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Isolation and Characterization of Mutant Strains of *Bordetella bronchiseptica* Lacking Dermonecrotic Toxin-Producing Ability

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Mutant strains of *Bordetella bronchiseptica*, named B-42, B-76, B-84, and B-119, were obtained after serial passages of a parent strain, L3, on Bordet-Gengou agar plates containing 20% horse blood and 200 μg of nalidixic acid per ml (BGN-20 agar plates) at 42°C. Mutant strains completely lacked dermonecrotic toxin-producing ability, and lethal activity of the strains for mice was apparently reduced compared with that of strain L3. Mutant strains were able to grow at 42°C, and the strains were nalidixic acid resistant. The mutant strains showed domed (Dom^+) colony morphology with smooth texture (Scs^+) and no production of zone of hemolysis (Hly^-), but the agglutinability of these strains to antiserum prepared with Dom^+ Scs^+ Hly^+ organisms of strain L3 was the same as that of strain L3. When strain B-42 was inoculated intramuscularly or intranasally into guinea pigs, all the animals survived without manifesting clinical signs and produced a high-level of serum agglutination antibodies against strain L3. These inoculated animals were protected against intranasal challenge with strain L3. These properties of mutant strains are hereditarily stable after 50 subcultures on BGN-20 agar plates or 20 passages in mice. These data suggest that the mutant strains lacking dermonecrotic toxin-producing ability can be used as a live attenuated vaccine against swine atrophic rhinitis.

Swine atrophic rhinitis (AR) is a disease characterized by severe necrosis in the epithelia of the upper respiratory tract and by deformity and reduction in both volume and size of the nasal turbinates and snouts (3, 5, 16, 17, 30, 38). Phase I (22) of *Bordetella bronchiseptica* causes a marked loss of cilia, accompanied by the characteristic morphological changes of the nasal mucosa, when the organisms are intranasally inoculated into gnotobiotic (3, 16) or specific-pathogen-free (SPF) (5, 38) neonatal pigs. Injection of pure cultures of *B. bronchiseptica* also results in severe nasal turbinate atrophy. Therefore, *B. bronchiseptica* has been considered a primary causative agent of swine AR. Thus, inactivated whole-cell vaccines were prepared from *B. bronchiseptica* (7, 8, 10, 25, 37) and applied in the field.

Dermonecrotic toxin (DNT) extracted from the phase I organisms of *B. bronchiseptica* has been suggested as one of the virulence factors responsible for the production of nasal turbinate atrophy in piglets (9), young rats (13), young rabbits (15), and young mice (32). For example, sonic extracts of *B. bronchiseptica* containing DNT have produced AR after intranasal instillation into SPF piglets (9). Cultured swine nasal tissue fragments were incubated in a medium containing a purified *B. bronchiseptica* DNT preparation (14), and Nakai et al. (19) reported that morphological damage induced in the nasal mucosa was consistent with that observed in *B. bronchiseptica*-infected pigs (18, 24). Recently, Roop et al. (29) demonstrated that the production of the turbinate lesions in neonatal piglets by some *B. bronchiseptica* strains directly correlated with the levels of DNT production by the strains.

The present paper deals with the isolation of mutant strains of *B. bronchiseptica* lacking DNT-producing ability. Biological, serological, and immunological properties of the strains are described in comparison with those of the parent strain, L3.

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MATERIALS AND METHODS

Bacterial strains and culture media. Strain L3 (9) of *B. bronchiseptica* was used as the parent strain. It was isolated from the nasal cavity of a Landrace pig affected with AR. Ten field isolates of *B. bronchiseptica* recently obtained by us from pigs affected with AR were used, as well as four mutant strains derived from strain L3 and named B-42, B-76, B-84, and B-119. All the strains were preserved as lyophilized cultures. For cultivation of strain L3 and field isolates, Bordet-Gengou (BG) (2) agar plates supplemented with 20% horse blood (BG-20 agar plates) were used. For isolation of mutant strains, BG-20 agar plates supplemented with 200 μg of nalidixic acid (Sigma Chemical Co., St. Louis, Mo.) per ml (BGN-20 agar plates) were used.

Colony phenotypes of mutant strains. Colony phenotypes of mutant strains were confirmed as follows. Mutant strains were incubated on BG or BGN agar plates supplemented with 13% horse blood (BG-13 and BGN-13 agar plates, respectively) (26). After incubation at 42°C for 3 to 5 days, colony phenotypes of mutant strains were examined with a stereomicroscope. Colonies were graded for domed or umbonate elevation (Dom^+ or Dom^-), smooth or rough colony texture (Scs^+ or Scs^-), and production or lack of production of zone of hemolysis (Hly^+ or Hly^-) (27).

