

Isolation and Characterization of Small-Colony Variants of *Ornithobacterium rhinotracheale*

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Ornithobacterium rhinotracheale is a Gram-negative bacterium associated with respiratory diseases in many avian species, with worldwide distribution, and it causes significant economic loss to the poultry industry. In this study, the isolation and characterization of *O. rhinotracheale* small-colony variants (SCVs) are described for the first time. *O. rhinotracheale* isolates ($n = 27$) were recovered from tracheal samples ($n = 321$) collected from different avian species with clinical signs of respiratory disease. Of the 27 *O. rhinotracheale* isolates, 21 (77.8%) showed SCVs in their primary cultures. Five *O. rhinotracheale* SCV isolates showed high levels of stability and were chosen for further characterization with their wild-type (WT) isolates. Stable *O. rhinotracheale* SCVs were oxidase negative, while their WT isolates were positive. Growth curves for stable *O. rhinotracheale* SCVs indicated lower growth rates and longer lag phases than for their WT isolates. Furthermore, it was possible to increase the efficacy of the broth medium in supporting the growth of *O. rhinotracheale* WT isolates by supplementing it with 5% fetal bovine serum (FBS) and 2% IsoVitaleX Enrichment. Antibiotic susceptibility tests showed that *O. rhinotracheale* SCVs had higher MIC values than their WT isolates. This study suggests that successful antibiotic treatment of respiratory diseases associated with *O. rhinotracheale* must take into consideration the resistance patterns of *O. rhinotracheale* SCVs. Intracellular persistence in murine RAW 264.7 macrophages revealed that *O. rhinotracheale* SCV28 had higher survival rates than its WT isolate. Finally, small-colony variants may be important contributors to the pathogenesis of *O. rhinotracheale*.

Small-colony variants (SCVs) represent a naturally occurring, slow-growing, auxotrophic subpopulation of bacteria with distinctive phenotypic and pathogenic traits (1). Phenotypically, SCVs have low growth rates and atypical colony morphology (SCVs grow in pinpoint colonies nearly one-tenth the size of their wild-type [WT] counterparts). They also typically have deficiencies or reductions in biochemical reaction activities (2). The biochemical basis of this phenotypic abnormality is auxotrophism caused by mutations of genes involved in the biosynthesis of thiamine, menadione, hemin, or thymidine (3). Two main domains of auxotrophic SCVs have been defined, i.e., SCVs that are deficient in electron transport and SCVs that are deficient in thymidine biosynthesis. However, SCVs for which the auxotrophism cannot be defined also have been described (1). Clinically, SCVs are more resistant to some classes of antibiotics than are their wild-type counterparts (1, 3, 4). They are often associated with persistent or recurrent infections (5, 6). Furthermore, a recent study on *Enterococcus faecalis* SCVs isolated from chickens indicates that SCVs have higher infection potency than their WT isolates (7).

SCVs have been described for a wide range of bacterial genera and species, including *Staphylococcus aureus* (8), *Pseudomonas aeruginosa* (9), *Salmonella* serovars (10), *Escherichia coli* (11), *Neisseria gonorrhoeae* (12), and *Enterococcus* spp. (7, 13). However, their uncommon morphological and physiological properties cause them to be frequently undetected or misidentified by standard clinical microbiology procedures (1).

Ornithobacterium rhinotracheale is a Gram-negative bacterium of the family *Flavobacteriaceae* in rRNA superfamily V (14). The pathogen was relatively recently discovered and is distributed worldwide in chickens and turkeys, as well as in other avian species (15–17). *O. rhinotracheale* is associated with respiratory

diseases and causes significant economic loss to the poultry industry (17–19).

In the present study, we report for the first time the isolation of *O. rhinotracheale* SCVs. Furthermore, the *O. rhinotracheale* SCV isolates obtained were characterized and compared with wild-type isolates with regard to auxotrophism, biochemical profiles, growth curves, hemagglutinating activity, antimicrobial susceptibility, autolytic activity, intracellular persistence, and phylogenetic patterns.

MATERIALS AND METHODS

Sample collection. Between December 2011 and August 2012, tracheal samples ($n = 321$) from different avian sources (chicken, $n = 246$; duck, $n = 42$; turkey, $n = 1$; pigeon, $n = 4$; goose, $n = 19$; pheasant, $n = 9$) with clinical signs of respiratory disease were submitted to the poultry diagnostic laboratories at China Agricultural University (Beijing, China), Animal Health Directorate (Beijing, China), and Shandong Agricultural University (Shandong, China). The tracheas were aseptically removed, put in separate plastic bags, and kept at -20°C until tested.

Isolation and identification of *O. rhinotracheale* WT isolates and SCVs. Tracheal swabs were streaked onto tryptic soy agar (BBL; Becton, Dickinson and Co., Sparks, MD) supplemented with 5% sheep blood (i.e., sheep blood agar [SBA]), with or without gentamicin (10 $\mu\text{g}/\text{ml}$), and

Received 22 May 2013 Returned for modification 13 June 2013

Accepted 12 July 2013

Published ahead of print 17 July 2013

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doi:10.1128/JCM.01337-13

were incubated for 24 to 48 h under microaerobic conditions in candle jars at 37°C (17, 20). After incubation, all colonies that were pinpoint/small, circular, opaque, and gray to gray-white were chosen for further identification (15, 21). Colonies suspected to be *O. rhinotracheale* were subcultured on SBA (under the same environmental conditions), tentatively identified on the basis of routine phenotypic and biochemical traits, and genetically confirmed by PCR and DNA sequencing (17).

During the primary course of cultivation of *O. rhinotracheale* isolates, most of the pure cultures of *O. rhinotracheale* isolates showed a vast difference in colony size after 48 h of incubation. This difference goes beyond size variance and discriminates all isolates into two main phenotypically different subpopulations, i.e., large colonies (~1 to 3 mm in diameter) and pinpoint small colonies (up to 10 times smaller). If the large colonies are cultured on SBA, they grow typical, uniformly large *O. rhinotracheale* colonies. In contrast, small colonies generally produce both types. To further investigate this observation, one colony from each type was picked and passaged on SBA several times, to assess stability. All *O. rhinotracheale* isolates (WT isolates and SCVs) then underwent sequence analysis of the complete 16S rRNA gene (22). Furthermore, all of the stable SCVs and the WT isolates were chosen for further studies, including Gram staining, scanning electron microscopy (SEM), autolysis assay, growth curve analysis, determination of biochemical characteristics, hemagglutination assay (HA), auxotrophism test, antimicrobial susceptibility test, and intracellular persistence assay. The *O. rhinotracheale* isolates with small colonies were termed SCVs if they maintained their phenotype on SBA for at least 10 passages, while the *O. rhinotracheale* isolates with typical colonies were termed wild-type (WT) isolates. Cryopreservation of the *O. rhinotracheale* isolates was done by swabbing the pure cultures directly from the SBA and storing the samples at -80°C in cryoprotection medium composed of trypticase soy broth (TSB) supplemented with 25% glycerol (23).

