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Salmonella typhimurium Deletion Mutants Lacking Adenylate Cyclase and Cyclic AMP Receptor Protein Are Avirulent and Immunogenic

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***Salmonella typhimurium* SR-11 mutants with *cya*::Tn*10* or *crp*::Tn*10* mutations were found to be avirulent and immunogenic for BALB/c mice. Fusaric acid-resistant derivatives with deletions of the Tn*10* and adjacent DNA sequences were constructed in *S. typhimurium* SR-11 strains with or without the virulence plasmid pStSR100. These Δ*cya* Δ*crp* strains grew more slowly than wild-type strains. They possessed wild-type ability to attach to, invade, and persist in gut-associated lymphoid tissue for up to a week but exhibited a diminished ability to reach mesenteric lymph nodes and the spleen. Mice 4 to 8 weeks old were resistant to oral infection with 10^9 cells of several different Δ*cya* and Δ*cya* Δ*crp* strains (the equivalent to 10^4 50% lethal doses of wild-type *S. typhimurium* SR-11) and 30 days after immunization became resistant to oral challenge with 10^3 to 10^4 50% lethal doses of wild-type *S. typhimurium* SR-11.**

Salmonella typhimurium and, presumably, other *Salmonella* species with invasive properties enter deep tissues after oral ingestion by attaching to, invading, and proliferating in cells of the gut-associated lymphoid tissue (GALT) (11). Delivery of an antigen to the GALT elicits generalized secretory (8, 12, 37, 43), humoral (2, 19a, 21), and cellular (10) immune responses. Therefore, avirulent *Salmonella* mutants that have lost the ability to cause disease without impairment of their ability to attach to and invade the GALT are likely to serve as effective means to deliver foreign antigens to the GALT and to induce protective immunity against the pathogen supplying such colonization or virulence antigens (13, 17, 19a, 22, 41, 42, 60). Such bivalent, avirulent *Salmonella* strains have been shown to elicit antibodies (10, 13, 19a, 41, 42, 60, 61) and, in one instance, a cellular immune response (10) to the expressed antigen, but data pertaining to induction of protective immunity against the pathogen supplying the colonization or virulence antigen are still scant.

Bacon et al. (3, 4) were first to investigate the avirulence of auxotrophic mutants of *S. typhi* and noted that mutants with requirements for purines, *p*-aminobenzoic acid (*p*ABA), and aspartate had reduced virulence for mice. Germanier and Fürer first investigated the use of *galE* mutants of *S. typhimurium* for avirulence and immunogenicity in mice (24) and then proposed the use of *S. typhi galE* mutant Ty21a as a vaccine against typhoid fever in humans (25). Hoiseth and Stocker (32) used transposon mutagenesis with Tn*10* (35), followed by selection for fusaric acid resistance that leads to deletional loss of Tn*10* and adjacent DNA sequences (9, 40), to produce deletion mutations unable to revert, a problem that was apparent in the mutants used by Bacon et al. (4). Hoiseth and Stocker initially isolated *aroA* deletion mutants impaired in the ability to synthesize the aromatic amino acid family of compounds, including *p*ABA, needed for folate biosynthesis, and dihydroxybenzoic acid, a precursor to enterochelin. Recently, they combined the Δ*aroA* mutation with a deletion mutation blocking adenine biosynthesis (Δ*purA*) (38a). We have used Tn*10*-induced and fusaric acid resistance-generated Δ*asd* mutations that impose a requirement for diaminopimelic acid to render *S. typhimurium* avirulent without impairing its ability to induce a generalized secretory immune response (19a). Since *S. typhimurium* possesses a plasmid that contributes to virulence (5, 27, 28, 29, 31, 34, 48), plasmid-cured derivatives have been investigated and proposed for use as live vaccines (46).

Although each means for rendering *Salmonella* spp. avirulent without impairing immunogenicity has merit, each has problems. *galE* mutants are difficult to grow to maintain immunogenicity, since they are galactose sensitive (24, 25) but must be grown in the presence of galactose to produce the normal lipopolysaccharide essential for immunogenicity (24, 26). However, growth in galactose selectively leads to galactose-resistant variants that are nonimmunogenic (24). Although Δ*aroA* mutants abolish the synthesis of both enterochelin and folic acid, the conclusions of Yancey et al. (62) on the necessity of enterochelin for *S. typhimurium* virulence have been called into question by the results of Benjamin et al. (7), who demonstrated that mutations that interfere with the ability of *S. typhimurium* to chelate and transport iron are without significant effect on virulence. Therefore, the avirulence of Δ*aroA* mutants is most likely due to inability to synthesize *p*ABA and, subsequently, folic acid. Since Bacon et al. (4) observed that administering *p*ABA in the diet of mice infected with mutants unable to synthesize *p*ABA led to wild-type levels of virulence, one must be concerned with phenotypic reversal of avirulence in vaccine strains due to dietary consumption of metabolites whose synthesis is blocked in the avirulent mutants. Although they do not have some of these other difficulties, Δ*asd* mutants rapidly die following oral feeding and invasion of the GALT. Thus, they are only effective at eliciting mucosal immunity and are partially and totally ineffective in inducing humoral and cellular immunities, respectively (19a).