Isolation of mutant strains lacking DNT-producing ability. Dom^+ Scs^+ Hly^+ organisms of strain L3 were streaked on two BG-20 agar plates and incubated at 37°C for 2 to 3 days. Three Dom^+ Scs^+ Hly^+ colonies grown on each plate were picked up, streaked on two new plates, and incubated again under the same culture conditions. Then, 20 colonies grown on each plate were transferred to two sets of BG-20 agar plates and incubated at 42°C for 5 to 7 days. Ten colonies that grew to 0.5 to 0.7 mm in diameter on each plate were picked up and subcultured 10 times under the same culture conditions. At this stage, all 100 colonies had grown to about 2 mm in diameter and showed the Dom^+ Scs^+ Hly^- phenotype. Then, 10 colonies were transferred to BGN-20 agar plates and incubated at 42°C for 5 to 7 days. Ten Dom^+ Scs^+ Hly^- colonies (0.5 to 0.7 mm in diameter) grown on each

plate were subcultured 10 times under the same culture conditions. Finally, four mutant strains showing the Dom$^+$ Scs$^+$ Hly$^-$ phenotype and lacking DNT-producing ability (named B-42, B-76, B-84, and B-119) were obtained.

Growth of colonies on BG-20 agar plates. Growth of colonies on BG-20 agar plates was compared by using L3 and B-42 strains. Each strain was streaked onto duplicate BG-20 agar plates; one plate was incubated at 37°C, and the other was incubated at 42°C. The diameters of 30 colonies grown on each plate were measured once a day during the observation period of 5 days.

Determination of MICs. MICs of nalidixic acid for L3 and the mutant strains were determined by a standardized broth dilution method. Organisms grown on BG-20 (strain L3) or BGN-20 (mutant strains) agar plates were harvested and suspended in phosphate-buffered saline solution (PBS [pH 7.2]), and the cell numbers were adjusted to 10^6 cells per ml by spectrophotometry (14). A portion (0.1 ml) of the bacterial suspension of each strain was inoculated into 1 ml of antibiotic broth medium no. 3 (Difco Laboratories, Detroit, Mich.) containing various concentrations of nalidixic acid (3.125 to 200 μg per ml) and incubated overnight at 37°C in stationary culture, and the MIC of nalidixic acid for each sample was determined.

Hemagglutination test. The hemagglutination test was carried out by a method described by Bemis and Plotkin (1) with a sonicated antigen as a hemagglutinating antigen. Titers of hemagglutinin were expressed as the reciprocal of the highest sample dilution showing complete agglutination of bovine or equine erythrocytes.

Agglutinability to antisera. Antisera were obtained by immunizing rabbits (22) with strain L3 Dom$^+$ Scs$^+$ Hly$^+$ organisms or strain B-42 Dom$^+$ Scs$^+$ Hly$^-$ organisms. A tube agglutination method was used with antiserum diluted from 1:20 to 1:20,480 in PBS and antigen adjusted to 10^{10} cells per ml by spectrophotometry. A 0.4-ml sample of each reactant was mixed in a test tube and left for 2 h at room temperature. After the samples had stood at 4°C overnight, titers of agglutinin were measured. They were expressed as the reciprocal of the highest serum dilution showing complete agglutination.

Assay of dermonecrotic activity. Dermonecrotic activity for guinea pigs was assayed by the method described by Nakase et al. (23). Guinea pigs weighing about 300 g each were depilated and injected intradermally with 0.1-ml portions of twofold dilutions (in distilled water) of each sample (5×10^{12} cells per ml). Titers of samples were expressed as the reciprocal of the highest sample dilution showing a positive necrotic lesion more than 5 mm in diameter at 48 h after injection.

Lethal toxicity for mice. Lethal toxicity for mice was determined by using 4-week-old SPF ddY female mice (10 mice for each dilution). Organisms grown on BG-20 (strain L3) or BGN-20 (mutant strains) agar plates were harvested and suspended in PBS (10^{11} cells per ml), and 0.5-ml samples of 10-fold dilutions (in PBS) were inoculated intraperitoneally into each mouse. The mice were observed for 3 weeks thereafter, and the 50% lethal dose (LD_{50}) was estimated by the method of Reed and Muench (28).