PCR and sequence analysis. Primary identification was conducted by extracting the DNA from the suspected colonies using the conventional boiling method. Subsequently, PCR was conducted using specific *O. rhinotracheale* primers (which amplify a 784-bp region of the 16S rRNA), as described previously (24). Following *O. rhinotracheale* PCR, identification was confirmed by single-strand sequencing of the amplified fragments, and comparison with the published GenBank sequences was performed.

For sequence analysis, the complete 16S rRNA genes from all *O. rhinotracheale* isolates (WT isolates and SCVs) were amplified and sequenced as described previously (22). The PCR products were purified and sequenced on an ABI 3730 automated sequencer at Beijing Sunbio-tech, Inc. (Beijing, China). The chromatographs obtained were verified by eye and aligned using the program MUSCLE (25), as implemented in MEGA5 (26). The program DnaSP v.5 (27) was used to compute haplotype diversity (h) (28) and nucleotide diversity (π) (29). Consequently, phylogenetic analysis for the discrimination of clades was performed with the neighbor-joining method (28) using the Tamura 3-parameter substitution model (30), as implemented in MEGA5 (26). The phylogenetic results obtained were verified by bootstrap tests (31), as well as Bayesian statistics in MrBayes 3.1 (32).

Biochemical characteristics. Pairs of *O. rhinotracheale* isolates (stable SCVs and their WT counterparts) were compared with respect to their biochemical profiles using API-20NE identification strips (bioMérieux, France), according to the manufacturer's instructions (15, 21). Moreover, isolates were tested for growth on MacConkey agar, catalase activity, and oxidase activity. Two methods were used to test oxidase activity, i.e., the oxidase reagent purchased with the API-20NE identification strips (bioMérieux, France) and oxidase disks (Sigma, India) to confirm the results.

Bacterial growth curves and enrichment on broth medium. Growth kinetics for all stable *O. rhinotracheale* SCVs and WT isolates were determined as described previously, with modification (13, 33). Single loopfuls of 48-h pure *O. rhinotracheale* cultures were inoculated in 225-ml Falcon tubes containing 20 ml of TSB (BBL; Becton, Dickinson and Co., Sparks,

MD) supplemented with 5% fetal bovine serum (FBS). The tubes were then sealed tightly and incubated for 24 h at 37°C with shaking (200 rpm). After incubation, 1-ml culture samples were transferred to new tubes containing 40 ml TSB supplemented with 5% FBS and were incubated under the same environmental conditions (initial optical density at 600 nm [OD₆₀₀] values were adjusted to be less than 0.1). Bacterial growth was assessed every 3 h, by measuring the OD₆₀₀ of the culture with a Multi-skan-MK3 microplate reader (Thermo Labsystems). Growth curves were constructed in triplicate. Additionally, the purity and stability (switching and reverting between SCV and WT forms) of the cultures were checked on SBA.

Furthermore, bacterial growth was determined with the standard plate counting method. Thus, serial 10-fold dilutions of bacterial cultures were made in phosphate-buffered saline (PBS), 100- μ l aliquots were spread on SBA in triplicate, and the plates were then incubated to quantify the viable CFU.

Hemagglutination assay. The hemagglutinating activity of all stable *O. rhinotracheale* SCVs and WT isolates was tested with fresh chicken, pigeon, and rabbit erythrocytes, as described previously (15, 34). Briefly, fresh bacterial suspensions of the *O. rhinotracheale* isolates were prepared from 48-h cultures on SBA; they were harvested in 1.5 ml of PBS (pH 7.0), adjusted to an OD₆₀₀ value of 1.5, and washed 3 times with PBS. Serial dilutions of each *O. rhinotracheale* isolate were then prepared in 0.85% NaCl. The hemagglutinating activity was determined with 50- μ l volumes of relevant fresh 1% erythrocyte suspension. Wells containing only suspensions of erythrocytes served as negative controls. The hemagglutination titer was the reciprocal of the highest dilution of antigen showing complete agglutination of the erythrocytes after 40 min of incubation at room temperature. Each test was performed in triplicate, and the hemagglutination assay (HA) results were regarded as positive when erythrocyte suspensions were agglutinated in the three repetitions.

Auxotrophism assay. Stable *O. rhinotracheale* SCV isolates underwent single auxotrophism tests as described previously (5). Briefly, colonies were taken from 48-h cultures and suspended in 1 ml TSB, 50- μ l aliquots were swabbed on SBA, and 10-mm disks (Sigma, India) loaded with 20 μ l of thymidine at 100 μ g/ml (Sigma), hemin at 100 μ g/ml (Sigma), or menadione at 25 μ g/ml (Sigma) were laid on the top of the SBA plates. An isolate was considered auxotrophic if a zone of WT colonies or enhanced growth surrounding the disks, compared to the periphery, was detected following 24 to 48 h of incubation at 37°C under microaerobic conditions (13).

Antimicrobial susceptibility testing. The *in vitro* susceptibility of stable *O. rhinotracheale* SCVs and wild-type isolates was determined by the agar disc diffusion method with SBA (15). The antibiotics tested included aminoglycosides (kanamycin, 30 μ g; gentamicin, 10 μ g), β -lactams (penicillin G, 10 IU; amoxicillin, 10 μ g; ampicillin, 10 μ g; cefuroxime sodium, 30 μ g; cefotaxime, 30 μ g), macrolides (clarithromycin, 15 μ g; erythromycin, 15 μ g), tetracyclines (doxycycline, 30 μ g; tetracycline, 30 μ g), norfloxacin (10 μ g), polymyxin B (300 IU), sulfamethoxazole-trimethoprim (23.75 μ g and 1.25 μ g), clindamycin (20 μ g), and chloramphenicol (30 μ g). Results were read after 24 and 48 h of incubation at 37°C under microaerobic conditions, according to the National Committee for Clinical Laboratory Standards procedure for fastidious Gram-negative organisms (35).

