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Virulence Attenuation and Live Vaccine Potential of *aroA*, *crp cdt cya*, and Plasmid-Cured Mutants of *Salmonella enterica* Serovar Abortusovis in Mice and Sheep

Sergio Uzzau,[1] Gavino Marogna,[2] Guido Sisinnio Leori,[2] Roy Curtiss III,[3] Giuseppe Schianchi,[2] Bruce A. D. Stocker,[4] and Salvatore Rubino[1]*

Dipartimento di Scienze Biomediche, Center for Biotechnology Development and Biodiversity Research, Università di Sassari, 07100 Sassari, Italy[1]; Istituto Zooprofilattico Sperimentale della Sardegna, 07100 Sassari, Italy[2]; Department of Biology, Washington University, St. Louis, Missouri 63130[3]; and Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305[4]

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Three live vaccine candidates of *Salmonella enterica* subspecies I serotype Abortusovis (*aroA*, *cya crp cdt*, and plasmid-cured strains) have been developed, and their efficacies in inducing humoral antibodies and protecting against abortion after challenge with wild-type strain SS44 were evaluated in sheep. Following estrus synchronization, animals were immunized 3 weeks after fertilization and boosted once 3 weeks later. Following challenge with wild-type SS44, pregnancy failure of vaccinated ewes was reduced compared to that of nonimmunized controls. Attenuation of each vaccine was also assessed in challenge experiments with nonimmunized pregnant ewes and in BALB/c mice. All three vaccine candidates appear to be safe for use in sheep and provide a model for the development of live vaccine candidates against naturally occurring ovine salmonellosis.

Salmonella enterica subspecies I serotype Abortusovis is an ovine-restricted serotype (O 4,12:c:1,6) that ranks among the main causes of ovine abortions in Europe and western Asia, where it represents a major pathological and economic problem in countries with an economy based on sheep herding (41). Typical signs of serovar Abortusovis infection of young ewes are the induction of abortion and death of newborn lambs, whereas nonpregnant ewes and older sheep naturally exposed to serovar Abortusovis show no symptoms (25, 26). In areas of endemicity, abortion occurs in 30 to 50% of sheep in a flock, generally during the first pregnancy and mainly during the last 2 months of gestation (a total of 5 months), through a mechanism that has yet to be elucidated (41).

After abortion, bacteria can be isolated from placental and fetal tissues (liver, spleen, brain, and stomach), which are the principal sites of multiplication. Infected ewes that do not abort deliver weak lambs that generally develop bacteremia and die within a few days. Lambs may also be born strong and become infected and die within the first 2 weeks after birth with signs of pneumonia. Serovar Abortusovis can be also isolated from the ewe vaginal discharges for up to 2 to 4 weeks after abortion (41), which contributes to increased transmission during the lambing season.

Little is known about the duration of effective immunity following *Salmonella* infection in sheep (6, 7). Yet, ewes infected with serovar Abortusovis usually abort once in their lifetime, with the cyclical evolution of abortive episodes inside a flock or a sheep-rearing region (26, 35).

Several strategies have been used to obtain attenuated *Salmonella* strains (12). A living attenuated serovar Abortusovis strain Rv6 was developed by Pardon and coworkers (36). This vaccine gives partial protection against abortion (36, 38), but it represents progress compared to vaccination using a killed vaccine (18, 40). *S. enterica* serovar Typhimurium attenuated strains have also been used to immunize sheep and were found to be highly protective (27, 29). These observations indicate the potential of live attenuated vaccine for the prevention of sheep abortion.

In particular, genetically defined *Salmonella* vaccines that have been shown to induce protective immunity in various animal models include *aroA* or *aroCD*, *crp cya*, *phoPQ*, *ompR*, and *htrA* mutants (4, 9, 11, 13, 14, 16, 32). Infection with such *Salmonella* vaccine strains effectively induced cell-mediated and humoral immunity to homologous (8, 21, 24) or heterologous (11, 33, 43) antigens.

In this study we describe *crp cdt cya*, *aroA*, and plasmid-cured serovar Abortusovis mutants with respect to their level of attenuation and ability to protect against a virulent challenge in mice and sheep.

