used as a positive control for complement-mediated bacteriolysis.

Eight-week-old female BALB/c mice obtained from Sasco Inc. (Omaha, Nebr.) were used for all infectivity and immunization experiments. The animal room was maintained at 22 to 23°C with 12 h of illumination daily. Methods for growth of the bacteria and inoculation of mice were as described by Curtiss and Kelly (13) and Kelly et al. (22). Generally, the mutants or wild-type strains were grown to late log phase in LB broth at 37°C with aeration. Prior to immunization mice were deprived of food and water for 4 h and then were orally given 30 μl of sodium bicarbonate (10% [wt/vol]) to neutralize stomach acid. Twenty minutes later, 20 μl of *S. typhimurium* cells suspended in BSG diluted to the desired density was administered through a pipette tip to the back of the mouth. Food and water were returned 30 min after inoculation.

The levels of virulence of the *S. typhimurium* wild-type and mutant strains were determined by oral inoculation of mice with various doses of the strains. Morbidity and mortality of mice were observed for 30 days postinoculation. Survivors were subsequently challenged with up to 2×10^5 times the oral 50% lethal dose (LD_{50}) of the wild-type parent, χ3761 or χ3339. Morbidity and mortality of the challenged mice were observed for an additional 30 days. LD_{50}s were calculated by the method of Reed and Muench (32).

Groups of three mice were inoculated with 10^9 CFU of the mutants and necropsied on days 1, 4, 7, and 10 after oral inoculation. Spleens, livers, Peyer's patches, and blood samples were collected, and the tissues were homogenized in BSG with

a homogenizer (Brinkmann, Westburg, N.Y.). *S. typhimurium* was recovered from the homogenates and blood samples by plating on MacConkey agar supplemented with 1% maltose for identification of the Crp^- mutants or with 1% lactose for identification of the Crp^+ strains. The CFU recovered from tissues were replica plated onto MacConkey agar supplemented with maltose and ampicillin to test the in vivo stability of plasmid pSD110. Group B antiserum (Difco) was used for verification of *S. typhimurium* CFU. CFU are presented as geometric means with standard errors.

Student's *t* test was used to compare differences in colonization of mice by the wild-type and mutant strains. $P < 0.05$ was regarded as significant.

Discovery of (*crp-cdt*) mutations and construction of the mutants. In an effort to construct a prototype deletion mutation of the *crp* region for all future vaccine strain constructions, several independent deletions of *crp-773*::Tn*10* were generated in *S. typhimurium* SL1344. One Δ*crp* mutant, designated χ3622, appeared to be particularly more attenuated, as mice receiving greater than 10^9 CFU did not develop any visible signs of illness. Upon oral challenge with the wild-type SL1344 parent strain 30 days later, the mice were protected against at least 10 times the wild-type LD_{50} (higher challenge doses were not tested). Further characterization revealed that this deletion, created by the imprecise excision of Tn*10*, conferred auxotrophy for arginine and cysteine and presumably caused deletion of the *argD* and *cysG* loci in addition to *crp*. This mutation was initially given the designation Δ(*arg-crp-cysG*)*10* (based on the then-reported gene order in the *S. typhimurium* chromosome (33). crp^+-containing plasmid pSD110 was transformed into χ3622 and into χ3623, which has another independently generated *crp* mutation, designated Δ*crp-11*, to restore *crp* function. The pSD110 plasmid was able to restore virulence to Δ*crp-11* χ3623, but χ3622, carrying the crp^+ plasmid, remained fully attenuated for mice at doses exceeding 6×10^8 CFU. Mutants with *argD*::Tn*10* and *cysG*::Tn*10* mutations were constructed in SL1344, and each displayed wild-type SL1344 virulence, with LD_{50}s of about 10^5 CFU. The Δ(*arg-crp-cysG*)*10* mutation was subsequently introduced into other *Salmonella* serotypes via P22- or P1L4-mediated transduction. In each case, the avirulence phenotype was associated with the genetic lesion generated in the 75-min map region of the *Salmonella* chromosome.