Subsequently, pairs of *O. rhinotracheale* isolates (stable *O. rhinotracheale* SCVs and their WT counterparts) that showed different patterns of susceptibility to certain antibiotics were further tested to identify the MIC values for those antibiotics, as described previously (36). Therefore, *O. rhinotracheale* isolates were grown on TSB supplemented with 5% FBS and 2% IsoVitalEx Enrichment at 37°C under microaerobic conditions (see below), and standard microdilution procedures were conducted using the same medium and conditions to determine MIC values.

Autolysis assay. The Triton X-100-induced autolysis assay was performed as described previously (37). Briefly, 48-h cultures of stable *O. rhinotracheale* SCVs and WT isolates were subcultured on TSB and incu-

TABLE 1 Bacterial isolates in this study

Isolate	Origin of isolate, age, and location	SCV ^a	Geographical region	Accession no. ^b
<i>Ornithobacterium rhinotracheale</i> isolates				
1	Chicken hen, 150 days, trachea	–	Hebei Province	KC454287
3	Chicken hen, 157 days, trachea	+	Hebei Province	KC454288, KC454289
4		–		KC454290
5		–		
6	Chicken broiler, 10 days, trachea	+	Shandong Province	KC454291
7	Chicken hen, 30 days, trachea	+	Shandong Province	KC454292
8	Chicken broiler, 37 days, trachea	+	Hebei Province	KC454293, KC454294
9		+		
10	Chicken hen, 300 days, trachea	+	Hebei Province	KC454295
11		+		KC454296, KC454297
12		–		
13	Chicken broiler, 45 days, trachea	+	Hebei Province	KC454298
14	Turkey, trachea	–	Hebei Province	KC454299
15	Chicken hen, 70 days, trachea	+	Shandong Province	KC454300
16		+		KC454301
17	Chicken hen, 21 days, trachea	+	Shandong Province	KC454302
18		+		KC454303
19	Domestic chicken, 70 days, trachea	+	Shandong Province	KC454304
20	Chicken hen, 67 days, trachea	–	Shandong Province	KC454305
21	Chicken hen, 50 days, trachea	+	Shandong Province	KC454306
22		+		KC454307
23		+		KC454308
24	Chicken hen, 70 days, trachea	+	Shandong Province	KC454309
25		+		KC454310
26	Chicken hen, 60 days, trachea	+	Shandong Province	KC454311
27	Chicken hen, 240 days, trachea	+	Shandong Province	KC454312
28	Chicken breeder, 70 days, trachea	+	Shandong Province	KC454313
Non- <i>O. rhinotracheale</i> isolates				
<i>Riemerella columbina</i> RC29	Chicken hen, 50 days, trachea	NA	Hebei Province	KC454317
<i>Coenonia anatina</i> CA39	Duck broiler, 25 days, trachea	NA	Shandong Province	KC454314
<i>Riemerella anatipestifer</i> RA37	Duck broiler, 31 days, trachea	NA	Shandong Province	KC454315
<i>Riemerella anatipestifer</i> RA38	Duck broiler, 37 days, trachea	NA	Shandong Province	KC454316

^a –, no SCV recovered; +, SCV recovered; NA, not applicable.

^b GenBank accession number of the 16S rRNA gene.

bated for 5 h at 37°C with shaking (220 rpm), and the OD₆₀₀ was adjusted to 1.0. The cells were then pelleted, washed twice with ice-cold PBS, and subsequently resuspended in PBS containing 0.04% Triton X-100. Suspensions were then incubated with shaking (220 rpm) at 37°C. OD₆₀₀ readings were taken at 0 min, at 15 min, and then at 30-min intervals up to 4 h. Triton X-100-induced autolysis was measured as a percentage of the initial OD₆₀₀ value.

Preparation of *O. rhinotracheale* isolates for the assay. Before testing, stable *O. rhinotracheale* SCVs and WT isolates were grown on TSB supplemented with 5% serum and 2% IsoVitalX Enrichment at 37°C under microaerobic conditions, with shaking (220 rpm), to the early exponential phase. CFU/ml values were enumerated using SBA plates, and stability was observed. For the intracellular persistence assay, bacterial suspensions were centrifuged at 8,000 rpm for 5 min, and the cell pellets were washed two times with PBS. Washed cells were then suspended in invasion medium (Dulbecco's modified Eagle's medium [DMEM]) and adjusted to cell densities that corresponded to a multiplicity of infection (MOI) of 100 CFU of *O. rhinotracheale* per cell for each isolate (38).

Intracellular persistence assay. To investigate the intracellular persistence of *O. rhinotracheale* SCVs and WT isolates, an intracellular survival assay was conducted as described previously, with modifications (38). Briefly, confluent monolayers of murine RAW 264.7 macrophages were seeded in 24-well tissue culture plates. Before testing, the medium was removed from the wells and 1 ml of invasion medium (MOI of 100 CFU/

cell) was added to each well except for the control. The plates were incubated for 2 h, the medium was removed, and 1 ml of DMEM containing chloramphenicol at 40 µg/ml was added to each well to kill extracellular bacteria. The medium was then removed, the cells were washed three times with PBS, 1 ml of DMEM with 2% FBS was added to each well, and the plates were incubated for 3, 24, and 48 h. At these time points, the monolayers were washed 3 times with PBS, followed by addition of 500 µl of ice-cold sterile water to disrupt the cells and to release the intracellular bacteria. After this, serial dilutions were made in PBS, and the numbers of intracellular CFU were determined by plating 100-µl aliquots on SBA in duplicate. Each test was done in triplicate. Statistical analysis of the differences in CFU between *O. rhinotracheale* SCV28 and its WT isolate after 3, 24, and 48 h of incubation was performed using Student's *t* test (*P* < 0.05).

RESULTS

Isolation of *O. rhinotracheale*. A total of 27 *O. rhinotracheale* isolates were recovered from the collected samples; 26 of the isolates were obtained from chickens and one *O. rhinotracheale* isolate was obtained from a turkey (Table 1). In contrast, no *O. rhinotracheale* could be isolated from the other avian species. Moreover, with the exception of *O. rhinotracheale* isolates 21, 22, and 23, all *O. rhinotracheale* isolates were picked from SBA supplemented with gentamicin.