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TABLE 1. Bacterial strains

Strain	Parent strain (plasmid)	Relevant genotype	Derivation (reference)[a]
χ3041	SR-11(pStSR100)	Virulent prototroph P22^i	William Benjamin
χ3181	SR-11(pStSR100)	Prototroph	Mouse-passaged χ3041
χ3306	SR-11(pStSR100)	*gyrA1816* P22^i	(27)
χ3337	SR-11	*gyrA1816* P22^i	χ3306 cured of 100-kilobase virulence plasmid (27)
χ3456	SR-11(pStSR101)	pStSR100::Tnmini*tet43*	P22 HT *int*(pStSR100::Tnmini*tet43*) ⇒ χ3181
PP1002	LT-2(pStLT100)	*cya*::Tn*10 trpB223*	P. W. Postma; P22(PP991) ⇒ SB3507 (52)
PP1037	LT-2(pStLT100)	*crp773*::Tn*10 trpB223*	P. W. Postma; P22(PP1011) ⇒ SB3507 (52)
χ3395	SR-11(pStSR100)	*gyrA1816 cya*::Tn*10*	P22 HT *int*(PP1002) ⇒ χ3306
χ3396	SR-11(pStSR100)	*gyrA1816 crp773*::Tn*10*	P22 HT *int*(PP1037) ⇒ χ3306
χ4032	SR-11(pStSR100)	*gyrA1816 Δcya-1*	FA^r Tc^s derivative of χ3395
χ4055	SR-11(pStSR100)	*gyrA1816 Δcya-1 crp773*::Tn*10*	P22 HT *int*(PP1037) ⇒ χ4032
χ4058	SR-11	*gyrA1816 cya*::Tn*10*	P22 HT *int*(PP1002) ⇒ χ3337
χ4060	SR-11	*gyrA1816 Δcya-3*	FA^r Tc^s derivative of χ4058
χ4061	SR-11	*gyrA1816 Δcya-3 crp773*::Tn*10*	P22 HT *int*(PP1037) ⇒ χ4060
χ4062	SR-11	*gyrA1816 Δcya-3 Δcrp-2*	FA^r Tc^s derivative of χ4061
χ4064	SR-11(pStSR100)	*gyrA1816 Δcya-1 Δcrp-1*	FA^r Tc^s derivative of χ4055
χ4065	SR-11	*gyrA1816 crp773*::Tn*10*	P22 HT *int*(PP1037) ⇒ χ3337

[a] FA, Fusaric acid resistance.

Therefore, we began to investigate the use of transposon-induced mutations in which the impairment leading to avirulence could not be repaired either by diet or by the animal host. We furthermore sought mutants that were stably avirulent, immunogenic, and easy to grow and store. Cyclic AMP (cAMP) and the cAMP receptor protein (CRP) are necessary for the transcription of many genes and operons concerned with the transport and breakdown of catabolites (1). Systems used for transporting fuel and carbon sources are all under positive control by cAMP, as are several amino acid permeases (1). In addition, the cAMP concentration in cells also influences lysogenization by temperate phages (33, 53), synthesis of pili and fimbriae (47, 54), synthesis of flagella (36, 49, 63), and synthesis of at least one outer membrane protein (45). Although cAMP is present in mammalian cells, the concentrations of 1.0 to 0.1 μM present in gastrointestinal tissues and fluids and other cells (30, 59) in which *S. typhimurium* can invade and multiply are well below the concentration of 0.1 mM (36) to 1.0 mM (1) cAMP necessary to allow *cya* mutants to exhibit a wild-type phenotype in vitro. Furthermore, the inclusion of a *crp* mutation should abolish any benefit that could accrue from uptake of cAMP in vivo by *cya* mutants.

We therefore constructed *S. typhimurium* strains with Δ*cya* and Δ*crp* mutations, eliminating the ability to synthesize adenylate cyclase (ATP pyrophosphate lyase [cyclizing] EC 4.6.1.1) and the CRP, respectively, and here we report our findings on the virulence and immunogenicity of these strains.

MATERIALS AND METHODS

Bacterial strains. The *S. typhimurium* SR-11 strains used are listed in Table 1. They were maintained as frozen cultures suspended in 1% Bacto-Peptone containing 5% glycerol and fast frozen in dry ice-ethanol for storage in duplicate at −70°C and also suspended in 1% Bacto-Peptone (Difco Laboratories, Detroit, Mich.) containing 50% glycerol for storage at −20°C for routine use.

Media. The complex media used for routine cultivation were L broth (38) and Luria broth (39). Difco agar was added to Luria broth at 1.2% for base agar and 0.65% for soft agar. Penassay agar (Difco Antibiotic Medium 3–1.5% Difco agar) was used for routine enumeration of bacteria. Fermentation was evaluated by supplementing Difco MacConkey base agar or eosin methylene blue agar (15) with an appropriate carbohydrate at 1% (final concentration). Nalidixic acid and tetracycline were added at concentrations of 40 and 12.5 μg/ml, respectively, when required. Motility was assayed by using motility test medium with triphenyltetrazolium chloride (Scott Laboratories, Inc., Richmond, Calif.) at 50 mg/liter.