MATERIALS AND METHODS

Bacterial strains and media. The serovar Abortusovis and serovar Typhimurium strains used in this study are listed in Table 1. Serovar Abortusovis wild-type strain SS44 was isolated from sheep fetal tissues and has been described elsewhere (10, 37). The strains were maintained as frozen cultures until use. For DNA recombination and genetic analysis, bacteria were grown in Luria-Bertani (LB) medium. The minimal medium used to determine nutritional characteristics was an inorganic salts mixture [KH_2PO_4, 8 g; K_2HPO_4, 28 g; $(NH_4)_2SO_4$, 4 g; $MgSO_4(7 \cdot H_2O)$, 0.4 g; water, 1 liter], with glycerol (4 g/liter) and trisodium citrate (1.7 g/liter) added as carbon and energy sources and solidified with agar (12 g/liter). Since wild-type serovar Abortusovis SS44 is auxotrophic for nicotinic acid and cystine (S. Uzzau and B. A. D. Stocker, unpublished data), these nutrients were added at a concentration of 50 μg per ml of medium. Sugar fermentation was evaluated by supplementing MacConkey base (Difco) with 1% of the appropriate carbohydrate. When necessary, antibiotics (Sigma) were added to the medium at the following concentrations: 20

* Corresponding author. Mailing address: Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica, Università degli Studi di Sassari, Viale S. Pietro, 43B, 07100 Sassari, Italy. Phone: 39.079.228303. Fax: 39.079.212345. E-mail: rubino@uniss.it.

TABLE 1. Bacterial strains

Strain	Relevant genotype and/or phenotype	Reference or source
S. enterica serovar Abortusovis		
SS44	Wild type	10
SU34	*aroA (serC)1121*::Tn*10*, Tet^+	This study
SU304	*aroA148* deletion, Tet^-	This study
SU40	Plasmid cured	42
SSM178	Δ*crp-10* Δ*cdt zhc1431*::Tn*10*, Tet^+	This study
SSM179	Δ*crp-10* Δ*cdt*, Fa^+ Tet^-	This study
SSM180	Δ*crp-10* Δ*cdt* Δ*cya-12 zid-62*::Tn*10* (pSD110), Tet^+ Ap^+	This study
SSM189	Δ*crp-10* Δ*cdt* Δ*cya-12*, Fa^+ Tet^- Ap^-	This study
S. enterica serovar Typhimurium		This study
aroA148	LT2 *aroA148*	34
TT472	LT2 *aroA (serC)1121*::Tn*10*, Tet^+	23
χ3711	LT2 Δ*cya-12 zid-62*::Tn*10*, Tet^+	28
χ3712	SL1344 Δ*crp-10* Δ*cdt zhc1431*::Tn*10*, Tet^+	44

μg/ml tetracycline, 50 μg/ml kanamycin, and 100 μg/ml ampicillin. When required for animal work, other media used were Trypticase soy agar, xylose-lysine-deoxycholate, and selenite broth (Difco).

Genetic manipulations and analysis. An *int* (integration negative) derivative of a high-transducing phage P22 variant, P22 HT105/1, was used to transduce genes from serovar Typhimurium donors to serovar Abortusovis recipients according to standard protocols (2). Lysates, usually around 1×10^{10} PFU/ml, were used to evoke transductants by the drop-on-lawn method (17). Putative transductant clones were purified by single-colony reisolation on the selective medium before characterization. The lipopolysaccharide antigen of each serotype and of each mutant strain obtained after genetic manipulation was tested by slide agglutination of freshly grown bacteria with group B *Salmonella* antiserum (Difco). The lipopolysaccharide pattern was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and visualized by silver staining as described previously (15). Fusaric acid selection for deletion derivatives of strains harboring a Tn*10* insertion was done as described previously (30).

Mouse infection. Infection of female BALB/c mice by oral inoculation was performed as previously described (19). Ten-week-old female mice were fed with 1×10^8 and with 7×10^7 CFU of serovar Abortusovis strains (mutants and wild type, respectively) after 4 h of food and water deprivation and feeding with 10% sodium bicarbonate (30 μl). Mice immunized with the vaccine strains and nonimmunized control animals were orally challenged 1 month later with 1×10^9 CFU of serovar Abortusovis SS44 wild-type strain.