We endeavored to isolate this virulence-associated allele independent of *crp* by generating another series of independent deletion mutations by imprecise excision of *cysG*::Tn*10* located near 75.5 min (34) downstream from *crp* and *arg*. This attempt produced a mutant, χ3931, that was Arg^+ Cys^- Crp^-. Complementation of the *crp* mutation with pSD110 did not restore virulence for mice. The mutation in χ3931 was initially designated Δ(*crp-cysG*)*14*. In this mutant search, we failed to recover Cys^- Crp^+ strains that were avirulent for mice. Persistence studies of mice with χ3622 [Δ(*arg-crp-cysG*)*10*] and χ3931 [Δ(*crp-cysG*)*14*] with and without pSD110 revealed that these mutants localized to the GALT but that the number of bacteria that reached deeper tissues were reduced compared to strains with complementable *crp* mutations. Thus, we termed this locus *cdt*, as *Salmonella* strains with this region of DNA deleted were defective in reaching and colonizing deep tissues of mice after oral inoculation. The mutant alleles were therefore redesignated Δ(*crp-cdt*)*10* and Δ(*crp-cdt*)*14*. Since *S. typhimurium* SL1344 is streptomycin resistant, which would make it less suitable as a vaccine, we generated Δ(*crp-cdt*)*10* and Δ(*crp-cdt*)*14* mutations in *S. typhimurium* UK-1, which is highly virulent for mice and totally antibiotic sensitive.

TABLE 2. Mortality of BALB/c mice 30 days after oral inoculation with mutant *S. typhimurium* strains[a]

Strain	Genotype	Inoculating dose (CFU)	No. of survivors/ total no. of mice
SL1344			
χ3622	Δ(*crp-cdt*)*10*	6.2×10^8	5/5
χ3623	Δ*crp-11*	2.2×10^9	1/5
χ3737	Δ(*crp-cdt*)*10* (pSD110)	5.0×10^8	5/5
χ3774	Δ*crp-11* (pSD110)	3.0×10^4	3/5
UK-1			
χ3779	Δ(*crp-cdt*)*10*	1.8×10^9	9/9
χ3828	Δ*crp-11*	2.6×10^9	8/9
χ4635	Δ(*crp-cdt*)*10* (pSD110)	1.0×10^9	9/9
χ4636	Δ*crp-11* (pSD110)	5.0×10^4	8/15

[a] Mice were inoculated perorally with the indicated strains. Morbidity and mortality were observed for 30 days.

Characterization of the mutant strains. The Δ*crp* and Δ(*crp-cdt*) derivatives failed to ferment or utilize maltose, mannitol, sorbitol, melibiose, rhamnose, or xylose as the sole carbon source but did grow on glucose and mannose. Wild-type UK-1 strain χ3761 and SL1344 strain χ3339 fermented all of the carbon sources assayed. The plasmid carrying the *Salmonella* crp^+ allele with its own promoter, pSD110, restored the carbohydrate utilization ability of the Δ*crp* and Δ(*crp-cdt*) mutants to the wild-type phenotype. The mutant strains did not display motilities equivalent to those displayed by the wild-type parents in the medium with triphenyltetrazolium chloride, but they agglutinated with *Salmonella* H antiserum. The synthesis of flagella is under cyclic AMP-cyclic AMP receptor protein control (42). Komeda et al. (23) have described a suppressor mutation, *cfs* (constitutive flagellar synthesis), which restores flagellation in Crp^- mutants. It is therefore likely that such suppressor mutations can be or are spontaneously selected to result in a Crp^- mutant with restored ability to synthesize flagella. The Δ*crp* and Δ(*crp-cdt*) mutants exhibited reduced growth rates, with mean generation times in LB broth decreased by 13% compared to that of the wild-type parent. Both wild-type parent and mutant strains had similar lipopolysaccharide compositions. Resistance to complement-mediated bacteriolysis when exposed to 90% normal human serum in a 5% CO_2 atmosphere was not affected by introducing the *crp* and/or *crp cdt* mutations into wild-type *S. typhimurium* χ3761.