Synthetic media were minimal liquid and minimal agar supplemented with nutrients at optimal levels as previously described (18). Buffered saline with gelatin (BSG) (14) was used routinely as a diluent.

Bacteriophage P22HT *int* was routinely used for transduction with standard methods and media (20, 56). The media and methods described by Maloy and Nunn (40) were used for fusaric acid selection for deletion mutations.

Mice. Female BALB/c mice (Sasco, Inc., St. Louis, Mo.) were used for all infectivity and immunization experiments. Animals 3 or 7 weeks old were kept for 1 week in a quarantined room before being used in experiments. Mice were placed in Nalgene filter-covered cages with wire floors. Food and water were given ad libitum. The animal room was maintained at 22 to 23°C with 12 h of illumination daily.

Animal infectivity. The virulence of *S. typhimurium* SR-11 strains was determined after peroral or intraperitoneal inoculation. Bacteria for inoculation in mice were grown overnight as standing cultures at 37°C in L broth. These cultures were diluted 1:50 into prewarmed L broth and aerated at 37°C for approximately 4 h to an optical density at 600 nm of about 0.8 to 1.0. The cells were concentrated 50-fold by centrifugation at 8,000 × *g* for 10 min at 4°C, followed by suspension in BSG. Dilutions were plated on Penassay agar for titer determination and on MacConkey agar with 1% maltose to verify the Cya or Cya-Crp phenotype.

For peroral inoculations, mice were deprived of food and water for 4 h before infection. They were then given 30 μl of 10% (wt/vol) sodium bicarbonate 5 min before being fed 20 μl of *S. typhimurium* cells suspended in BSG. Food and water were returned 30 min after peroral inoculation.

Intraperitoneal inoculation of unfasted mice was performed by using a 26-gauge needle to deliver 100 μl of bacteria diluted in BSG.

TABLE 2. Growth properties of bacterial strains

Strain	Relevant genotype	pStSR100	Colony diam[a]	Generation time[b] (min)
χ3306	Wild type	+	2.0	78
χ3337	Plasmid cured	−	2.0	82
χ3395	*cya*::Tn*10*	+	1.5	128
χ3396	*crp773*::Tn*10*	+	1.0	120
χ4032	Δ*cya-1*	+	1.0	150
χ4058	*cya*::Tn*10*	−	1.5	163
χ4060	Δ*cya-3*	−	0.9	128
χ4062	Δ*cya-3* Δ*crp-2*	−	0.8	199
χ4064	Δ*cya-1* Δ*crp-1*	+	1.0	163
χ4065	*crp773*::Tn*10*	−	1.0	139

[a] Cells distributed at ~100 per plate on minimal agar medium–0.5% glucose and incubated at 37°C for 40 h.
[b] Growth in minimal liquid medium–0.5% glucose.

Enumeration of viable *S. typhimurium* cells in mice. Mice were euthanized by CO_2 asphyxiation. Colonization and persistence of wild-type and mutant *S. typhimurium* SR-11 strains were determined, at 4, 24, 48, 72, and 96 h and at 7 days after peroral inoculation, from the titers in the contents of the small intestine and colon. Titers were also determined in homogenates of Peyer's patches (8 to 10 patches), a 10-cm section of small intestinal wall located at the distal end of the ileum with Peyer's patches removed, the colon wall, mesenteric lymph nodes, and spleen. The mesenteric lymph nodes, spleen, small intestine, and colon were immediately placed in minimal liquid medium (18)–15% sucrose (MLS) and kept on ice. A petri dish containing 4.0 ml of chilled MLS was used to hold the small intestine while the Peyer's patches were removed. Peyer's patches were rinsed twice with 1.0 ml (each time) of chilled BSG before being vortexed with 1.0 ml of chilled MLS and glass beads. The small intestine was then cut longitudinally, the contents and wall were placed in a 50-ml disposable conical tube, and chilled MLS was added to a 5.0-ml volume before vortexing for 1 min. A 10-cm section of small intestine was rinsed twice in 5.0 ml of BSG with 2.5 ml of chilled MLS and glass beads. The contents of the small intestine were collected from the longitudinal dissection and the washing and then added to the 50-ml disposable conical tube containing the small intestinal wall and contents. A petri dish containing 4.0 ml of chilled MLS was used to hold the colon while it was cut longitudinally through the cecum. The contents of the colon and the colon wall were placed in a 50-ml disposable conical tube and vortexed for 1 min. The colon wall was removed and rinsed twice in 5.0 ml of BSG before being vortexed with 2.5 ml of chilled MLS and glass beads. The contents of the colon were collected from the MLS used during longitudinal dissection and the washings. The mesenteric lymph nodes and connecting fatty tissue were vortexed with 1.0 ml of chilled MLS and glass beads. Spleens in 2.5 ml of chilled MLS were processed in a Dounce pestle-type tissue homogenizer. Suspensions were diluted in BSG and plated on MacConkey agar containing 1% lactose and 40 μg of nalidixic acid per ml. MacConkey agars containing various carbohydrates at 1% were used to verify that the recovered organisms had the expected phenotype.