Sheep challenge experiments. Berrichon crossbred ewes, with no cultural or serological evidence of serovar Abortusovis or other causative agent of sheep abortion, were used in these experiments. Groups of 5 ewes were randomly chosen. All bacterial inocula were tested for purity and number of viable microorganisms by plating the bacteria on Trypticase soy agar medium.

Vaccine strain virulence in pregnant sheep was evaluated by subcutaneous (s.c.) administration in ewes at 100 to 110 days of pregnancy as determined by ultrasound examination. Groups of five animals were inoculated with 1.6×10^9 bacteria. Animal appetite and behavior were monitored daily for a week following infection. Fecal and vaginal swabs were taken from each sheep daily for a week before and 4 weeks after immunization. Swabs were cultured in 10 ml selenite broth for 8 h at 37°C before plating on xylose-lysine-deoxycholate agar.

Protection induced by the vaccine strains against challenge with wild-type serovar Abortusovis was evaluated with four groups of ewes. Groups A, B, and C were immunized with vaccine strains SU34 (*aroA*), SU40 (plasmid cured), and SSM189 (*crp cdt cya*), respectively. The fourth group (D) was left as a nonimmunized control. Ewes were subjected to hormonal synchronization by use of vaginally applied fluorogesterone sponges (Intervet) and immunized (1×10^9 bacteria s.c.) 3 weeks after fertilization. A second boost of 10^9 bacteria was administered s.c. 3 weeks later. Ewes were monitored for pregnancy by ultrasound examination at 50 days of gestation. Animals that were found not pregnant were withdrawn from the study. All four groups were challenged s.c. with 1×10^9 CFU of wild-type strain SS44 at 110 days of pregnancy. Animal appetite and behavior were monitored daily for the entire experiment. Febrile responses and serum immunoglobulin M (IgM) and IgG anti-outer membrane protein (OMP) values were monitored weekly for 5 months after the first immunization. Rectal and vaginal swabs were collected weekly from each ewe after delivery or abortion until three consecutive swabs were negative for serovar Abortusovis. Aborted fetuses were examined for the presence of serovar Abortusovis as previously described (10).

Preparation of serovar Abortusovis OMPs. Major OMPs of wild-type strain SS44 were prepared as an SDS-insoluble fraction (1). A 1-liter culture of serovar Abortusovis wild-type strain SS44 was harvested by centrifugation, and the pellet was washed in ice-cold phosphate-buffered saline (PBS). Cells were lysed by sonication, and unbroken bacteria were removed by centrifugation at $2{,}000 \times g$ for 10 min. The supernatants were then centrifuged at $100{,}000 \times g$ for 30 min at 4°C. The pellets (containing the envelope fraction) were finely resuspended in 10 ml of cold PBS by passage through 25-gauge needles. Ten milliliters of 2% SDS in PBS was added and incubated for 30 min at room temperature with gentle agitation. Finally, suspensions were centrifuged at $100{,}000 \times g$ for 30 min at 4°C. The pellets (crude outer membrane protein preparation) were thoroughly resuspended in 3 ml of PBS. Protein yield was determined by the method of Bradford (5). The purity of each OMP preparation was analyzed by 10% SDS-polyacrylamide gel electrophoresis with prestained standards (Bio-Rad).

ELISA detection of sheep antibody to serovar Abortusovis OMPs. Anti-OMP antibodies in sheep sera were determined by an indirect enzyme-linked immunosorbent assay (ELISA) using the serovar Abortusovis OMP crude preparation described above as antigen. Each well of a 96-well microtiter plate (Maxisorp; Nunc) was coated overnight at 4°C with 0.5 μg of OMP preparation. The wells were washed three times between each step with PBS containing 0.05% Tween 20. The wells were blocked by adding 1% bovine serum albumin (BSA) in PBS and incubating them at 37°C for 2 h. Animal sera were diluted 1:500 in PBS, added to the wells, and left for 90 min at room temperature. After the wells were washed, serum IgG bound to OMP was measured by incubating for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Kirkegaard & Perry Laboratories) diluted 1:1,500 in PBS containing 1% BSA. To measure serum IgM bound to serovar Abortusovis OMP, horseradish peroxidase-conjugated rabbit anti-sheep IgM antibodies (Kirkegaard & Perry Laboratories) diluted 1:500 in 1% BSA-PBS were used. After washing, 0.1 ml of 3,3′,5,5′-tetramethylbenzidine–dimethyl sulfoxide–0.02% hydrogen peroxide solution was added to each well and left for 30 min. The plate was read by use of an automated ELISA reader (VersaMax; Molecular Devices) set at 450 nm. The positive cutoff value was calculated as the mean optical density (OD) of the preimmune sheep sera plus twice the standard deviation.