Virulence of mutant strains in BALB/c mice. The oral LD_{50}, determined for 8-week-old BALB/c mice, of wild-type SL1344 χ3339 was 6×10^4 CFU and of UK-1 χ3761 was 8.5×10^3 CFU. All the mutant strains had LD_{50}s more than 10^5 times higher than those of the wild-type parents, χ3339 and χ3761 (Table 2). The Crp^- Cdt^- mutants were completely avirulent, as all mice survived oral infection with 6×10^8 CFU of Crp^- Cdt^- SL1344 strain χ3622 [Δ(*crp-cdt*)*10*] and 1.8×10^9 CFU of Crp^- Cdt^- UK-1 strain χ3779 [Δ(*crp-cdt*)*10*] (Table 2). Mice that received these mutants did not show any signs of illness and remained healthy throughout the 30-day observation period after inoculation. The Crp^- Cdt^+ SL1344 and UK-1 mutants (χ3623 and χ3828 with Δ*crp-11*, respectively) were less attenuated than the Crp^- Cdt^- strains, as some mice died in both treatment groups (Table 2). In addition, mice inoculated with UK-1 Crp^- Cdt^+ mutant χ3828 (Δ*crp-11*) developed splenomegaly 10 days postinoculation, whereas the Crp^- Cdt^- mutants of both SL1344 and UK-1 did not induce any spleen enlargement.

Introduction of plasmid pSD110 carrying the wild-type *crp*

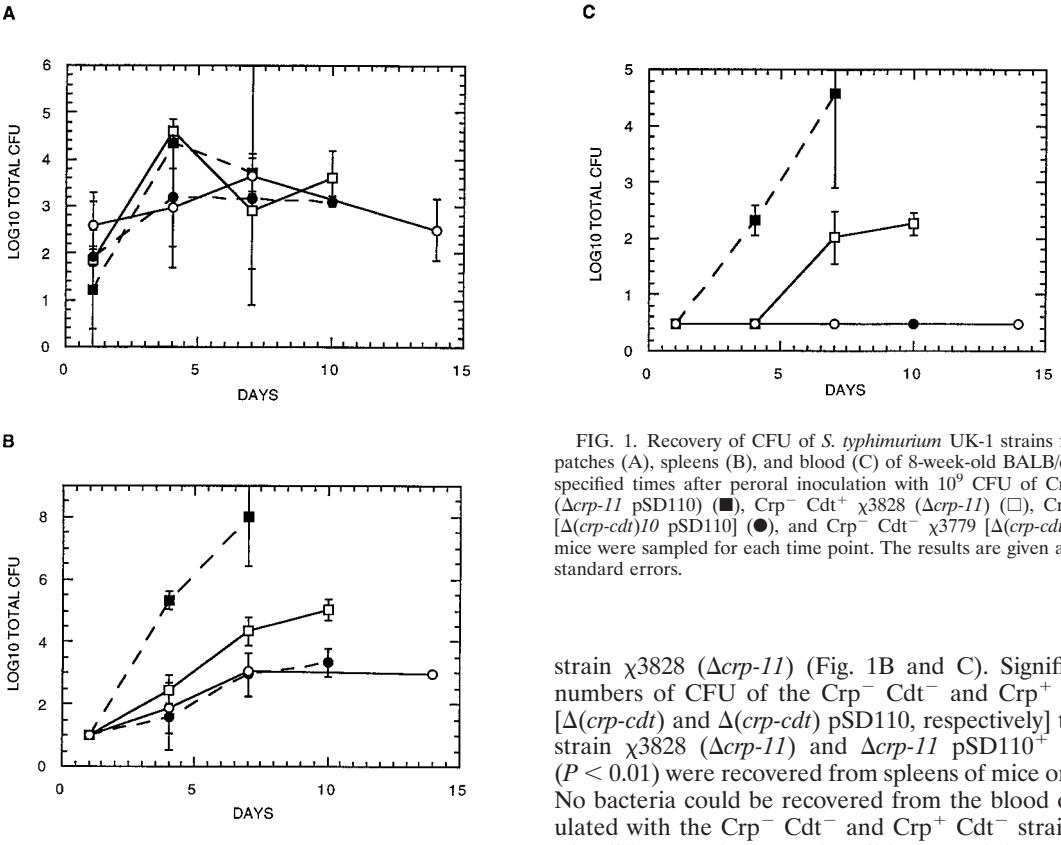

FIG. 1. Recovery of CFU of *S. typhimurium* UK-1 strains from the Peyer's patches (A), spleens (B), and blood (C) of 8-week-old BALB/c female mice at specified times after peroral inoculation with 10^9 CFU of Crp^+ Cdt^+ χ4636 (Δ*crp-11* pSD110) (■), Crp^- Cdt^+ χ3828 (Δ*crp-11*) (□), Crp^+ Cdt^- χ4635 [Δ(*crp-cdt*)*10* pSD110] (●), and Crp^- Cdt^- χ3779 [Δ(*crp-cdt*)*10*] (○). Three mice were sampled for each time point. The results are given as $\log_{10}$ means ± standard errors.