Splenotoxicity for mice. For the splenotoxicity test, 3-week-old SPF ddY female mice (20 mice for each strain) were used. Each bacterial suspension was adjusted to a sublethal dose (10^4 viable cells of strain L3 per 0.5 ml or 10^8 viable cells of each mutant strain per 0.5 ml), and 0.5 ml of each sample was inoculated intraperitoneally into each mouse. Ten days after inoculation the body weight of each mouse was recorded; all the mice were then sacrificed. Spleens were collected from the sacrificed mice, and weight of each spleen was also recorded. The statistical significance of the differences was determined by a t test.

Serial passages on agar plates or in mice. Organisms of strain B-42 were serially subcultured on BGN-20 agar plates. For passage in mice, organisms of strain B-42 cultured on BGN-20 agar plates were harvested, suspended (10^7 viable cells per ml) in PBS, and inoculated intraperitoneally in 0.5-ml portions into 10 4-week-old SPF ddY female mice. The mice were sacrificed at day 4, postinoculation, and cotton swab samples of their peritoneal fluids were collected. The samples were streaked on BGN-20 agar plates and incubated at 42°C for 3 days, and Dom$^+$ Scs$^+$ Hly$^-$ colonies were selected. The organisms were used as a second inoculum, and the recovered organisms were serially passaged in mice. Biological and serological properties of the resulting organisms were studied after 50 subcultures on BGN-20 agar plates or 20 passages in mice.

Immunization and challenge. Immunogens were prepared as follows. Organisms of strain B-42 grown on BGN-20 agar plates were harvested, suspended in PBS, and adjusted to a concentration of about 10^{10} viable cells per ml by spectrophotometry. In total, 40 SPF Hartley guinea pigs weighing about 300 g each (10 animals in each group) were inoculated intramuscularly (0.5 ml per animal) or intranasally (0.025 ml per animal). After immunization, the effect on the guinea pigs was determined by intranasal challenge with Dom$^+$ Scs$^+$ Hly$^+$ organisms of strain L3 (4×10^8 viable cells per 0.025 ml per animal) 3 weeks after the inoculation. These guinea pigs were observed for 2 weeks after challenge, and the number of survivors was recorded. Serum samples were obtained at the time of challenge for titration of antibody.

Antibody determination. Titers of antibody in serum were determined by a tube agglutination method involving the use of a commercialized AR antigen (AR antigen Kitasato; Kitasato Institute, Tokyo, Japan) prepared with *B. bronchiseptica* H-16. The procedure recommended by the manufacturer was used.

RESULTS

Isolation of mutant strains lacking DNT-producing ability. Organisms of strain L3 produced Dom$^+$ Scs$^+$ Hly$^+$ colonies (about 1 mm in diameter) on BG-13 and BG-20 agar plates after incubation at 37°C for 2 to 3 days, whereas they sometimes produced Dom$^+$ Scs$^+$ Hly$^-$ colonies (most of them less than 0.4 mm in diameter) on BG-20 agar plates after incubation at 42°C for 5 to 7 days. However, the colonies rarely grew to 0.5 to 0.7 mm in diameter, and so they were subcultured 10 times under the same culture conditions. During this period, they produced relatively large Dom$^+$ Scs$^+$ Hly$^-$ colonies (about 1.5 mm in diameter) within 3 days when incubated at 42°C. Dermonecrotic titers of these derivatives (1:2 to 1:8) were significantly reduced ($P < 0.01$) in comparison with that of the parent strain, L3 (1:8,192). These derivatives were transferred to BGN-20 agar plates and incubated at 42°C for 5 to 7 days. A few small colonies (about 0.5 mm in diameter) were found on the plates, and these were subcultured 10 times under the same culture conditions. They produced relatively large Dom$^+$ Scs$^+$ Hly$^-$ colonies (about 2 mm in diameter) within 3 days when incubated at 42°C. Finally, we obtained four mutant strains completely lacking DNT-producing ability; these were named B-42, B-76, B-84, and B-119.

Colony phenotypes. Strain L3 formed colonies with a Dom$^+$ Scs$^+$ Hly$^+$ phenotype on BG-13 agar plates, whereas

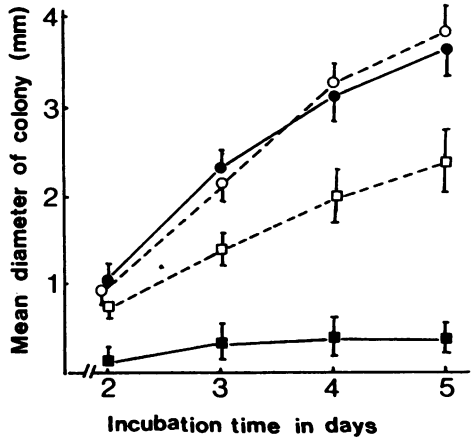

FIG. 1. Growth of *B. bronchiseptica* colonies cultured on BG-20 agar plates. Symbols: ●, strain B-42 incubated at 42°C; ○, strain B-42 incubated at 37°C; ■, strain L3 incubated at 42°C; □, strain L3 incubated at 37°C.

the four mutant strains produced colonies with a Dom^+ Scs^+ Hly^- phenotype on BGN-13 agar plates. However, these mutant strains produced colonies surrounded by narrow and weak hemolytic zones on BG-5 agar plates after incubation at 37°C for 3 days.