RESULTS

Construction of serovar Abortusovis *crp cdt cya* mutant. A *crp cdt* serovar Abortusovis mutant strain (SSM178) was constructed by transducing a Δ*crp* Δ*cdt zhc1431*::Tn*10* insertion from serovar Typhimurium donor χ3712 to wild-type serovar Abortusovis SS44. Isolation of transductants with a Crp^- phenotype (maltose negative, tetracycline resistant) was followed by selection of fusaric acid-resistant, tetracycline-sensitive,

TABLE 2. Attenuation of serovar Abortusovis vaccine strains after oral administration to BALB/c mice and effectiveness in protecting against oral challenge with wild-type serovar Abortusovis strain SS44

Strain (group/no. of mice)	Relevant genotype	Vaccine		Wild type	
		Dose (CFU)	No. of survivors/total	Dose[a] (CFU)	No. of survivors[b]/total
SU304 (A/10)	*aroA*	1×10^8	10/10	1×10^9	10/10
SSM189 (B/10)	*crp cdt cya*	1×10^8	10/10	1×10^9	10/10
SU40 (C/10)	Plasmid cured	1×10^8	10/10	1×10^9	10/10
SS44 (D/10)	Wild type	7×10^7	0/10		

[a] The serovar Abortusovis SS44 50% lethal dose was 1×10^7 CFU by oral administration.
[b] All surviving mice were healthy up to 1 month after challenge with wild-type strain SS44.

Crp$^-$ mutants. A selected mutant, named SSM179, was complemented with plasmid pSD110 (39), which carries the serovar Typhimurium *crp* gene. Then, a Δ*cya-12 zid-62*::Tn*10* insertion was introduced into strain SSM179/pSD110 (maltose positive, ampicillin resistant) by transduction with P22HT *int* grown in serovar Typhimurium χ3711 to produce serovar Abortusovis mutant SSM180, which scored maltose negative and tetracycline and ampicillin resistant. Finally, a *cya crp cdt* serovar Abortusovis derivative, named SSM189, was obtained by selecting fusaric acid-resistant, tetracycline- and ampicillin-sensitive (pSD110-cured) mutants on Bochner medium.

Construction of serovar Abortusovis *aroA* mutant. A serovar Abortusovis *aroA* mutant has been obtained by two-step transduction of wild-type strain SS44, using as the first donor strain serovar Typhimurium TT472 [*aroA* (*serC*)*1121*::Tn*10*] and as the second donor a serovar Typhimurium LT2 *aroA148* mutant that carries a deletion of the *aroA* gene C-terminal end (34). P22 grown on serovar Typhimurium TT472 evoked tetracycline-resistant colonies from the wild-type SS44 strain; such colonies were both serine and aromatic dependent, since *aroA* is promoter distal to *serC*::Tn*10* in the *serC aroA* operon (23). Tiny colonies, inferred to be abortive transductants, were also obtained. A representative transductant, designated SU34, was treated with P22 grown on the LT2 *aroA148* mutant and plated on minimal medium including aromatic amino-acids, nicotinic acid, and L-cystine but devoid of serine and pyridoxine. About 55% of the SerC$^+$ clones were aromatic dependent but tetracycline sensitive and were inferred to have resulted from replacement of the *serC*::Tn*10* segment of the recipient chromosome by *serC*$^+$ *aroA148* of the serovar Typhimurium donor. A representative transductant was named SU304.

Safety and immunogenicity of recombinant strains in mice. The three vaccine candidates were inoculated orally into BALB/c mice to assess their virulence. As shown in Table 2, all three serovar Abortusovis mutants were clearly avirulent, whereas all mice injected with wild-type SS44 were moribund after 5 to 6 days. Furthermore, mice immunized with SSM189, SU40, and SU304 survived oral challenge with wild-type SS44 by a number of organisms equivalent to 10^2 times the 50% lethal dose (1×10^7) for the oral administration route. Survivors did not show any sign of disease and remained healthy 30 days after challenge.