gene and promoter into the mutant strains restores *crp* gene function in *trans*. Inclusion of pSD110 in the SL1344 and UK-1 Crp^- strains with the Δ*crp-11* allele complemented the mutation to generate strains with near-wild-type virulence (Table 2). However, inclusion of the crp^+ plasmid in the SL1344 and UK-1 Crp^- Cdt^- strains did not restore full virulence, as all mice survived oral infection with the highest dose given (Table 2). These results indicate that pSD110 does not carry the DNA sequences needed to complement the missing *cdt* allele(s) in the *crp* region of the chromosome to restore full virulence.

Substitution of the Δ(*crp-cdt*)*14* allele for the Δ(*crp-cdt*)*10* allele in both SL1344 and UK-1 backgrounds generated strains that displayed identical phenotypes with regard to avirulence, inability to induce splenomegaly, and inability to have virulence restored by the addition of the crp^+ allele in pSD110 (data not shown).

Tissue tropism and persistence of avirulent mutants in mice. Figure 1 presents data on the recovery of the UK-1 mutants from tissues of mice after oral infection with 10^9 CFU of the strains. Strain χ4636 (Δ*crp-11* pSD110) was used as a wild-type control, as the *crp* deletion mutation was complemented by the *crp* gene carried by pSD110. Figure 1 shows high numbers of χ4636 CFU recovered on day 7 from the Peyer's patches of the intestinal tract, spleens, and blood of mice prior to death due to disease. The Crp^- χ3828 (Δ*crp-11*) strain colonized Peyer's patches (Fig. 1A) as well as did wild-type χ4636 (Δ*crp-11* pSD110). χ3828 was also found in the spleen and blood (Fig. 1B and C). Mice inoculated with this strain displayed signs of illness and had enlarged spleens. Crp^- Cdt^- strain χ3779 colonized the Peyer's patches (Fig. 1A) as efficiently as the Δ*crp-11* and wild-type strains but was limited in its ability to colonize spleen and blood compared to Crp^- strain χ3828 (Δ*crp-11*) (Fig. 1B and C). Significantly lower numbers of CFU of the Crp^- Cdt^- and Crp^+ Cdt^- strains [Δ(*crp-cdt*) and Δ(*crp-cdt*) pSD110, respectively] than of Crp^- strain χ3828 (Δ*crp-11*) and Δ*crp-11* $pSD110^+$ strain χ4636 ($P < 0.01$) were recovered from spleens of mice on day 7 or 10. No bacteria could be recovered from the blood of mice inoculated with the Crp^- Cdt^- and Crp^+ Cdt^- strains (Fig. 1C). The differences between the wild-type and the Crp^- Cdt^- and Crp^+ Cdt^- strains were statistically significant ($P < 0.01$). Inclusion of the *crp* gene carried on pSD110 in the Crp^- Cdt^- strains did not completely restore the ability of the mutants to colonize deep tissues as did complementation of the Δ*crp-11* mutation in χ3828. The phenotype associated with the *cdt* mutation has been identified as a marked decrease in the efficiency of the bacteria to reach and colonize deep tissues compared to the parent strain. Crp^+ Cdt^- strain χ4635 [Δ(*crp-cdt*)*10* $pSD110^+$] (Fig. 1B and C) colonized deeper tissues at significantly reduced levels ($P < 0.05$ and $P < 0.01$, respectively) compared with Crp^- Cdt^+ Δ*crp-11* strain χ3828. An evaluation of *S. typhimurium* UK-1 strains with the Δ(*crp-cdt*)*14* allele in place of the Δ(*crp-cdt*)*10* allele gave equivalent results in terms of the abilities of the strains to colonize Peyer's patches and deep tissues in orally infected female BALB/c mice (data not shown).