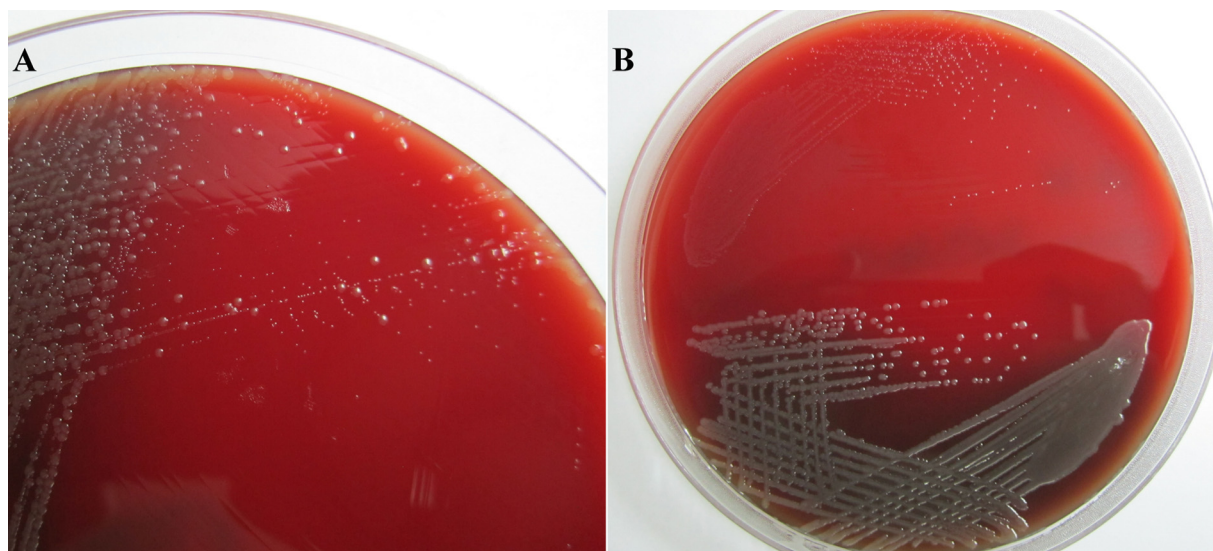


FIG 1 (A) Primary plate with a mixed culture of pinpoint small-colony variants and typical normal-sized wild-type colonies of *Ornithobacterium rhinotracheale*. (B) Colony morphology of a stable *O. rhinotracheale* SCV (top) and its WT isolate (bottom). Note the weak hemolytic activity of the wild-type isolate.

Cultivation and morphological characteristics of *O. rhinotracheale* WT isolates and SCVs. The best results for isolation of *O. rhinotracheale* were achieved by picking pinpoint colonies on SBA (with or without gentamicin) after incubation for 24 h at 37°C under microaerobic conditions (39). Furthermore, unique characteristics of the *O. rhinotracheale* colonies were their paraffinic structure and their poor adherence to agar (40).

After 48 h of incubation on SBA, 21 of the 27 *O. rhinotracheale* isolates showed heterogeneous populations of both typical *O. rhinotracheale* colonies (~1 to 3 mm, circular, opaque, and gray to gray-white) and potential *O. rhinotracheale* SCV pinpoint colonies (Fig. 1A). In addition, the stability of these potential *O. rhinotracheale* SCVs was checked by passaging every isolate on SBA for 10 generations. Five *O. rhinotracheale* SCVs (SCV3, SCV13, SCV16, SCV21, and SCV28) showed high stability and were chosen for further studies.

The characteristics of these stable *O. rhinotracheale* SCV isolates included slow growth (requiring up to 48 h of incubation at 37°C to be detected), tiny pinpoint (approximately 10 times smaller than their WT isolates), less-opaque, gray-white colonies on SBA, and failure to present any type of hemolytic activity after 48 h (Fig. 1B and Table 2). No differences between *O. rhinotracheale* SCVs and their WT isolates were detected under the light microscope; both types showed Gram-negative, pleomorphic, rod-shaped bacteria. In contrast, SEM observations showed major differences in the amounts of intercellular substances, with *O.*

rhinotracheale WT isolates showing larger amounts than the SCV isolates (Fig. 2A to D).

Furthermore, auxotrophism assays for stable *O. rhinotracheale* SCV isolates on SBA revealed enhanced growth rates only around thymidine discs for SCV16 and around menadione discs for SCV3. In contrast, isolates SCV13, SCV21, and SCV28 showed no differences in growth rates around any of the impregnated discs.

Biochemical characteristics. The results with API-20NE strips were assessed initially after 24 h of incubation at 30°C and did not change after 48 h. All five stable *O. rhinotracheale* SCV isolates gave identical API-20NE codes (code 0020000). In contrast, the WT isolates showed two different codes; isolates WT3, WT13, WT21, and WT28 gave code 0020004 and WT16 gave code 0220004. All five pairs of isolates gave positive results in the β -galactosidase assay. In contrast to their WT isolates, all *O. rhinotracheale* SCV isolates were oxidase negative. Additionally, only WT16 was positive for urease. Furthermore, all of the tested isolates were catalase negative and did not grow on MacConkey agar.

Bacterial growth curves. Growth curves for the stable *O. rhinotracheale* SCVs and their WT isolates were drawn (Fig. 3A to E). The five tested pairs of *O. rhinotracheale* isolates showed two different patterns in growth on TSB supplemented with FBS. Whereas the WT isolates with the first pattern (WT3, WT16, and WT21) showed prolonged lag phases (up to 27 h) and lower exponential-phase peaks (OD_{600} of 0.361 for WT16), the WT isolates with the second pattern (WT13 and WT28) had very short lag

TABLE 2 Colony diameters for stable *O. rhinotracheale* SCVs and their wild-type isolates on SBA

Incubation time (h)	Colony diameters (mm) for the SCV or WT isolate ^a :									
	SCV3	WT3	SCV13	WT13	SCV16	WT16	SCV21	WT21	SCV28	WT28
24	— ^b	0.6	—	0.3	0.1	0.4	—	0.3	—	0.1
48	0.2	1.9	0.1	1.5	0.2	2.4	0.2	1.8	0.1	0.6
72	0.3	3.4	0.3	2.5	0.5	4	0.3	3.2	0.2	1.1

^a Values are means of 30 measured colonies for each isolate.