Safety of recombinant strains in pregnant sheep. To assess the possibility of pregnancy failure after challenge with the three vaccine strains, groups of five sheep were made pregnant (see Materials and Methods) and inoculated s.c. at 90 to 110 days of pregnancy. After subcutaneous administration of the live vaccine strains (groups A, B, and C) or the wild-type strain (group D), infected sheep showed a local cutaneous inflammatory reaction that persisted for 1 to 2 weeks, with increase of skin thickness at the site of injection; inflammation was more intense in all the animals inoculated with the plasmid-cured strain SU40 and eventually evolved in an abscess. Animal behavior was normal throughout the experiment, without prostration, spoiled appetite, or fecal softness.

As shown in Table 3, serovar Abortusovis strains SU304 (group A), SU40 (group B), and SSM189 (group C) did not cause abortion. Conversely, three out of five pregnant ewes challenged with SS44 aborted (group D). One sheep in group A and one in group C delivered a normally developed dead lamb. These lambs did not show any sign of serovar Abortusovis infection as assessed by bacteriological analysis. One of the two ewes in group D that did not abort delivered a healthy lamb, whereas the other one delivered a dead lamb without any sign of infection. All ewes that experienced abortion excreted serovar Abortusovis from the reproductive tract (up to 4 weeks later) and had infected fetuses. Taken together, these data suggest that the three vaccine strains tested are highly attenuated in the natural host for serovar Abortusovis.

Protection against abortion induced by recombinant serovar Abortusovis strains in sheep. Since the three vaccine candidates showed no virulence in mice and in sheep, we tested their ability to induce effective protection in pregnant ewes against challenge with wild-type serovar Abortusovis. Thus, three groups of 13 ewes each were immunized s.c. with SU304 (group A), SU40 (group B), or SSM189 (group C) vaccine strains at 3 weeks of pregnancy; a fourth group of 16 ewes was not immunized (group D). As observed in the previous exper-

TABLE 3. Safety of serovar Abortusovis vaccine strains after subcutaneous administration to pregnant ewes

Strain[a] (group/no. of ewes)	Relevant genotype	No. of abortions	No. of lambs (healthy/dead[b])	No. of ewes with infection at parturition
SU304 (A/5)	*aroA*	0	4/1	0
SSM189 (B/5)	*crp cdt cya*	0	5/0	0
SU40 (C/5)	Plasmid cured	0	4/1	1
SS44 (D/5)	Wild type	3	1/1	3

[a] Serovar Abortusovis wild-type and live vaccine strains were administered at the dose of 1.6×10^9 CFU.
[b] Delivered dead lambs had no signs of serovar Abortusovis infection at necropsy.

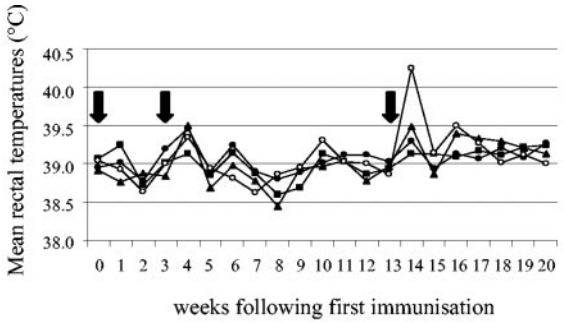

FIG. 1. Mean rectal temperatures of ewes immunized with *aroA* strain SU304 (solid circles), plasmid-cured strain SU40 (squares), or *crp cdt cya* strain SSM189 (triangles) or nonimmunized (open circles). Temperatures were recorded weekly from the day of first immunization up to 7 weeks postchallenge with wild-type strain SS44.