Evaluation of protective immunity. In initial experiments, mice that survived infection with an *S. typhimurium* mutant strain for 30 days were challenged on day 31 postinfection with 10^3 to 10^4 times the 50% lethal dose (LD_{50}) of the wild-type mouse-virulent strain χ3306. Subsequently, groups of mice were perorally immunized with various doses of avirulent mutants and then challenged with various doses of χ3306 cells at 30 days after the initial immunization.

RESULTS

Construction of *S. typhimurium* SR-11 strains with *cya* and *crp* mutations. The mouse-virulent *S. typhimurium* SR-11 strain χ3306 (27) possesses a *gyrA* mutation that confers resistance to nalidixic acid to facilitate recovery from mice and enumeration. This strain was used as the parent to the strains described in this report (Table 1).

Since *S. typhimurium* isolates cured of the 100-kilobase virulence plasmid, pStSR100, are able to attach to, invade, and persist in Peyer's patches but are defective in traversing to the mesenteric lymph nodes and spleen (27, 48), we chose to introduce *cya*::Tn*10* and *crp*::Tn*10* mutations (52) into the plasmid-cured strain χ3337 (27), as well as into the plasmid-containing strain χ3306. It should be emphasized that reintroduction of the pStSR100 plasmid into χ3337 completely restores its virulence (27), thus ensuring that no secondary mutation affecting virulence arose in χ3337 during curing of the virulence plasmid.

The *cya* mutants χ3395, χ4032, and χ4060, the *crp* mutants χ3396 and χ4065, and the *cya crp* double mutants χ4062 and χ4064 failed to ferment maltose, mannitol, sorbitol, and melibiose and gave slow fermentation of galactose. These strains failed to grow on maltose, mannitol, sorbitol, melibiose, citrate, succinate, and glycerol but did grow on glucose, fructose, and galactose as the sole energy sources. The wild-type strain χ3306 fermented and grew on all of the carbon sources. The phenotypes were as expected on the basis of published reports of the requirement for cAMP and the CRP for catabolic activities (49, 50, 51, 57, 58).

Flagellum synthesis is under cAMP control (63). Tests for motility using medium with triphenyltetrazolium chloride and observation of cells by light microscopy revealed considerable variation from strain to strain. All χ3306 and χ3337 cells were motile. Although PP1002 and χ3395 were uniformly nonmotile, their *cya* derivatives χ4032, χ4058, χ4060, χ4062, and χ4064 all had some motile cells. On the other hand, PP1037 had some motile cells, as did its *crp* derivative χ3396; its other *crp* derivative, χ4065, had uniformly nonmotile cells. Komeda and others (36) have described a suppressor mutation, *cfs* (constitutive flagellar synthesis), which restored flagellation in Crp^- and Cya^- mutants. It is

TABLE 3. Virulence of *S. typhimurium* SR-11 *cya*::Tn*10* and *crp773*::Tn*10* in BALB/c female mice 30 days after peroral inoculation[a]

Strain	Relevant genotype	Inoculating dose (CFU)	Survivors/ total	Mean no. of days to death[b]	Health[c]
χ3306	Wild type	2.5×10^5	2/5	7	Scruffy
χ3395	*cya*::Tn*10*	1.3×10^9	2/5	17	Scruffy
χ3396	*crp773*::Tn*10*	2.4×10^8	5/5		Healthy

[a] All of the mice were 8 weeks old at the time of immunization.
[b] For animals that died.
[c] Healthy, No noticeable signs of disease; scruffy, noticeably ill.

TABLE 4. Morbidity and mortality 30 days after peroral challenge of BALB/c mice with *S. typhimurium* SR-11 Δ*cya*, Δ*crp*, or both strains

Strain	Relevant genotype	pStSR100	Inoculating dose (CFU)	Age of mice[a] (wk)	Survivors/ total[b]	Health[c]
χ3306	Wild type	+	2.5×10^5	8	3/6	Scruffy
χ3337	Plasmid cured	−	5.0×10^8	8	6/6	Scruffy
χ4032	Δ*cya-1*	+	1.3×10^9	4	6/6	Healthy
χ4032	Δ*cya-1*	+	1.3×10^9	8	6/6	Healthy
χ4060	Δ*cya-3*	−	7.8×10^8	4	6/6	Healthy
χ4060	Δ*cya-3*	−	7.8×10^8	8	6/6	Healthy
χ4062	Δ*cya-3* Δ*crp-2*	−	1.2×10^9	4	6/6	Healthy
χ4062	Δ*cya-3* Δ*crp-2*	−	1.2×10^9	8	6/6	Healthy
χ4064	Δ*cya-1* Δ*crp-1*	+	1.2×10^9	4	6/6	Healthy
χ4064	Δ*cya-1* Δ*crp-1*	+	1.2×10^9	8	6/6	Healthy

[a] At the time of challenge.
[b] Mean number of days to death, 7.
[c] Healthy, No noticeable signs of disease; Scruffy, noticeably ill.

therefore likely that such suppressor mutations might accumulate during cultivation of *cya* and *crp* mutants. The presence of the pStSR100 plasmid had no effect on expression of motility or on carbon source fermentation and use (Table 2).