Growth of colonies on BG-20 agar plates. Growth of colonies on BG-20 agar plates was compared by using L3 and B-42 strains (Fig. 1). Strain B-42 grew well and produced large colonies at both 37°C and 42°C. On the other hand, strain L3 and all the 10 field isolates (data not shown) of *B. bronchiseptica* grew well at 37°C but produced only a few small colonies at 42°C. Therefore, the ability to produce large colonies at 42°C appears to be one of the markers to distinguish the mutant strains from the field isolates.

Biological properties. All four mutant strains and strain L3 produced urease, oxidase, and catalase, but they did not produce indole, hydrogen sulfide, or lysine decarboxylase. They neither utilized citrate nor fermentated glucose, mannose, sucrose, or arabinose (data not shown). There was no difference in these biological properties between strain L3 and the four mutant strains.

Susceptibility to nalidixic acid. Strain L3 and all 10 field isolates (data not shown) were susceptible to nalidixic acid (MIC, <3.125 μg/ml), whereas the four mutant strains were resistant (MIC, >200 μg/ml) (Table 1).

TABLE 1. Biological properties of *B. bronchiseptica* parent and mutant strains

Strain	Colony phenotype[a]	MIC of nalidixic acid (μg/ml)	Titers[b] of hemagglutinin against erythrocytes collected from:		Titers[c] of DNT (5×10^{11} cells/ml)
			Cattle	Horses	
B-42	Dom^+ Scs^+ Hly^-	>200	<1	8	<1
B-76	Dom^+ Scs^+ Hly^-	>200	<1	4	<1
B-84	Dom^+ Scs^+ Hly^-	>200	<1	4	<1
B-119	Dom^+ Scs^+ Hly^-	>200	<1	8	<1
L3	Dom^+ Scs^+ Hly^+	3.125	128	4	8,192

[a] Nomenclature of Peppler and Schrumpf (27) is used for describing colony morphology of *B. bronchiseptica* on BGN-13 or BG-13 agar plates. Dom designates domed colony type, Scs designates smooth colony surface, and Hly designates production of a zone of hemolysis surrounding the colony.

[b] The titers are expressed as the reciprocal of the highest sample dilution showing complete hemagglutination.

[c] The titers are expressed as the reciprocal of the highest sample dilution showing a positive necrotic lesion for guinea pigs.

TABLE 2. Agglutinability of *B. bronchiseptica* parent and mutant strains to antisera prepared with L3 and B-42

Strain[a]	Titer[b] of agglutinin against antisera prepared with following strain:	
	B-42	L3
B-42	10,240	10,240
B-76	5,120	5,120
B-84	10,240	10,240
B-119	5,120	5,120
L3	10,240	10,240

[a] Strains were used at 10^{10} cells per ml.

[b] The titers are expressed as the reciprocal of the highest serum dilution showing complete agglutination.

Hemagglutinating activity. A sonicated cell suspension of strain L3 agglutinated both bovine and equine erythrocytes, whereas those of four mutant strains agglutinated only equine erythrocytes (Table 1).

Agglutinabilities to antisera. Agglutinabilities of parent and mutant strains to anti-L3 and anti-B-42 rabbit sera were compared (Table 2). There was no difference in agglutinability to the antisera between the agglutinating antigens of strain L3 and the four mutant strains.

Toxic activities. When a sonicated cell extract of strain L3 was injected intradermally into a guinea pig, it produced severe necrotic lesions and the titer of DNT for strain L3 was 1:8,192 (Table 1). On the other hand, no toxic activity was detected for the four mutant strains in this assay system, even when undiluted samples were used.