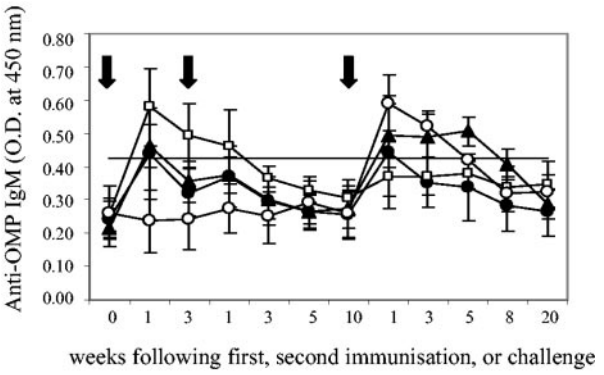

FIG. 2. Anti-OMP IgM values in pregnant ewes immunized with vaccine strain SU304 (solid circles), plasmid-cured strain SU40 (squares), or *crp cdt cya* strain SSM189 (triangles) and in nonimmunized ewes (open circles). All animals were challenged with wild-type SS44 10 weeks after the second vaccine administration. Arrows indicate times of first immunization, second immunization, and challenge with wild-type strain SS44. Values are expressed as means and standard deviations of OD readings at 450 nm. The horizontal line represents the cutoff value (0.424), calculated as described in Materials and Methods.

iment, all animals presented a slight and persistent inflammatory reaction at the site of injection, including those animals vaccinated with the plasmid-cured strain SU40, in which the development of an abscess was observed. Three weeks after the first immunization, the animals were boosted with the second dose of vaccine. The animals remained alert, produced normal fecal pellets, and retained their appetite throughout the experiment. Pregnancy was confirmed by ultrasound examination at 30 days after the first immunization for 9 ewes of group A, 13 of group B, 6 of group C, and 14 of group D. At 110 days of pregnancy (12 weeks plus 5 days after the first immunization), the animals were challenged s.c. with 10^9 CFU of wild-type strain SS44. The mean rectal temperature of the vaccinated sheep did not increase after they were exposed to virulent wild-type serovar Abortusovis SS44 (Fig. 1). On the other hand, the nonvaccinated sheep (group D) experienced a moderate but significant febrile response during the first week after challenge with SS44 (Fig. 1).

Evolution of anti-OMP IgG and IgM antibodies was monitored weekly by indirect ELISA. Sera collected from preimmunized sheep gave mean absorbance readings, at 450 nm, of 0.188 ± 0.033 (IgG) and 0.270 ± 0.077 (IgM). The calculated cutoffs were 0.254 (IgG) and 0.424 (IgM). All immunized animals responded with a significant production of anti-OMP IgM antibody by 7 days after the first vaccine dose (Fig. 2). IgM returned below the cutoff value 7 days later and did not rise again following the second vaccine dose, with the exception of group B sera, which were positive for up to a month after the first vaccine dose. Following challenge with wild-type strain SS44, anti-OMP IgM did not increase in group B animals, and sera from group A animals were positive only at 7 days postchallenge. Anti-OMP IgM production increased significantly in the nonvaccinated animal control group (D) and in group C animals, up to 17 and 32 days postchallenge, respectively. Sera from group A, B, and C immunized sheep were positive for anti-OMP IgG from 7 days after the first vaccine dose, with a peak at 3 weeks after the second vaccine dose (Fig. 3). Sera from group C animals were an exception and scored below the cutoff value at the time of challenge (70 days after the second vaccine dose). As for the anti-OMP IgM values, anti-OMP IgG also increased more dramatically in nonimmunized (group D) animals and *crp cdt cya* mutant-vaccinated (group C) animals than in animals of groups A and B (Fig. 3).

Decreased lambing performance was observed for the ewes of group D (nonvaccinated) compared to those of those of groups A, B, and C (Table 4). In group D, 8 out of 14 sheep aborted near term and 1 sheep delivered a weak lamb that died after 3 days. Only five sheep delivered healthy lambs (pregnancy failure of 64%). Our data suggest the acquisition of some degree of protection against challenge with virulent wild-type strain SS44 for the animals immunized with SU304 (group A) and SSM189 (group C), in whom pregnancy failure was 44% and 50%, respectively. Ewes vaccinated with SU40 (group B) showed an even better lambing performance (pregnancy failure of 23%; $P < 0.05$), suggesting that plasmid-cured serovar Abortusovis is able to induce an effective immune response against ovine salmonellosis. Serovar Abortusovis SS44 was isolated from the fetal brain of each aborted fetus, demonstrating that the wild-type strain was the cause of abortion.