At each step in the construction following selection of a fusaric acid-resistant, tetracycline-sensitive derivative, we investigated whether spontaneous tetracycline-resistant revertants or mutants or both could be recovered at frequencies higher than observed for the parental χ3306 strain. In all cases, tetracycline-resistant revertants and mutants were not observed. This indicated that fusaric acid resistance was most likely not due to point mutations in the Tn*10* *tet* gene.

The colony diameters on minimal agar medium containing 0.5% glucose and the generation times in minimal liquid medium containing 0.5% glucose of several of the mutant strains and their parents are presented in Table 2. The Δ*cya* strains, with or without the Δ*crp* mutation and independent of the pStSR100 plasmid, grew more slowly than the parent χ3306 and χ3337 cultures.

Virulence of mutant strains in mice. Preliminary information on the virulence of mutant strains was obtained by infecting mice with either 10^5 mutant cells intraperitoneally or 10^8 mutant cells perorally and recording morbidity and mortality. Since mice infected either perorally or intraperitoneally with χ3395 with the *cya*::Tn*10* mutation and χ3396 with the *crp*::Tn*10* mutation survived and became immune to challenge with wild-type χ3306 cells, we embarked on more studies on the virulence and immunogenicity of χ3395 and χ3396. Mice survived infection with about 10^3 times the wild-type LD_{50} of either the *cya*::Tn*10* mutant or the *crp*::Tn*10* mutant, but some mice died when infected with 10^4 times the wild-type LD_{50} of the χ3395 *cya*::Tn*10* strain (Table 3). We then commenced studies with strains in which Tn*10* and adjacent DNA sequences had been deleted. Table 4 presents data on the morbidity and mortality of mice infected perorally with pStSR100-containing and pStSR100-cured *S. typhimurium* wild-type, Δ*cya*, and Δ*cya* Δ*crp* strains. Irrespective of age at the time of infection, all of the mice survived infection with approximately 10^9 cells of any of the deletion mutant strains (i.e., 1,000 to 4,000 wild-type LD_{50}s). The information in Table 4 pertaining to avirulence of the pStSR100-cured derivative χ3337 is in accord with more thorough studies reported by Gulig and Curtiss (27).

Data on intraperitoneal challenge with the *S. typhimurium* mutants are presented in Table 5. Mice survived infection

TABLE 5. Morbidity and mortality 30 days after intraperitoneal challenge of 8-week-old BALB/c female mice with *S. typhimurium* SR-11 Δ*cya*, Δ*crp*, or both strains

Strain	Relevant genotype	pStSR100	Challenge dose (CFU)	Survivors/ total	Mean no. of days to death[a]	Health[b]
χ3306	Wild type	+	~40	3/6	7	Scruffy
χ3395	*cya*::Tn*10*	+	1.0×10^4	0/5	12	
			1.0×10^5	1/5	7	Scruffy
			1.0×10^6	0/5	3	
χ3396	*crp773*::Tn*10*	+	1.9×10^4	5/5		Healthy
			1.9×10^5	4/5	18	Moderate
			1.9×10^6	1/5	12	Scruffy
χ4062	Δ*cya-3* Δ*crp-2*	−	1.0×10^4	5/5		Healthy
			1.0×10^5	5/5		Healthy
			1.0×10^6	0/5	10	
χ4064	Δ*cya-1* Δ*crp-1*	+	1.6×10^4	5/5		Healthy
			1.6×10^5	2/5	14	Scruffy
			1.6×10^6	0/5	16	

[a] For animals that died.
[b] Healthy, No noticeable signs of disease; moderate, moderately ill; scruffy, noticeably ill.

TABLE 6. Spontaneous reversion or mutation of Δ*cya* Δ*crp* strains as revealed by plating on minimal agar medium with various carbohydrates

Strain	pStSR100	Relevant genotype	Reversion frequency with the following at 0.5% (final concn):						
			Mannitol	Sorbitol	Maltose	Citrate	Succinate	Melibiose	Glycerol
χ4032	+	Δ*cya-1*	3.0×10^{-5}	3.0×10^{-5}	<10	<10	<10	<10	3.0×10^{-5}
χ4060	−	Δ*cya-3*	TMTC[a]	TMTC	2.0×10^{-7}	2.0×10^{-7}	2.0×10^{-7}	TMTC	TMTC
χ4062	−	Δ*cya-3* Δ*crp-2*	2.7×10^{-8}	<10	<10	<10	<10	<10	3.0×10^{-5}
χ4064	+	Δ*cya-1* Δ*crp-1*	6.0×10^{-8}	<10	<10	<10	<10	<10	3.0×10^{-5}

[a] TMTC, Too many to count.

with 10^2 to 10^3 times the wild-type LD_{50} with the mutant strains χ3396, χ4062, and χ4064. This was not the case for mice infected with χ3395. In initial experiments, mice were much more tolerant of infection with χ3395 by either the peroral or intraperitoneal route than in the experiments reported in Tables 3 and 5. Since *cya* mutants grow more slowly than the wild-type (Table 2), they readily accumulate suppressor mutations (23, 44, 57) which could restore virulence, as well as catabolic abilities. Therefore, studies were conducted to evaluate the genetic stability of the various mutant strains.