Similarly, in evaluating *S. typhimurium* SL1344 strains, there was no significant difference in colonization of Peyer's patches between wild-type χ3339, Crp^- Cdt^- strain χ3622 (Δ*crp-cdt*), and Crp^+ Cdt^- strain χ3737 [Δ(*crp-cdt*) $pSD110^+$]. Crp^+ Cdt^- strain χ3737 did not colonize spleens as efficiently as wild-type χ3339. No splenomegaly was observed in mice inoculated with SL1344 Crp^+ Cdt^- or Crp^- Cdt^- mutants. Mice that received 10^9 CFU of χ3622 or χ3737 did not show signs of illness; however, all mice that received wild-type χ3339 died by day 7 (data not shown).

The in vivo stability of plasmid pSD110 was assessed by replica plating CFU recovered from the tissue homogenates plated on MacConkey agar with either maltose or lactose onto MacConkey agar plus maltose and ampicillin. All CFU recovered from mice inoculated with mutant strains containing pSD110 were Ap^r and Mal^+, indicating that each colony visualized on the MacConkey plates plus maltose had Ap^r and crp^+

TABLE 3. Effectiveness of single oral immunization with *S. typhimurium* SL1344 mutants in protecting BALB/c mice against challenge with wild-type SL1344[a]

Strain	Genotype	Immunizing dose (CFU)	Challenging dose (CFU)	No. of survivors/ total no. of mice
χ3622	Δ(*crp-cdt*)*10*	1.5×10^9	3.2×10^8	5/5
		6.2×10^8	3.6×10^8	5/5
		4.2×10^8	8.8×10^8	5/5
χ3737	Δ(*crp-cdt*)*10* (pSD110)	5.8×10^8	8.4×10^8	5/5

[a] Thirty days after mice were immunized perorally with a single dose of the indicated attenuated strains, they were challenged with wild-type SL1344 strain χ3339. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge.

portions of the plasmid and thus demonstrating the stability of the *crp* gene complementing the *crp* deletion.

Efficacy of avirulent mutants in conferring protective immunity. Table 3 contains data on the ability of SL1344 mutant strains χ3622 and χ3737 to induce protective immunity. All mice immunized with a single dose of Crp^- Cdt^- strain χ3622 were protected against challenge with 5×10^3 to 1.5×10^4 times the LD_{50} of the wild-type parent, χ3339. Crp^+ Cdt^- strain χ3737 protected mice against wild-type challenge to the same degree. These encouraging results prompted us to conduct a more extensive series of experiments to evaluate the avirulence and immunogenicity of the mutant derivatives of the highly virulent and antibiotic-sensitive UK-1 strain.

Data on the efficacy of a single oral immunization with *S. typhimurium* UK-1 mutant strains for protecting BALB/c mice against challenge with wild-type strains are shown in Table 4. Since colonization of deep tissues in mice by Crp^- Cdt^- strain χ3779 was significantly lower than for Crp^- strain χ3828, it follows that mice immunized with the Crp^- Cdt^- mutant might not be as protected against wild-type challenge as mice immunized with χ3828. Thus, although mice immunized with 10^8 or 10^9 CFU of Crp^- strain χ3828 displayed signs of illness as evidenced by splenomegaly and bacteremia and although 2 of 18 mice died prior to challenge, consistent with the above reasoning, 23 of 25 (92%) mice immunized with Crp^- strain χ3828 survived challenge with various doses of wild-type χ3761 (Table 4). In contrast, only 10 of 27 (37%) mice immunized with either 10^7, or 10^8, or 10^9 CFU of χ3779 [Δ(*crp-cdt*)*10*] survived challenge with the wild-type parent strain, χ3761. Complementation of the *crp* mutation in Crp^- Cdt^- strains by pSD110 yielded strains that induced greater protective immunity in mice, as mice immunized with Δ(*crp-cdt*)*10* $pSD110^+$ strain χ4635 displayed higher frequencies (14 of 27; 52%) of survival after challenge with the wild-type parent than mice immunized with the Crp^- Cdt^- strain (Table 4). Δ(*crp-cdt*)*14* strain χ4464 showed protective efficacy results similar to those of Δ(*crp-cdt*)*10* strain χ3779 and failed to provide complete protection (data not shown).