DISCUSSION

Pathogenesis of serovar Abortusovis infection in sheep is a complex phenomenon, since the major clinical and pathological traits of the disease concern systemic dissemination and invasion of the fetus in pregnant ewes. In this regard, ovine salmonellosis due to serovar Abortusovis is strikingly different from natural serovar Typhimurium and serovar Dublin infections in sheep. These serotypes cause both enteritis and systemic dissemination; should abortion occur, this is normally associated with death of the ewe (26, 31). By contrast, natural and experimental infections of sheep with ovine-restricted serovar Abortusovis do not cause enteritis, and abortion occurs without apparent illness in the ewe (3, 41). The sheep model used in the present study is aimed to evaluate vaccine candidates ability to colonize and survive in the ovine deeper tissues to an extent sufficient to effectively prime the host immune

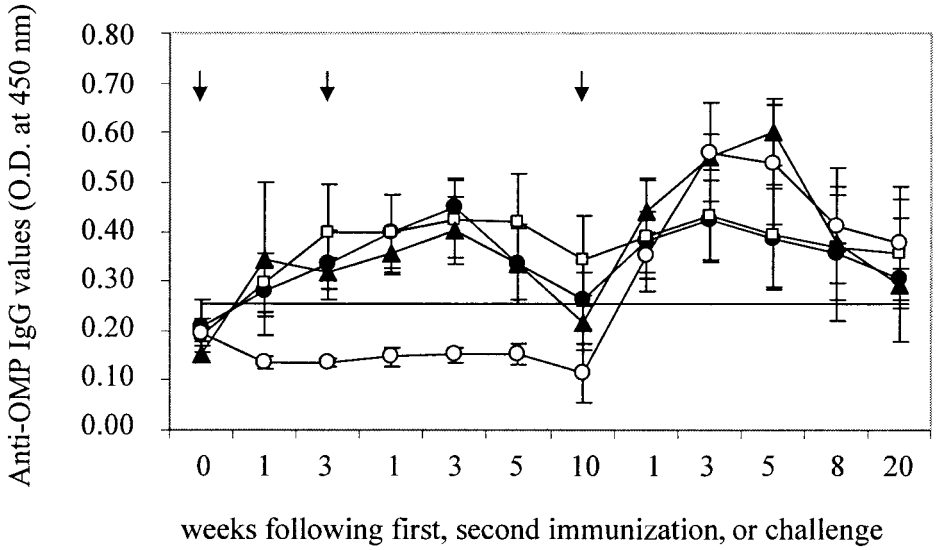

FIG. 3. Anti-OMP IgG values in pregnant ewes immunized with vaccine strain SU304 (solid circles), plasmid-cured strain SU40 (squares), or *crp cdt cya* strain SSM189 (triangles) and in nonimmunized ewes (open circles). All animals were challenged with wild-type SS44 10 weeks after the second vaccine administration. Arrows indicate times of first immunization, second immunization, and challenge with wild-type strain SS44. Values are expressed as means and standard deviations of OD readings at 450 nm. The horizontal line represents the cutoff value (0.254), calculated as described in Materials and Methods.

system without affecting the fetal tissues (i.e., causing abortion). Considering serovar Abortusovis vaccine development under this perspective, the ideal vaccine strain would require minimal attenuation of the bacterial capability to invade and persist in internal lymphoid organs.

We have evaluated the degrees of attenuation and immunogenicity, in the murine and ovine models, of three serovar Abortusovis mutants, SU304, SSM189, and SU40, whose development involved the alteration of unrelated growth and virulence factors and therefore were expected to show different extents of attenuation. Serovar Abortusovis SU304 was made auxotrophic by deletion of the *aroA* gene. SSM189 was deleted of the *crp*, *cdt*, and *cya* genes and therefore was made unable to control the transcription of many genes involved in catabolite transport and breakdown. SU40 was obtained by elimination of the virulence plasmid.

When the effect of such mutations on virulence and stimulation of a protective immunological response was examined in BALB/c mice (Table 2), all three mutants were unable to provoke disease even at high oral doses (10^8 bacteria) and elicited full protection against oral challenge with wild-type serovar Abortusovis. Thus, despite the high level of attenuation, the mutants were protective in mice.