Genetic stability of avirulent mutants. Live-vaccine strains should have complete stability with regard to both avirulence and their immunogenic attributes. Several of the candidate vaccine strains were grown in L broth to late-log phase, concentrated 50-fold, and plated at various dilutions on minimal agar media containing carbon sources that should not support their growth. A duplicate set of plates was exposed to UV light at an intensity that caused 70% cell death. Revertants and mutants were observed at low but measurable frequencies (Table 6) in the Δ*cya* strain χ4032 and at higher frequencies in the Δ*cya* strain χ4060; many of these revertants were able to grow on most carbon sources and therefore were likely to be *crp** (23, 57) or *csm* (44) mutations that permit the CRP to activate transcription in the absence of cAMP. Repeat tests with χ4032 and χ4060, starting with single-colony isolates, gave results similar to those observed in the experiment reported in Table 6. Revertants or mutants or both from the Δ*cya* Δ*crp* strains such as χ4064 were extremely rare (Table 6). Although the revertants or mutants or both were able to grow on either mannitol or glycerol, they failed to grow on any of the other

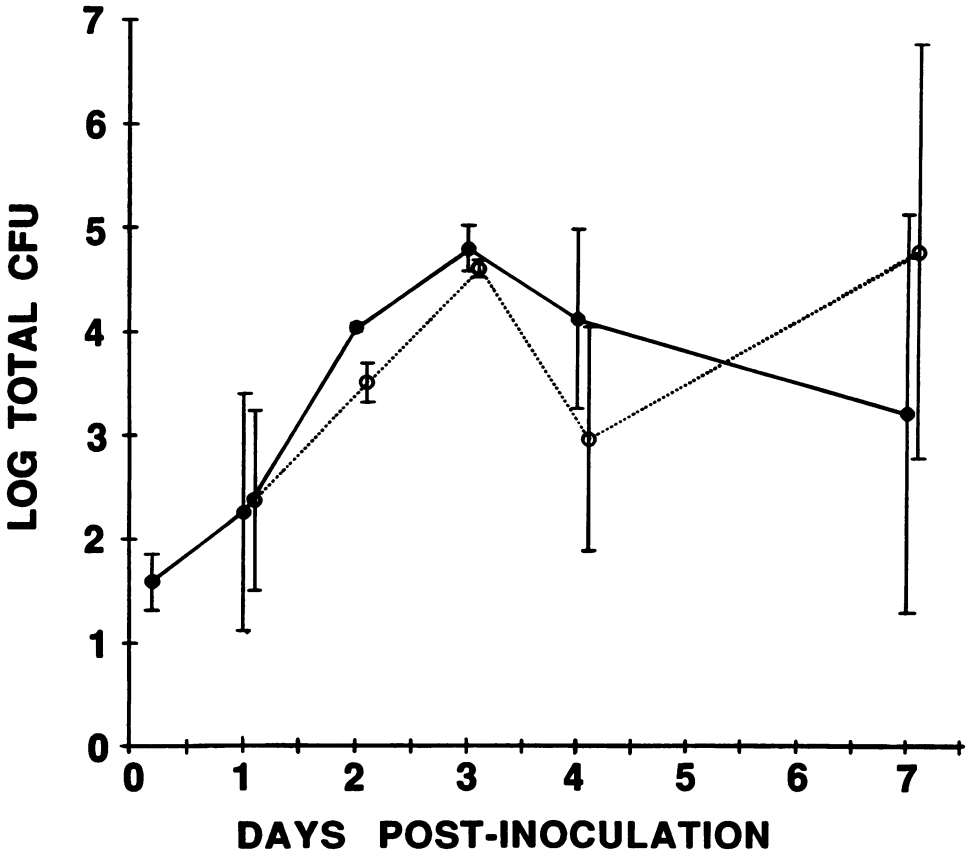

FIG. 1. Recovery of *S. typhimurium* Δ*cya* Δ*crp* χ4064 (●) and wild-type χ3456 (○) from Peyer's patches at specified times after peroral inoculation with 7.4×10^8 CFU of χ4064 and 4.0×10^8 CFU of χ3456. Three mice were sacrificed for each time point. The vertical bars denote standard deviations.