The lethal toxicity of the four mutant strains for mice ($LD_{50} = 6.31 \times 10^8$ to 3.48×10^9 viable cells) was apparently lower than that of strain L3 ($LD_{50} = 1.11 \times 10^5$ viable cells) (Table 3). When a sublethal dose (10^4 viable cells per mouse) of strain L3 was inoculated intraperitoneally into 20 mice, all the inoculated mice showed clinical signs such as depression and bristle for 7 to 10 days after the inoculation, and their spleens showed atrophy at necropsy. On the other hand, all 80 mice (20 mice for each strain) inoculated with four mutant strains (10^8 viable cells of each strain per mouse) appeared to be normal during the 10-day observation period, and their spleens appeared to have normal morphology. Significant differences ($P < 0.05$) both in body weight and in spleen weight at 10 days after the inoculation were observed between the mice inoculated with strain L3 and those inoculated with four mutant strains (Table 3).

Stability of mutant strain B-42. Dermonecrotic activity of strain B-42 did not return after 20 passages in mice or after 50

TABLE 3. Lethal toxicity and splenotoxicity of *B. bronchiseptica* parent and mutant strains for mice

Strain	LD_{50} (viable cells)	Mean body wt (g)[a]	Mean spleen wt (mg)[a]
B-42	3.48×10^9	24.1 ± 0.27	132.6 ± 6.73
B-76	1.19×10^9	24.7 ± 0.51	136.8 ± 12.37
B-84	6.31×10^8	24.3 ± 0.48	129.1 ± 6.83
B-119	9.53×10^8	24.9 ± 0.54	129.9 ± 8.58
L3	1.11×10^5	15.7 ± 1.02[b]	74.8 ± 12.68[b]
Uninjected control		25.2 ± 0.57	115.7 ± 6.66

[a] A sublethal dose of each strain was inoculated intraperitoneally into 20 mice. The body weights and spleen weights of all the mice were measured at 10 days after inoculation.

[b] These values were significantly different from those of other groups ($P < 0.05$).

TABLE 4. Protective effect of *B. bronchiseptica* B-42 against intranasal challenge with parent strain L3

Immunization route	Immunization dose of B-42 (viable cells/animal)	Geometric mean (range) of serum agglutination titers	No. of survivors[a]
Intranasal	2.3×10^8	390.1 (160–1,280)	10
	2.3×10^7	118.9 (<10–320)	9
Intramuscular	2.3×10^8	312.2 (40–1,280)	9
	2.3×10^7	46.7 (<10–1,280)	9
Unimmunized control		<10	0

[a] All the guinea pigs were challenged intranasally with 4.0×10^8 viable cells of strain L3 (770× LD_{50}) per animal at 3 weeks after immunization and observed for 2 weeks after challenge. There were 10 animals in each group.

serial subcultures on BGN-20 agar plates. The other biological and serological properties and colony phenotype of strain B-42 after the serial passages both in vitro and in vivo were same as those of the starting organisms (data not shown).

Protective effect of strain B-42 against challenge. Most of the guinea pigs inoculated intranasally or intramuscularly with 2.3×10^7 or 2.3×10^8 viable cells of strain B-42 produced serum agglutination antibodies at 3 weeks after the inoculation, and most of them survived intranasal challenge with strain L3 (770× LD_{50}) during the observation period (Table 4). All the unimmunized control guinea pigs were killed by the challenge dose.

DISCUSSION

There is much evidence that *B. bronchiseptica* is a major cause of swine AR (3, 5, 17–19, 24, 30, 31), although many bacteria and/or factors responsible for the occurrence of the disease have been suggested. Hanada et al. (9) suggested that *B. bronchiseptica* DNT may act as an active component to produce nasal turbinate atrophy in neonatal pigs. Recently, Nakai et al. (19) demonstrated that a purified DNT preparation from *B. bronchiseptica* (14) was capable of producing the mucosal damage characteristic of AR on cultured swine nasal tissue fragments. In general, *B. bronchiseptica* DNT was not produced by actively growing cells, but low levels of DNT were released from the cells following autolysis (20). Therefore, proliferation of organisms with a high DNT-producing ability on the infected nasal mucosa of swine might be essential for the production of DNT in amounts sufficient to cause nasal turbinate atrophy in neonatal pigs (18, 19, 24). The aim of the present study was to isolate mutant strains having a high immunogenicity but lacking DNT-producing ability as a means of developing a live attenuated vaccine for control of swine AR in the field.