^b —, no growth detected at the measured time point.

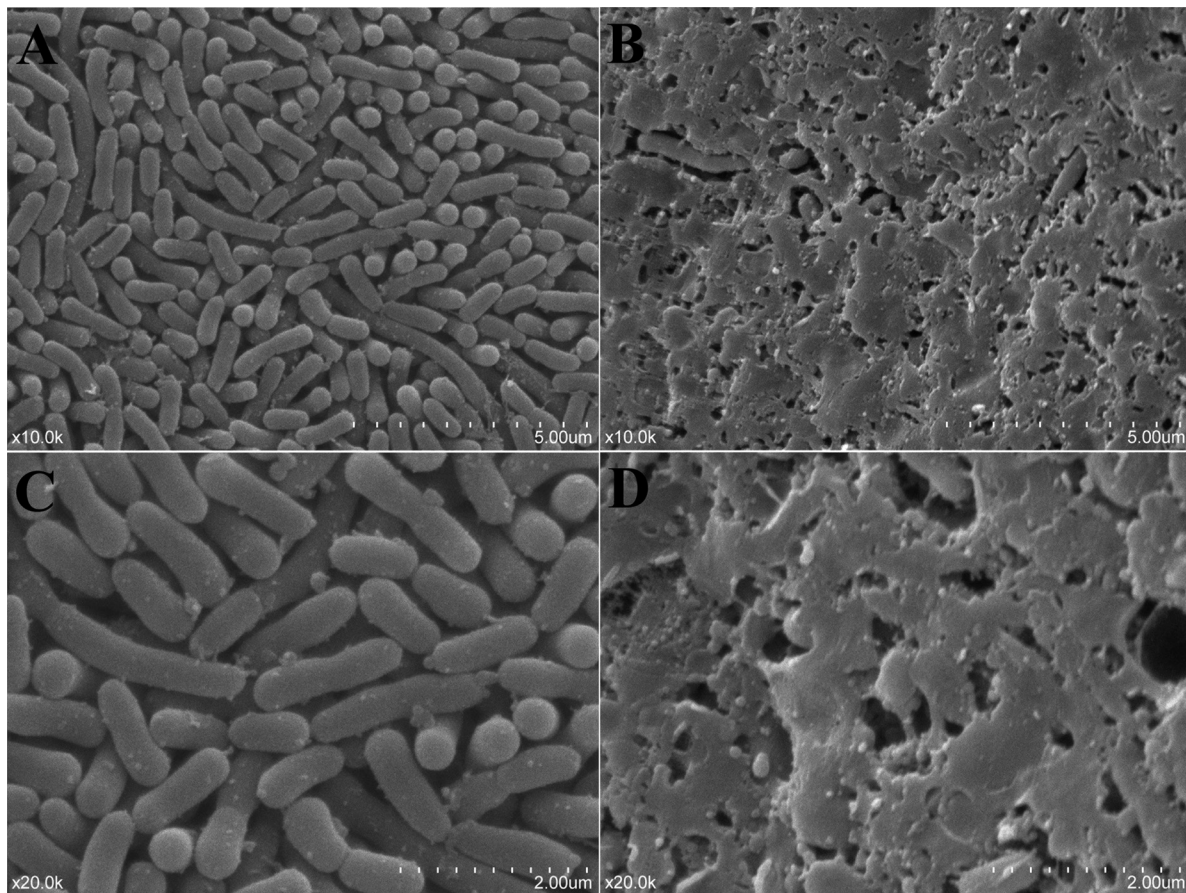


FIG 2 Scanning electron microscopy (SEM) photographs of a stable *O. rhinotracheale* SCV (A and C) and its wild-type isolate (B and D) on SBA after 48 h of incubation. The *O. rhinotracheale* WT isolate showed larger amounts of intercellular substances than did its SCV. Note the pleomorphic and rod-shaped bacteria. Magnification, $\times 10,000$ (A and B) or $\times 20,000$ (C and D).

phases and higher exponential-phase peaks (OD_{600} of 0.844 for WT28). Furthermore, the SCV isolates of both patterns showed extended lag phases, compared with the WT isolates. The plate counting method revealed that SCV13 grew nearly 4 times more slowly than its WT counterpart (the generation time for SCV13 was 205 min, compared to 54 min for WT13). Moreover, *O. rhinotracheale* SCVs and their WT isolates showed high levels of stability on TSB with 5% FBS. It should be noted that the efficacy of the TSB and other enrichment broth media (brain-heart infusion medium and Mueller-Hinton broth) in the enrichment and cultivation of *O. rhinotracheale* isolates was significantly enhanced by providing the broth with both 5% FBS and 2% IsoVitalEx Enrichment (BBL; Becton, Dickinson and Co., Sparks, MD) (data not shown).

Hemagglutination assay. No differences in HA activity between stable *O. rhinotracheale* SCVs and the WT isolates were detected. Furthermore, except for isolate 21 (SCV21 and WT21) with pigeon erythrocytes, all tested *O. rhinotracheale* isolates showed HA activity with the tested erythrocytes. Thus, all five pairs of *O. rhinotracheale* isolates tested demonstrated HA activity with chicken and rabbit erythrocytes.

Antimicrobial susceptibility testing. Similar patterns of antibiotic susceptibility were recorded for the stable *O. rhinotracheale* SCVs and their WT isolates with clindamycin, kanamycin, tetra-

cycline, ampicillin, chloramphenicol, cefotaxime, doxycycline, erythromycin, and penicillin G (data not shown). In contrast, all *O. rhinotracheale* SCV isolates were resistant to norfloxacin, unlike their WT isolates, which were susceptible to that drug. Furthermore, variable differences between some *O. rhinotracheale* SCVs and their WT isolates were recorded for amoxicillin, sulfamethoxazole, clarithromycin, polymyxin B, cefuroxime sodium, and gentamicin. *O. rhinotracheale* SCVs showed higher MIC values than their WT isolates (Table 3).

Autolysis assay. Two patterns were observed for the autolytic activity of the five pairs of *O. rhinotracheale* isolates (Fig. 4A to E). All of the stable *O. rhinotracheale* SCV isolates showed higher autolytic activity, as indicated by a decrease in the OD_{600} of about 53% within 4 h, compared to 25% for the WT isolates.