TABLE 4. Effectiveness of immunization with avirulent serovar Abortusovis vaccine strains in ewes in protecting against pregnancy failure after challenge with wild-type serovar Abortusovis strain SS44

Strain (group)	No. of animals	Relevant genotype	No. of abortions	No. of lambs (healthy/dead[a])	% of pregnancy failure	P[c]
SU304 (A)	9	*aroA*	4	8[b]/0	44	NS
SU40 (B)	13	Plasmid cured	3	13[b]/0	23	<0.05
SSM189 (C)	6	*crp-cya-cdt*	3	3/0	50	NS
SS44 (D)	14	Wild type	8	5/1	64	

[a] Lambs died within 3 weeks, and wild-type serovar Abortusovis SS44 was isolated at necropsy.
[b] Certain ewes that did not abort delivered twins.
[c] Chi-square analysis was used to compare abortion occurrence among immunized animals versus nonimmunized animals. NS, not significant.

On the basis of the experimental sheep model, our results also indicate that the attenuation of serovar Abortusovis obtained by *aroA* or *crp cdt cya* mutagenesis or by plasmid curing completely abolished the ability to cause abortion. In addition, plasmid-cured mutant SU40 (group B) appeared to evoke a more effective protection against serovar Abortusovis SS44-induced abortion in pregnant sheep than SU304 (*aroA*, group A) and SSM189 (*crp cdt cya*, group C). The protection efficacy in immunized ewes was paralleled by the values of vaccine-induced anti-OMP IgG antibodies at the time of challenge, with group C sera showing the lowest values (below the cutoff). Following challenge with wild-type strain SS44, serum IgG values from group C animals were similar to those from nonimmunized group D animals and significantly higher than those of groups A and B. The difference observed could be due to more dramatic changes in the antigen profile of SSM189 than in those of SU40 and SU304. In addition, SU304 and SU40 may have induced stronger protection against challenge with wild-type SS44 than the SSM189 vaccine strain because of a higher rate of replication in vivo. This would result in a more intense as well as a more prolonged period of stimulation with *Salmonella* antigen by the SU304 and SU40 strains. In particular, the IgM response induced in ewes by SU40 was stronger than that induced by SU304 and SSM189 and was very similar to that induced by wild-type strain SS44 in nonimmunized ewes, suggesting that the SU40 attenuated strain maintains a high level of immunogenicity. *Salmonella aroA* strains are not able to replicate in absence of *para*-aminobenzoic acid and dihydroxybenzoic acid, and such nutrients are not available in

mammalian tissues. On the other hand, although the *Salmonella* virulence plasmid has a well demonstrated role in the rate of intracellular replication in vivo (20), there could be less reliance on the virulence plasmid for tissue infection after s.c. inoculation in sheep, as described in case of intraperitoneal administration of plasmid-cured derivatives of serovar Typhimurium and serovar Abortusovis strains in the murine model (19, 42). Finally, we have consistently found a strong and persistent skin reaction and abscess formation at the site of inoculation of SU40. Skin reactions were milder following s.c. injection of the other serovar Abortusovis mutants, and even when parental strain SS44 was injected, we did not observed development of subcutaneous abscesses. The reason for such a nonspecific inflammatory response limited to the plasmid-cured strain is unclear. Interestingly, sheep immunized with plasmid-cured serovar Abortusovis SU40 also showed a slight increase of rectal temperature in the first 3 days after challenge, whereas no febrile reaction was detected in the other groups of animals (data not shown). Although the *Salmonella* mechanism(s) of immunosuppression has not been elucidated to date, the lack of abscess formation in plasmid-containing serovar Abortusovis might be due to the presence of a plasmid-encoded factor(s) that negatively modulates inflammatory cell recruitment at the site of bacterial injection. In addition, serovar Typhimurium-induced immunosuppression in mice has been reported to require the virulence plasmid (22). Furthermore, the increase of inflammatory cells in the site of inoculation and abscess formation in SU40-immunized ewes may be responsible for slower release of *Salmonella* antigens and lead to a more effective priming of the sheep lymphoid tissues. Therefore, SU40 may be able to attain higher bacterial numbers in the ovine tissues as well as persist longer than *aroA* and *crp cdt cya* vaccine candidates.

In conclusion, we evaluated the safety and the protective efficacy of three different serovar Abortusovis mutants in pregnant ewes and found that the plasmid-cured derivative SU40 was as safe and more protective than Δ*crp* Δ*cdt* Δ*cya* or Δ*aroA* derivatives. Taken together, these data demonstrate that serovar Abortusovis virulence plasmid-cured derivatives are vaccine candidates for protection against serovar Abortusovis-induced abortion in sheep.

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