In total, four mutant strains having a high immunogenicity (Table 4) but completely lacking DNT-producing ability by the present assay system (Table 1) were obtained from the parent strain, L3 (9), of *B. bronchiseptica*. In addition, lethal toxicity and splenotoxicity of the four mutant strains for mice were significantly ($P < 0.05$) reduced compared with those of the parent strain (Table 3) and field isolates of *B. bronchiseptica* (data not shown). These mutant strains were characterized as follows: (i) they grew well and formed relatively large colonies at 42°C, (ii) they formed Dom^+ Scs^+ Hly^- colonies, (iii) they were resistant to nalidixic acid, and (iv) they lacked the ability to produce DNT in guinea pigs. These mutant strains were very stable, since they did not change their original characteristics after 50 serial subcultures on BGN-20 agar plates or 20 passages in mice. In contrast, parent strain L3 and the field isolates of *B. bronchiseptica* were characterized as follows: (i) they grew well at 37°C but produced only a few small colonies at 42°C, (ii) they formed Dom^+ Scs^+ Hly^+ colonies, (iii) they were susceptible to nalidixic acid, and (iv) they had the ability to produce DNT to a certain high level. The other biological and serological properties of the mutant strains were same as those of parent strain L3 and the field isolates of *B. bronchiseptica*. Thus, the properties particular to the mutant strains described herein can be used as useful markers to distinguish them from field isolates of *B. bronchiseptica*. At present, we have no defined proof of whether the mutant strains completely lack DNT or cannot produce DNT by mutation.

The ability of *B. bronchiseptica* to agglutinate erythrocytes has been recognized since the early report of Keogh et al. (12). The organisms of pig origin agglutinated equine, sheep, dog, pig, and guinea pig erythrocytes (1), as well as bovine ones (11, 33). The existence of multiple hemagglutinins in the cells of *B. bronchiseptica* was previously suggested (1). In the present study, we found that strain L3 and the field isolates of *B. bronchiseptica* had at least two hemagglutinins to agglutinate equine and bovine erythrocytes (Table 1). However, the four mutant strains lacked hemagglutinin to agglutinate bovine erythrocytes. Previously, Éliás et al. (6) reported that hemagglutinin was lost when *B. bronchiseptica* strains were subcultured and that *B. bronchiseptica* strains lost their ability to adhere by loss of hemagglutinin. Mutant strain B-42 had the ability to adhere to and colonize the nasal cavities of intranasally inoculated guinea pigs or pigs without manifesting clinical signs (unpublished data), despite losing hemagglutinin to agglutinate bovine erythrocytes, although hemagglutinin to agglutinate equine ones was retained, suggesting that the former hemagglutinin may be related to virulence and that the latter one is related to adherence.

During the last decade, some attenuated or avirulent strains of *B. bronchiseptica* have been used as a live vaccine for control of canine bordetellosis (4, 34) or swine AR (31, 35, 36). Previously, Shimizu (35) isolated a temperature-sensitive mutant strain, ts-S34, by using nitrosoguanidine. The temperature-sensitive mutant is characterized as follows: it (i) cannot grow at or above 34°C, (ii) it has moderate growth in the nasal cavities of guinea pigs, and (iii) it is unable to grow in the lungs of guinea pigs. Recently, Shimizu and Ishikawa (36) isolated a urease-negative temperature-sensitive mutant (strain ts-S34-u^-) from strain ts-S34 by joint treatment with two mutagens, UV irradiation and nitrosoguanidine (35). The characteristics of the temperature-sensitive mutant strain were completely different from those of the present mutant strains in the following respects: (i) the temperature-sensitive mutant still retains a little DNT activity, (ii) it cannot grow well above 34°C, and (iii) it is urease negative. The first point is very important in view of safety, although Shimizu and Ishikawa (36) stated that the reversion frequency related to u^- or temperature sensitivity of strain ts-S34-u^- phase I was lower than $10^{-9.8}$.

Shimizu and Ishikawa (35, 36) noted that the temperature-sensitive mutant strains ts-S34 and ts-S34-u^- conferred strong immunity on guinea pigs. Recently, Sakano et al. (31) evaluated the effects of vaccinal strain ts-S34-u^- on immunogenicity and safety for piglets. They reported that all pigs inoculated intranasally at 7 days of age with more than $3 \times$

10^7 viable cells of the strain ts-S34-u$^-$ were protected against intranasal challenge, although the inoculated organisms were recovered from the immunized pigs at 1 week after inoculation but never at 3 weeks after the inoculation. They also stated that serum agglutination antibody was not detected in most of the immunized pigs before challenge but that titers increased after challenge (31), suggesting that, at best, there was slight proliferation of the vaccinal strain in the nasal cavities of the inoculated pigs. The reason why such inoculated pigs are protected against challenge (31) remains uncertain. As shown here, guinea pigs inoculated intranasally or intramuscularly with mutant strain B-42 produced high serum agglutination antibodies as well as being protected against intranasal challenge. This mutant strain, B-42, with high immunogenicity but without DNT-producing ability, might be useful as a live attenuated vaccine for control of swine AR in the field.