Intracellular persistence assay. One of the five pairs of *O. rhinotracheale* isolates tested was investigated for intracellular persistence using murine RAW 264.7 macrophages at three time points, i.e., 3, 24, and 24 h (Fig. 5). WT28 showed higher CFU values than did its SCV counterpart after 3 h of incubation, indicating greater bacterial uptake by the macrophages for WT28. In contrast, the next two time points showed higher CFU values for SCV28 than for its WT isolate, which showed noticeable CFU decreases after 48 h of incubation. Furthermore, reversion was observed only in the SCV28 cultures after 24 and 48 h of incuba-

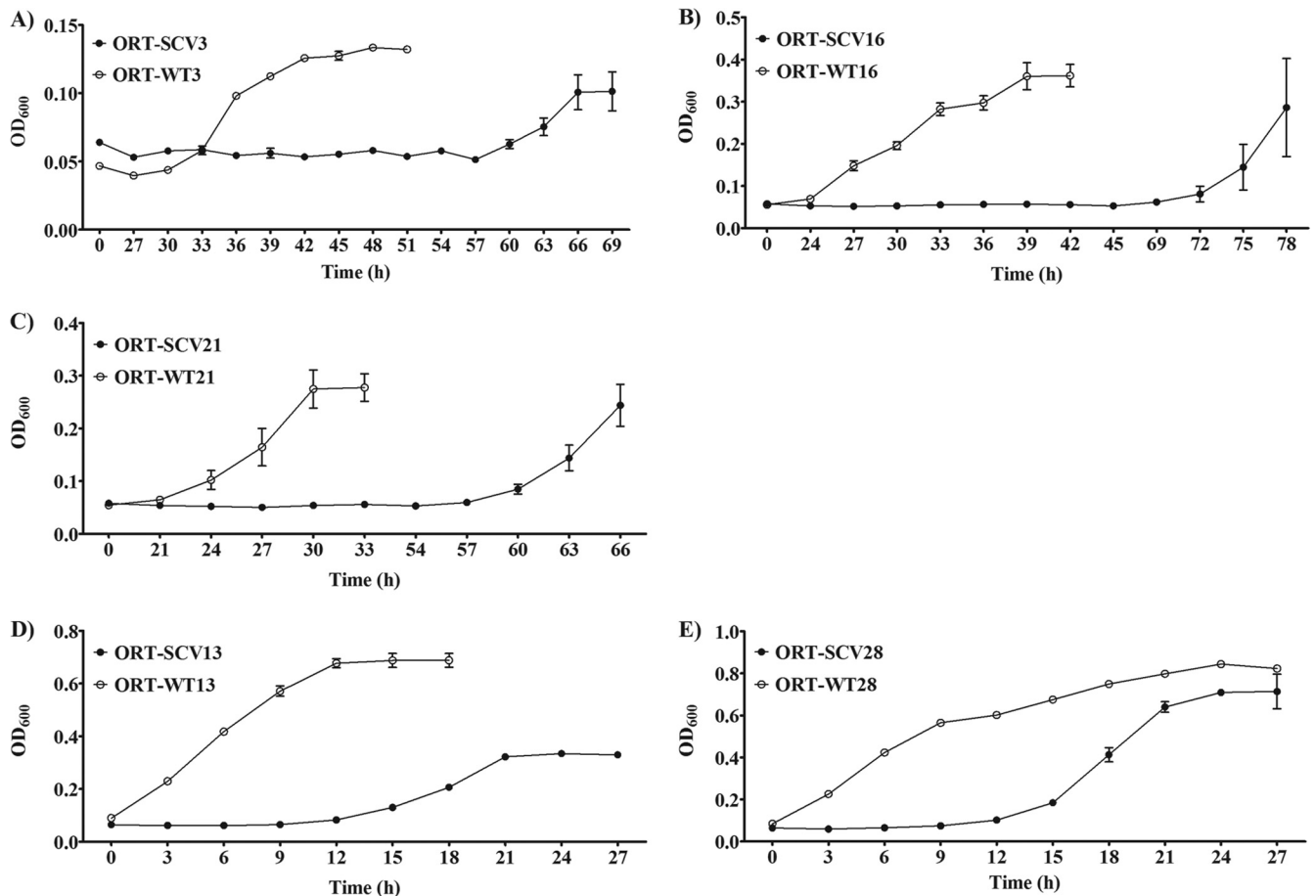


FIG 3 Growth curves for the five stable *O. rhinotracheale* (ORT) SCVs in TSB supplemented with 5% FBS, in comparison with their wild-type isolates.

tion (24.5% and 33% of the enumerated CFU, respectively). Lower switching rates were observed for the WT28 cultures (8.6% of the enumerated CFU after 48 h of incubation).

Phylogenetic analysis. In the entire data set ($n = 27$), 15 haplotypes were identified, and all nucleotide sites were monomorphic for SCV and WT forms of the same isolate. These 15 haplotypes clustered into two main well-supported clades. Clade 1 consisted of 23 isolates that clustered into three subclades and clade 2 was formed by four isolates (Fig. 6). The five stable SCVs did not show a phylogenetic pattern. Data set diversity indices revealed high levels of haplotype diversity in *O. rhinotracheale* ($h = 0.9373$) and low nucleotide diversity ($\pi = 0.00496$). The

average number of nucleotide differences (k) was 6.76923. Phylogeny inferred from 16S rRNA findings showed independent patterns for geographical region and host.

DISCUSSION

In this study, we report for the first time the isolation and characterization of small-colony variants of *O. rhinotracheale* from chickens with clinical signs of respiratory disease. *O. rhinotracheale*, which is considered an endemic pathogen associated with respiratory diseases in domestic poultry, causes significant economic loss in the poultry industry, with worldwide distribution in almost all avian species (16, 17, 19).

TABLE 3 MICs of selected antibiotics for stable *O. rhinotracheale* SCVs and their wild-type isolates

Antibiotic	Antibiotic MICs ($\mu\text{g/ml}$) for the SCV or WT isolate ^a :									
	SCV3	WT3	SCV13	WT13	SCV16	WT16	SCV21	WT21	SCV28	WT28
Amoxicillin	4	4	8	8	8	4	4	4	32	8
Gentamicin	128	128	>256	256	128	64	>256	8	256	256
Sulfamethoxazole	128	128	256	256	>256	128	256	4	256	16
Clarithromycin	32	8	128	16	8	4	64	64	8	8
Polymyxin B	64	64	256	8	64	64	64	8	256	8
Cefuroxime sodium	64	16	16	16	128	8	8	2	64	4
Norfloxacin	32	8	64	16	32	2	32	8	64	2

^a Values are median MICs from three tests.