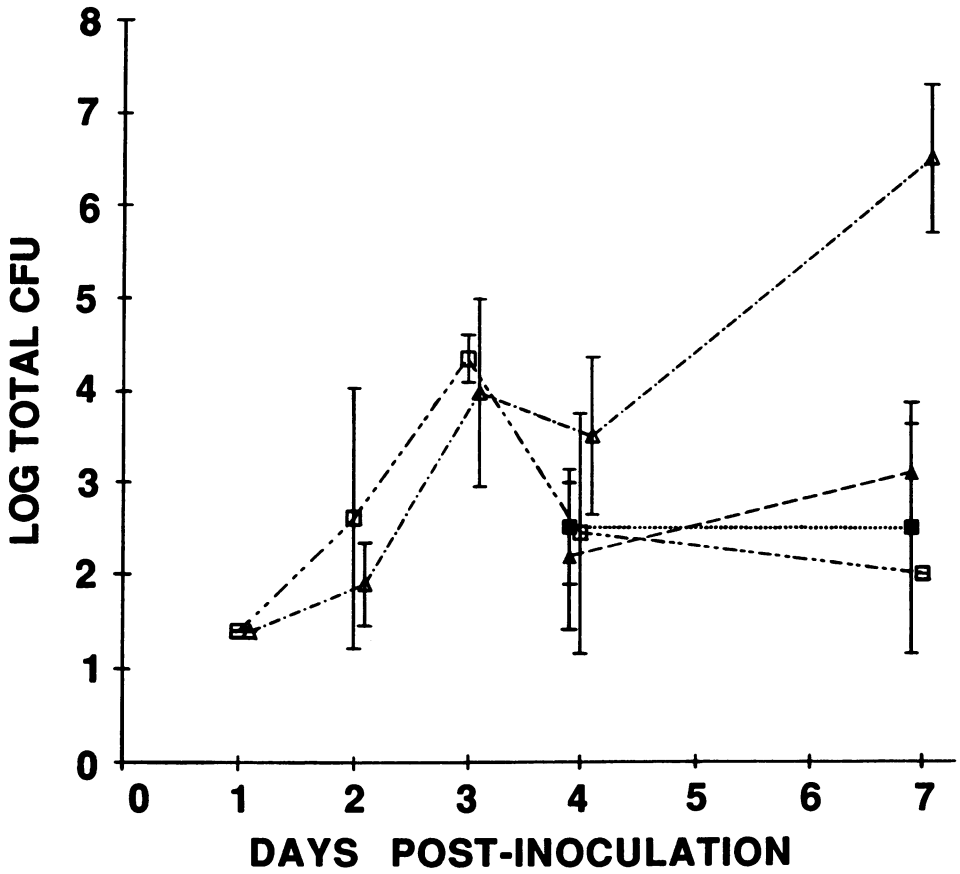

FIG. 2. Recovery of *S. typhimurium* Δ*cya* Δ*crp* χ4064 (■ and ▲) and wild-type χ3456 (□ and △) from mesenteric lymph nodes (■ and □) and spleens (▲ and △) at specified times after peroral inoculation. For experimental conditions, see the legend to Fig. 1.

carbon sources, as did the original strains. These revertants and mutants probably have promoter mutations that cause transcription of the specific gene or operon to be independent of CRP. UV light exposure did not enhance mutation and reversion frequencies.

Six revertants and mutants from the experiments reported in Table 6 were evaluated for virulence. Four mice were used to test each revertant. One each received an oral dose of $\sim 10^5$, or $\sim 10^8$ cells or an intraperitoneal dose of $\sim 10^2$ or $\sim 10^5$ cells. In all instances, the four revertants from Δ*cya* mutants and the two revertants of Δ*cya* Δ*crp* strains retained the avirulence of their parents since all 24 mice remained healthy with no noticeable illness. Therefore, it appears that the avirulence of Δ*cya* strains cannot be solely due to defective carbohydrate catabolism. However, it appears either that the degrees of genotypic stability of *cya*::Tn*10* and Δ*cya* mutations differ with regard to virulence or that long-term cultivation of *cya* mutants might lead to genetic changes not associated with carbohydrate use.

Tissue tropism and persistence of avirulent mutants in mice. Figures 1 and 2 present data on recovery of the Δ*cya* Δ*crp* strain χ4064 in comparison with recovery of the nalidixic acid-sensitive, tetracycline-resistant, virulent strain χ3456 after oral inoculation. It is evident that the Δ*cya* and Δ*crp* mutations do not significantly impair the ability of *S. typhimurium* to attach to, invade, and persist in Peyer's patches (Fig. 1) but do impair ability to reach or survive in the spleen (Fig. 2). χ4064 also attached to the walls and persisted in the contents of the small intestine and colon for 4 to 7 days at levels analogous to those observed for wild-type strains (data not shown). Titers of χ4062 and χ4064 were low to undetectable in Peyer's patches and spleens of animals sacrificed 30 days after oral inoculation with about 10^9 bacteria (data not shown).

Effectiveness of immunization with avirulent mutants. Table 7 presents data on the ability of the different *S. typhimurium* Δ*cya* and Δ*cya* Δ*crp* mutants to induce immunity to subsequent oral challenge with 10^4 times the LD_{50} of virulent *S. typhimurium* SR-11 χ3306 cells. Many of the mice displayed moderate illness, except for mice immunized with χ4064, which remained healthy. Sacrifice of animals (even those immunized with χ4064) 30 days postchallenge revealed splenomegaly and the ability to recover 10^2 to 10^3 cells of the χ3306 wild-type challenge strain. The avirulent Δ*cya* or Δ*cya* Δ*crp* strains were not detectable. In contrast, splenomegaly and recovery of the challenge strain were not observed when mice immunized with approximately 10^9 χ4064 cells were challenged with only 100 to 1,000 LD_{50}s of the fully virulent χ3306 parent strain (data not shown).