LITERATURE CITED

1. **Bemis, D. A., and B. J. Plotkin.** 1982. Hemagglutination by *Bordetella bronchiseptica*. J. Clin. Microbiol. **15**:1120–1127.
2. **Bordet, J., and O. Gengou.** 1906. Le microbe de la conqueluche. Ann. Inst. Pasteur (Paris). **20**:731–741.
3. **Brassinne, M., A. Dewaelle, and M. Gouffaux.** 1976. Intranasal infection with *Bordetella bronchiseptica* in gnotobiotic piglets. Res. Vet. Sci. **20**:162–166.
4. **Chaldek, D. W., J. M. Williams, D. L. Gerber, L. L. Harris, and F. M. Murdock.** 1981. Cannine parainfluenza-*Bordetella bronchiserptica* vaccine: immunogenicity. Am. J. Vet. Res. **42**:266–270.
5. **Duncan, J. R., R. F. Ross, W. P. Switzer, and F. K. Ramsey.** 1966. Pathology of experimental *Bordetella bronchiseptica* infection in swine: atrophic rhinitis. Am. J. Vet. Res. **27**:457–466.
6. **Éliás, B., M. Krüger, and F. Rátz.** 1982. Epizootiologishe Untersuchungen der Rhinitis atrophicans des Schweines. II. Biologishe Eigenschaften der von Schweinen isolierten *Bordetella bronchiseptica*-Stämme. Zentralbl. Veterinaermed. Reihe B **29**:619–635.
7. **Farrington, D. O., and W. P. Switzer.** 1979. Parenteral vaccination of young swine against *Bordetella bronchiseptica*. Am. J. Vet. Res. **40**:1347–1351.
8. **Goodnow, R. A., F. J. Shade, and W. P. Switzer.** 1979. Efficacy of *Bordetella bronchiseptica* bacterin in controlling enzootic atrophic rhinitisoin swine. Am. J. Vet. Res. **40**:58–60.
9. **Hanada, M., K. Shimoda, S. Tomita, Y. Nakase, and Y. Nishiyama.** 1979. Production of lesions similar to naturally occurring swine atrophic rhinitis by cell-free sonicated extract of *Bordetella bronchiseptica*. Jpn. J. Vet. Sci. **41**:1–8.
10. **Harris, D. L., and W. P. Switzer.** 1972. Immunization of pigs against *Bordetella bronchiseptica* infection by pareteral vaccination. Am. J. Vet. Res. **33**:1975–1984.
11. **Ishikawa, H., and Y. Isayama.** 1987. Evidence for sialyl glycoconjugates as receptors for *Bordetella bronchiseptica* on swine nasal mucosa. Infect. Immun. **55**:1607–1609.
12. **Keogh, E. V., E. A. North, and M. F. Warburton.** 1947. Hemagglutinins of the hemophilus group. Nature (London) **160**: 63.
13. **Kimman, T. G., and E. M. Kamp.** 1986. Induced atrophic rhinitis in rats. Am. J. Vet. Res. **47**:2426–2430.
14. **Kume, K., T. Nakai, Y. Samejima, and C. Sugimoto.** 1986. Properties of dermonecrotic toxin prepared from sonic extracts of *Bordetella bronchiseptica*. Infect. Immun. **52**:370–377.
15. **Maeda, M., and T. Shimizu.** 1975. Nasal infection of *Alcaligenes bronchisepticus* (*Bordetella bronchiseptica*) and lesions in newborn rabbits. Natl. Inst. Anim. Health Q. **15**:29–37.
16. **Miniatis, O. P., and J. A. Johnson.** 1980. Experimental atrophic rhinitis in gnotobiotic pigs. Can. J. Comp. Med. **44**:358–365.
17. **Nakagawa, M., T. Shimizu, and Y. Motoi.** 1974. Pathology of experimental atrophic rhinitis in swine infected with *Alcaligenes bronchisepticus* or *Pasteurella multocida*. Natl. Inst. Anim. Health Q. **14**:61–71.
18. **Nakai, T., K. Kume, H. Yoshikawa, T. Oyamada, and T. Yoshikawa.** 1986. Changes in the nasal mucosa of specific-pathogen-free neonatal pigs infected with *Pasteurella multocida* or *Bordetella bronchiseptica*. Jpn. J. Vet. Sci.**48**:693–701.
19. **Nakai, T., K. Kume, H. Yoshikawa, T. Oyamada, and T. Yoshikawa.** 1988. Adherence of *Pasteurella multocida* or *Bordetella bronchiseptica* to the swine nasal epithelial cell in vitro. Infect. Immun. **56**:234–240.