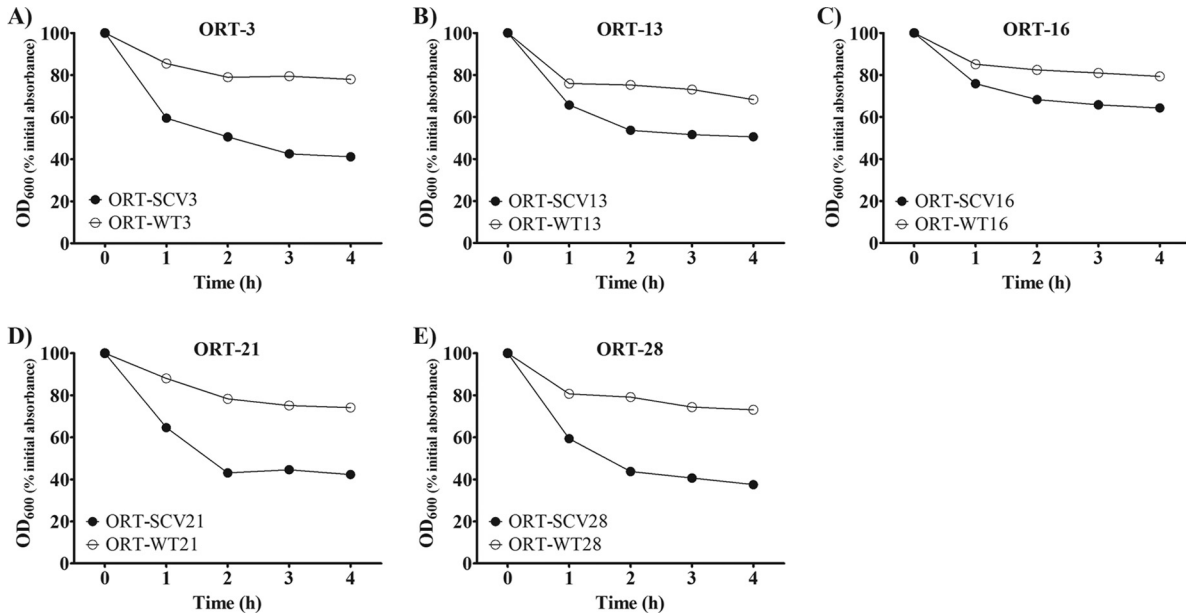


FIG 4 Triton X-100-induced autolysis for all stable *O. rhinotracheale* (ORT) SCVs and their WT isolates. Each test was done three times, and mean results are presented.

All of the *O. rhinotracheale* isolates gave a clear band at 784 bp by using the PCR with *O. rhinotracheale*-specific primers under the recommended conditions, but the primer pair also amplified fragments of *Riemerella anatipestifer* RA37 and RA38 (500 bp), *Riemerella columbina* RC29 (1,200 bp), and *Coenonia anatina* CA39 (200 bp). That finding is in agreement with a previous study that noted that false-positive results were recorded using this set of primers (41). Therefore, it is recommended that the identification of *O. rhinotracheale* be confirmed by sequencing the amplified fragment.

Forming small-colony variants appears to be a natural phenomenon characteristic of the *O. rhinotracheale* isolates; 21 (77.8%) of 27 isolates of *O. rhinotracheale* proved to have SCVs in their primary cultures. A typical *O. rhinotracheale* WT isolate appeared to be very stable and showed uniform colony size on SBA. However, different *O. rhinotracheale* WT isolates showed different colony sizes (Table 2). This is in accordance with a previous study

that reported that different *O. rhinotracheale* isolates had significant differences in their colony sizes (15). In contrast to the WT

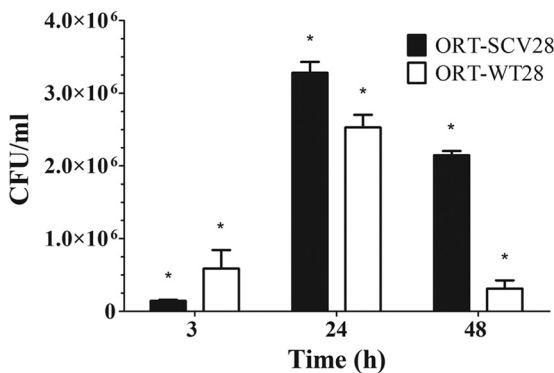


FIG 5 *O. rhinotracheale* (ORT) SCV28 (CFU/ml) recovered from RAW 264.7 cells at three time points, compared with its WT isolate (MOI of 100 CFU/ml). *, significant difference ($P < 0.05$).

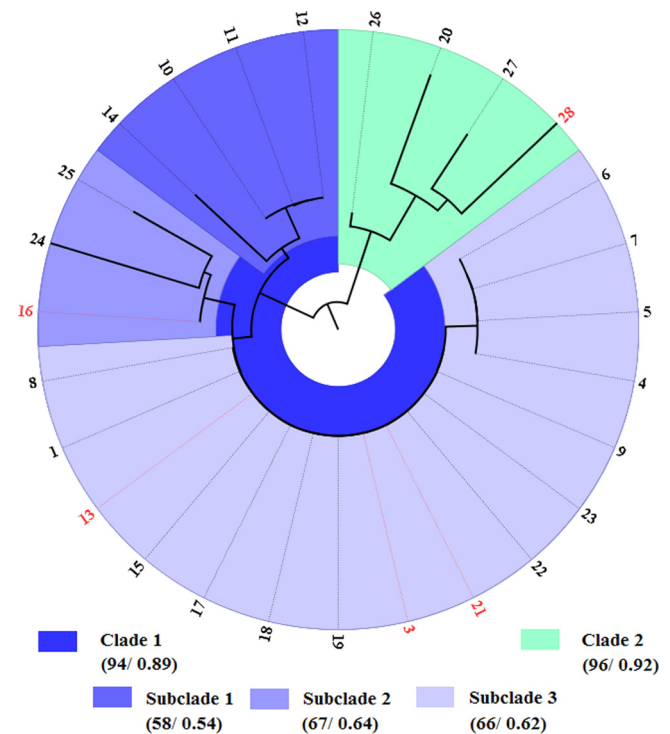


FIG 6 Phylogenetic relationships of the 27 isolates of *Ornithobacterium rhinotracheale* inferred from 16S rRNA gene sequence analysis of a 1,366-bp sequence. The five pairs of *O. rhinotracheale* isolates (stable ORT-SCV isolates and their WT) are marked in red. The first and second values in the key are the bootstrap and Bayesian values, respectively. Branch length, represented by the black lines, corresponds to the number of substitutions.