Since a single oral immunization with UK-1 Crp^- Cdt^- or Crp^+ Cdt^- mutants did not induce complete protection, it was of interest to determine whether multiple immunizations with the mutants would give enhanced protection and whether these UK-1 mutants can induce complete protection to challenge with less-virulent wild-type strains such as SL1344. Mice were perorally given two immunizations with 10^9 CFU of UK-1 Crp^- Cdt^- strain χ3779 [Δ(*crp-cdt*)*10*] or Crp^+ Cdt^- strain χ4635 [Δ(*crp-cdt*)*10* pSD110] one month apart and were challenged 30 days after the second immunization with either wild-type UK-1 or SL1344 (Table 5). Of 10 mice in each of the two groups immunized with either UK-1 attenuated strain, 8 (80%) survived challenge with wild-type UK-1; interestingly, all mice (4 of 4; 100%) challenged with the highest dose (10^9 CFU) survived, while one from each group challenged with the lower doses (10^6 and 10^7 CFU) died (Table 5). Complete protection was apparent when mice immunized with the UK-1 mutant strains survived challenge with 10^6 to 10^9 CFU of the wild-type SL1344 strain (Table 5). None of the immunized mice showed signs of illness either before or after challenge.

It has been shown that *S. typhimurium* Δ*crp* Δ*cya* mutants are avirulent and immunogenic in mice, chickens, and swine (9, 13, 19) but provide diminished attenuation to *S. typhi* for humans (37). An extended *crp* deletion mutation termed *crp-cdt* was discovered in *S. choleraesuis*, and bacteria carrying this mutation were found to be avirulent and protective in mice (22) and in swine (41). Subsequently, an *S. typhi* Δ(*crp-cdt*) strain was tested in adult volunteers and shown to be avirulent and immunogenic (38). In this study *S. typhimurium* SL1344 and UK-1 Δ*crp-cdt* and Δ(*crp-cdt*) mutants were constructed and characterized. The SL1344 wild-type strain, χ3339 (18), has an LD_{50} of 6×10^4 CFU. *S. typhimurium* UK-1 is a strain of extremely high virulence, having an LD_{50} for one-day-old chicks of 3×10^3 CFU (15) and an LD_{50} of 8.5×10^3 CFU for mice (this manuscript), and is capable of causing lethal infections in swine, cattle, and horses. It was therefore reasoned that if the strain could be effectively attenuated, then its ability

TABLE 4. Effectiveness of single oral immunization with *S. typhimurium* UK-1 mutants in protecting BALB/c mice against challenge with wild-type UK-1[a]

Strain	Genotype	Immunizing dose (CFU)	Challenging dose (CFU)	No. of survivors/ total no. of mice
χ3779	Δ(*crp-cdt*)*10*	1.8×10^9	1.8×10^9	1/3
			1.8×10^8	1/3
			1.8×10^7	2/3
		1.8×10^8	1.8×10^9	0/3
			1.8×10^8	0/3
			1.8×10^7	2/3
		1.8×10^7	1.8×10^9	0/3
			1.8×10^8	1/3
			1.8×10^7	3/3
Total				10/27
χ3828	Δ*crp-11*	2.6×10^9	1.8×10^9	3/3
			1.8×10^8	1/3
			1.8×10^7	2/2
		2.6×10^8	1.8×10^9	3/3
			1.8×10^8	3/3
			1.8×10^7	2/2
		2.6×10^7	1.8×10^9	3/3
			1.8×10^8	3/3
			1.8×10^7	3/3
Total				23/25
χ4635	Δ(*crp-cdt*)*10* (pSD110)	1.0×10^9	1.2×10^9	1/3
			1.2×10^8	2/3
			1.2×10^7	0/3
		1.0×10^8	1.2×10^9	2/3
			1.2×10^8	2/3
			1.2×10^7	2/3
		1.0×10^7	1.2×10^9	2/3
			1.2×10^8	3/3
			1.2×10^7	0/3
Total				14/27

[a] Thirty days after mice were immunized perorally with a single dose of the indicated attenuated strains, they were challenged with wild-type UK-1 strain χ3761. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge.