20. **Nakai, T., A. Sawata, and K. Kume.** 1985. Intracellular locations of dermonecrotic toxins in *Pasteurella multocida* and in *Bordetella bronchiseptica*. Am. J. Vet. Res. **46**:870–874.
21. **Nakai, T., A. Sawata, M. Tsuji, and K. Kume.** 1984. Characterization of dermonecrotic toxin produced by serotype D strains of *Pasteurella multocida*. Am. J. Vet. Res. **45**:2410–2413.
22. **Nakase, Y.** 1957. Studies on *Hemophilus bronchisepticus*. I. Antigenic structures of *H. bronchisepticus* from guinea pig. Kitasato Arch. Exp. Med. **30**:57–72.
23. **Nakase, Y., K. Takatsu, and M. Takeishi.** 1969. Heat-labile toxin of *Bordetella pertussis* purified by preparative acrylamide gel electrophoresis. Jpn. J. Microbiol. **13**:359–366.
24. **Oyamada, T., T. Yoshikawa, H. Yoshikawa, M. Shimizu, T. Nakai, and K. Kume.** 1986. Lesions induced in the nasal turbinates of neonatal pigs inoculated with *Pasteurella multocida* and/or *Bordetella bronchiseptica*. Jpn. J. Vet. Sci. **48**:377–387.
25. **Pedersen, K. B., and K. Barfod.** 1977. Effect of vaccination of sows with *Bordetella bronchiseptica* on the incidence of atrophic rhinitis in swine. Nord. Veterinaermed. **29**:369–375.
26. **Peppler, M. S.** 1982. Isolation and characterization of isogenic pairs of domed hemolytic and flat nonhemolytic colony types of *Bordetella pertussis*. Infect. Immun. **35**:840–851.
27. **Peppler, M. S., and M. E. Schrumpf.** 1984. Phenotypic variation and modulation in *Bordetella bronchiseptica*. Infect. Immun. **44**:681–687.
28. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating 50 per cent endpoits. Am. J. Hyg. **27**:493–497.
29. **Roop, R. M., II, H. P. Veit, R. J. Sinsky, S. P. Veit, E. L. Hewlett, and E. T. Kornegay.** 1987. Virulent factors of *Bordetella bronchiseptica* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. Infect. Immun. **55**:217–222.
30. **Ross, R. F., J. R. Duncan, and W. P. Switzer.** 1963. Turbinate atrophy in swine produced by pure cultures of *Bordetella bronchiseptica*. Vet. Med. **58**:566–570.
31. **Sakano, T., K. Sakai, T. Furutani, and T. Shimizu.** 1984. Immunogenicity and safety of an attenuated *Bordetella bronchiseptica* vaccine in pigs. Am. J. Vet. Res. **45**:1814–1817.
32. **Sawata, A., and K. Kume.** 1982. Nasal turbinate atrophy in young mice inoculated with *Bordetella bronchiseptica* of pig origin. Am. J. Vet. Res. **43**:1845–1847.
33. **Semjen, G., and T. Magyar.** 1985. A bovine hemagglutinin of *Bordetella bronchiseptica* responsible for adherence. Acta Vet. Hung. **33**:129–136.
34. **Shade, F. J., and R. A. Goodnow.** 1979. Intranasal immunization of dogs against *Bordetella bronchiseptica*-induced tracheobronchitis (kennel cough) with modified live-*Bordetella bronchiseptica* vaccine. Am. J. Vet. Res. **40**:1241–1243.
35. **Shimizu, T.** 1978. Prophylaxis of *Bordetella bronchiseptica* infection in guinea pigs by intranasal vaccination with live strain ts-S34. Infect. Immun. **22**:318–321.
36. **Shimizu, T., and H. Ishikawa.** 1982. Some characteristics of a urease-negative, temperature-sensitive strain of *Bordetella bronchiseptica* as a live, attenuated vaccine. Infect. Immun. **36**: 198–201.
37. **Smith, I. M., C. J. Giles, and A. J. Baskerville.** 1982. Immunisation of pigs against experimental infection with *Bordetella bronchiseptica*. Vet. Rec. **110**:488–494.
38. **Yokomizo, Y., and T. Shimizu.** 1979. Adherence of *Bordetella bronchiseptica* to swine nasal epithelial cells and its possible role in virulence. Res. Vet. Sci. **27**:15–21.