isolates, the stability of *O. rhinotracheale* SCVs varied greatly, and 16 *O. rhinotracheale* SCV isolates showed partial reversion upon subculturing on SBA. Notably, five *O. rhinotracheale* SCV isolates proved to maintain their phenotype and did not revert in 10 generations. However, subcultures on SBA from the stocks of these five frozen stable *O. rhinotracheale* SCVs revealed partial reversion and were stable again after subculturing on SBA. This is in agreement with a previous study on *Enterococcus faecium* SCVs that reported that reversion of SCVs was observed after freezing and thawing (13).

With respect to the biochemical profiles, tested *O. rhinotracheale* isolates (SCV and WT isolates) showed poor positive rates using API-20NE identification strips, in agreement with previous studies that reported low positive rates for biochemical reactions (15, 41). Two codes were obtained for *O. rhinotracheale* WT isolates (codes 0020004 and 0220004), in accordance with previous studies that reported that these two codes are prevalent and are found for about 99.5% of *O. rhinotracheale* isolates (17, 20, 21). Moreover, it was possible to discriminate between the *O. rhinotracheale* SCVs and their WT isolates by using API-20NE strips, because *O. rhinotracheale* SCVs gave one code (code 0020000). All of the *O. rhinotracheale* SCV isolates tested were oxidase negative. Additionally, WT16 was positive and SCV16 was negative for urease, in accordance with previous studies that reported that SCV bacteria have reductions in biochemical reaction activities (1, 3).

One of the major problems in enriching and testing *O. rhinotracheale* is that isolates do not grow equally on different broth media. Therefore, preparing and optimizing a broth medium that can support the growth of *O. rhinotracheale* isolates should be highlighted (15, 17, 20, 42–44). This can be clearly seen in the results presented; three of the tested *O. rhinotracheale* WT isolates showed longer lag phases and lower exponential-phase peaks than did the other two isolates. Furthermore, *O. rhinotracheale* SCVs showed extremely low growth rates, compared to their WT isolates, and the length of their lag phases correlated with the lag phases of their WT isolates. This is in agreement with studies of other SCV bacteria that indicated that SCVs show low growth rates on broth media, compared to their WT isolates (13, 38).

Supplementing the broth medium with 5% FBS and 2% Iso-VitaleX Enrichment (BBL; Becton, Dickinson and Co., Sparks, MD) proved to have a great impact on the growth rates of *O. rhinotracheale* isolates. Hence, such media can be of great use in future studies on *O. rhinotracheale*. However, the stability of *O. rhinotracheale* isolates (SCVs and WT isolates) was greatly affected with this medium, and high rates of reversion and switching were observed.

The results presented indicate that *O. rhinotracheale* SCV isolates have higher MIC values for antibiotics than do their WT isolates. These findings support previous studies that indicated that SCVs were more resistant to antibiotics than were their WT counterparts (1, 3). However, one study on *Enterococcus faecium* SCVs reported identical antibiotic resistance profiles for the SCVs and their WT isolates (13). Challenges in the treatment of respiratory diseases associated with *O. rhinotracheale* could be partially explained by the ability of *O. rhinotracheale* to exist as SCVs.

The Triton X-100-induced autolysis assay revealed decreases in the autolytic activity of the *O. rhinotracheale* WT isolates, compared to their SCVs (Fig. 4A to E); this could be explained by the accumulation of cell wall material and the abundance of intercellular substances observed in the *O. rhinotracheale* WT isolates,

which covered the bacterial cells and might protect them from the effects of Triton X-100 (Fig. 2A to D). A previous study reported that *S. aureus* SCVs and their WT isolates differed in the amounts of intercellular substances (45). In that study, however, intercellular substances were more abundant in the SCVs.

Previous studies reported that SCVs are phenotypes adapted for intracellular persistence (1, 2, 46, 47). In our study, the results of the intracellular persistence assay indicated that SCV28 had the ability to survive intracellularly for a prolonged time, compared to its WT isolate (Fig. 5). One explanation for the improved intracellular survival of SCVs is their decreased overall metabolism and dampened production of cytotoxins, which may downregulate the induction of cell lysis or apoptosis (1, 48). These data support the hypothesis that the SCV phenotype might be one of the survival strategies that bacteria use for optimal internalization and survival in the host (1).

In contrast to the phenotypic and antibiotic test results, all of the *O. rhinotracheale* SCVs and their *O. rhinotracheale* WT isolates showed identical 16S rRNA sequences. This is in accordance with a previous study on *E. faecalis* SCVs that reported identical 16S rRNA sequences for SCVs and their wild-type isolates (7). This finding indicates that other phylogenetic methods should be used to discriminate between the *O. rhinotracheale* SCVs and their WT isolates.

To the best of our knowledge, this is the first study to report the isolation and characterization of small-colony variants of *Ornithobacterium rhinotracheale*. Low growth rates, negative oxidase results, the presence in mixed cultures with the wild-type phenotype, and the strong tendency to exhibit unstable phenotypes explain why *O. rhinotracheale* SCVs are misidentified and overlooked in routine veterinary laboratory diagnostic procedures. The results presented indicate that *O. rhinotracheale* SCV isolates possess higher resistance to antibiotics than do their wild-type isolates, which emphasizes that successful antibiotic treatment of respiratory diseases associated with *O. rhinotracheale* needs to take into consideration the resistance patterns of *O. rhinotracheale* SCVs. In general, there is little understanding of the pathophysiology of SCVs and their clinical importance in veterinary medicine. Therefore, further studies are needed for better understanding of the pathogenic role and underlying mechanisms of the SCV phenotype. *O. rhinotracheale* can be a model for that purpose, considering the high probability of isolation of SCVs from cultures.

ACKNOWLEDGMENTS

This research was supported by China Ministry of Science and Technology Project 973 (grant 2012CB518702), the China Ministry of Education (grant 313054), and the National Natural Science Foundation of China (grant 31272623).

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