DISCUSSION

We have constructed derivatives of the mouse-virulent *S. typhimurium* SR-11 strain χ3306 that lack the ability to synthesize adenylate cyclase and the CRP and do or do not possess the pStSR100 virulence plasmid. Although all of the strains are avirulent and induce a high level of protective immunity against subsequent challenge with virulent *S.*

TABLE 7. Effectiveness of oral immunization with avirulent *S. typhimurium* SR-11 Δ*cya* Δ*crp* mutants in protecting against challenge with wild-type virulent *S. typhimurium* SR-11[a]

Strain	Relevant genotype	pStSR100	Dose (CFU) of immunizing strain	Age of mice[b] (wk)	Survivors/ total	Mean no. of days to death[c]	Health[d]
χ4032	Δ*cya-1*	+	1.3×10^9	4	6/6		Moderate
χ4032	Δ*cya-1*	+	1.3×10^9	8	5/6	46	Moderate
χ4060	Δ*cya-3*	−	7.8×10^8	4	6/6		Moderate
χ4060	Δ*cya-3*	−	7.8×10^8	8	6/6		Moderate
χ4062	Δ*cya-3* Δ*crp-2*	−	1.2×10^9	4	5/6	46	Moderate
χ4062	Δ*cya-3* Δ*crp-2*	−	1.2×10^9	8	5/6	48	Moderate
χ4064	Δ*cya-1* Δ*crp-1*	+	1.2×10^9	4	6/6		Healthy
χ4064	Δ*cya-1* Δ*crp-1*	+	1.2×10^9	8	6/6		Healthy

[a] Thirty days after immunization of 4- or 8-week-old mice with the strains indicated, mice were challenged perorally with 2.0×10^9 CFU of χ3306 wild-type virulent *S. typhimurium* SR-11.
[b] At the time of immunization.
[c] Following immunization of the animal that died.
[d] Healthy, No noticeable signs of disease or illness; moderate, moderately ill.

typhimurium, we will restrict future efforts to χ4062 and χ4064 or other strains which contain both the Δ*cya* and Δ*crp* mutations. The *cya* and *crp* genes are at minutes 83 and 72, respectively, on the *S. typhimurium* chromosome (55) and therefore could not be jointly lost except by gene transfer with an Hfr-type donor. Deletion of the *crp* gene precludes recovery of *crp*$^+$ (23, 57) mutations that permit transcription of genes and operons requiring activation by the CRP in the absence of cAMP. Similarly, the absence of the *crp* gene also presumably precludes recovery of *csm* suppressor mutations (44) that are closely linked to the *crp* gene (6) and which can also obviate the need for cAMP in *cya* mutants. Even though the Δ*cya* revertant strains remained avirulent, we have not tested enough independent revertant isolates of *cya*::Tn*10* and Δ*cya* strains to conclude that virulence could never be restored. In fact, the enhanced virulence of the χ3395 *cya*::Tn*10* mutant observed in the experiments for the data in Tables 3 and 5 over that observed in experiments with χ3395 conducted immediately after its construction suggests that such changes may be possible. Therefore, the introduction of a Δ*crp* mutation into Δ*cya* strains should, most likely, preclude restoration of virulence. In addition, the *crp*::Tn*10* mutation significantly reduces the virulence of *S. typhimurium* (Tables 3 and 5). Thus, in the unlikely event that the Δ*cya* mutation was lost by some type of gene transfer event (16, 19), the Δ*crp* mutation would still render the strain avirulent.

Both χ4062 and χ4064 merit closer evaluation as potential vaccine strains. It appears that χ4064, which still possesses the pStSR100 plasmid, may induce a higher level of immunity than does the plasmid-free strain χ4062 (Table 7). This may be due to the increased ability of χ4064 (Fig. 2) and plasmid-containing strains in general (27, 48) to achieve higher titers and persist longer than plasmid-free strains in mesenteric lymph nodes and spleen. χ4062 and χ4064 are not isogenic for their Δ*cya* and Δ*crp* mutations; therefore, we will have to compare a derivative of χ4062 re-endowed with pStSR100 and a plasmid-cured derivative of χ4064 with χ4062 and χ4064 to fully document the relative importance of specific Δ*cya* and Δ*crp* mutations and the pStSR100 plasmid in rendering *S. typhimurium* avirulent yet immunogenic. If differences in virulence and immunogenicity are found that are not solely ascribable to the presence or absence of pStSR100, we would then need to investigate the potential influence of deletions that might extend into genes on either side of the *cya*, *crp*, or both genes.

A potential and desired use of strains such as χ4062 and χ4064 would be to serve as vectors to target colonization or virulence antigens expressed by genes from other pathogens to the GALT to induce generalized secretory (13, 19a, 41, 42), humoral (13, 19a, 41, 42, 60, 61) and cellular (10) immunity, if that should be desired. Therefore, we have introduced several cloning vectors into χ4062 and χ4064 and found that these plasmids usually exhibit a stability equal to or greater than that of the wild-type parent strains. The slower rate of growth of χ4062 and χ4064 (Table 2) may enhance the ability of plasmid replicons to keep up with chromosomal replication to ensure that progeny cells contain plasmids. In addition, a variety of recombinant derivatives have been constructed, and we have found that the antigen, SpaA, necessary for colonization of *S. sobrinus* to the salivary glycoprotein-coated tooth surface (17) is expressed at a high level in the Δ*cya* Δ*crp* mutants. The results of these studies, as well as studies to evaluate the duration of protective immunity induced by Δ*cya* Δ*crp* strains, will be the subject of subsequent reports.

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