TABLE 5. Effectiveness of two oral immunizations with *S. typhimurium* UK-1 mutants in protecting BALB/c mice against challenge with wild-type UK-1 or SL1344[a]

Immunizing strain or compound	Genotype	Challenging dose (CFU)	No. of survivors/total no. of mice after challenge with:	
			UK-1	SL1344
χ3779	Δ(*crp-cdt*)*10*	1×10^9	4/4	4/4
		1×10^7	2/3	3/3
		1×10^6	2/3	3/3
χ4635	Δ(*crp-cdt*)*10* (pSD110)	1×10^9	4/4	4/4
		1×10^7	2/3	3/3
		1×10^6	2/3	3/3
BSG	NA	1×10^6	ND	0/3
		1×10^5	0/3	2/3

[a] Mice were given two immunizations, 1 month apart, with 10^9 CFU of the indicated attenuated strains or with BSG and challenged 30 days after the second immunization with the wild-type UK-1 χ3761 or SL1344 χ3339. Morbidity and mortality observations were recorded daily for an additional 30 days postchallenge. NA, not applicable; ND, not determined.

to induce protective immunity against *Salmonella* challenge might be enhanced relative to those of attenuated derivatives of less-virulent *S. typhimurium* strains.

All the SL1344 and UK-1 mutants were attenuated, with mice surviving greater than 10^4 times the wild-type LD_{50}. SL1344 Crp^- strain χ3623 and UK-1 Crp^- strain χ3828 were more virulent for mice than the Cdt^- or Crp^- Cdt^- strains since mice receiving doses higher than 10^8 CFU became ill and some died. χ3828 and χ3623 (data not shown) also colonized and persisted at higher levels in spleen and blood than did the more attenuated Δ(*crp-cdt*) strains. It is significant that this least-attenuated strain induced the highest level of protective immunity in mice surviving the immunizing dose. The Δ(*crp-cdt*) mutation did not significantly impair the abilities of the mutants to attach to, invade, and persist in GALT but significantly reduced the abilities of the strains to colonize deeper tissues, such as the spleen, compared to the Δ*crp-11* and the UK-1 wild-type strains. These data indicate that the level of protective immunity in mice is dependent on the degree of colonization by the immunizing strain, especially with respect to internal lymphoid organs. Similar data by others have shown that mutations such as *pur* (27), *asd* (12), *thy* (30), and *phoP* (17) in *Salmonella* impair the ability of the wild type to invade and persist in internal lymphoid organs and that protection was not complete after immunized mice received a significant oral challenge with the wild-type parent. Interestingly, when the *crp* deletion mutation in UK-1 strains was complemented by crp^+ plasmid pSD110, the Crp^+ Cdt^- strains induced a higher degree of protective immunity in immunized mice, as evidenced by a higher frequency of survival after challenge with the wild-type parent than that for mice immunized with the Crp^- Cdt^- strains. Complementation of the *crp* mutation in the Δ*crp-cdt* mutant strains did not restore the ability of the Crp^+ Cdt^- strains to colonize spleen tissue to the level observed for Crp^- strain χ3828. The Δ(*crp-cdt*) mutation is highly attenuating, possibly too much so in UK-1, but in some strains, such as SL1344, yields vaccines that are completely safe and that confer high-level protective immunity against infection by virulent *S. typhimurium* wild-type strains except for hypervirulent strains such as UK-1. On the other hand, the UK-1 Crp^- Cdt^- and Crp^+ Cdt^- strains, although not fully protecting mice against challenge with the highly virulent UK-1 strain, can induce excellent protective immunity to challenge with a less-virulent *S. typhimurium* strain such as SL1344. Also, since Cdt^- *S. typhimurium* strains are much more efficient at colonizing the GALT than internal lymphoid organs, such as the spleen, it can be anticipated that such attenuated strains might be more effective in inducing generalized mucosal immune responses than in inducing either systemic or cellular immune response.

The results of these studies help to improve the understanding of the phenotype associated with a locus close to the *crp* gene that results in stable avirulent *Salmonella* strains that are able to efficiently colonize the GALT but that do not significantly invade deep tissues. Studies are in progress to delineate the functions of this genetic locus.

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