EDTA, 0·04 g L^{-1} thallous acetate, 30 000 units L^{-1} polymyxin and 300 000 units L^{-1} lysozyme. The presence of anthrax spores in the samples was examined.

RESULTS

DNA templates were directly prepared from 1 g of soil artificially contaminated with 1–10^3 spores of *B. anthracis* using a FastDNA SPIN Kit for Soil, and nested PCR was performed. A DNA fragment of the correct size was amplified from the soil samples containing 10^3 spores (Fig. 1a, lanes 6), but not from samples containing less than 10^2 spores (Fig. 1, lanes 2–5). Therefore, to increase the sensitivity of the detection of *B. anthracis* DNA, each 1 g soil sample was cultivated twice in TSB after it was washed with ethanol and DDW. DNA templates were then prepared using the FastPrep™ FP120 instrument. DNA fragments were amplified from the first enrichment cultures with over 10^2 spores but not with 10 spores or less (Fig. 1c, lanes 2–5). However, DNA fragments were amplified from all DNA extracts from the second enrichment cultures by nested PCR (Fig. 1d, lanes 3–5). Soil samples are usually heated at 65–80°C for 30 min to kill nonsporulated bacterial cells, but we found that such heat treatment generated false-positive and unstable products in the PCR (Fig. 1b). We then collected several kinds of soil from different sources, artificially added anthrax spores to them, prepared DNA templates from their second enrichment cultures, and performed nested PCR, and a DNA fragment of the right size was detected in each case (data not shown). Thus, the origin of the soil samples did not affect our ability to detect the anthrax.

As nested PCR and agarose gel electrophoresis take about 4 h, we sought a more rapid and sensitive detection method. DNA templates from the first and second enrichment cultures were subjected to real-time PCR by monitoring the fluorescence signals of specific PCR-amplified products in real time and also by monitoring the melting curves of the PCR products at a target temperature of 95°C using the Light Cycler software version 4·2 (Roche Diagnostics;

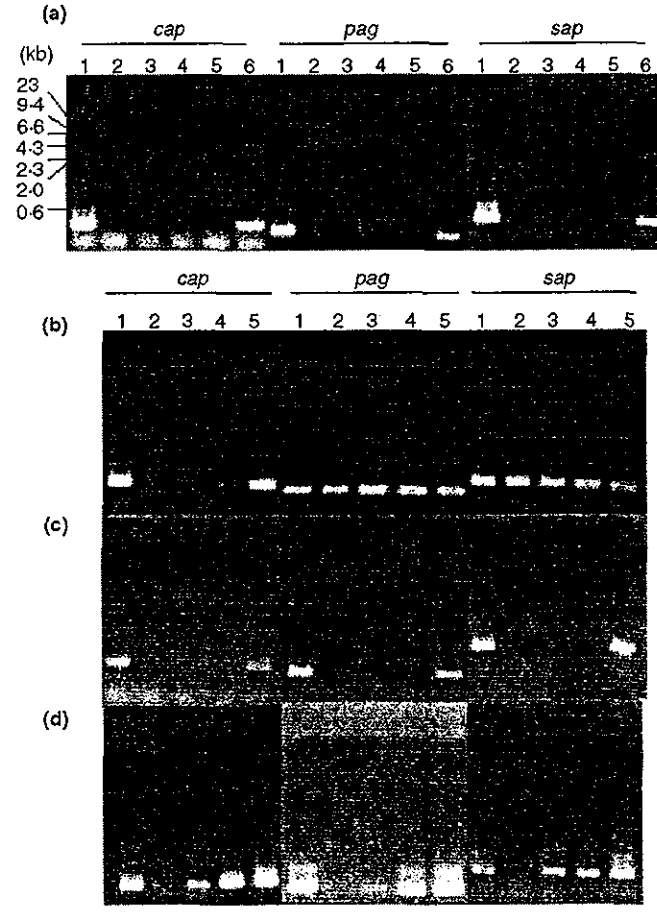

Fig. 1 Detection of anthrax DNA in DNA templates prepared from soil samples using nested PCR. Lane 1, DNA as a positive control; lanes 2–6, soil samples with 0, 1, 10, 10^2, 10^3 spores per 1 g respectively. The molecular sizes of λ *Hin*dIII DNA are shown at left. (a) DNA templates were directly prepared from soil samples, (b) DNA templates were prepared from the second enrichment cultures after heat treatment, (c) and (d) DNA templates were prepared from the first and second enrichment cultures, respectively after ethanol washing

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Tokyo, Japan), which differentiates between signals obtained from specific PCR products and nonspecific DNA fragments such as primer-dimers. In addition, the size of the products was checked by agarose gel electrophoresis. In the real-time PCR, using only the first primer set (Table 1), specific amplifications were detected in all samples from the second enrichment cultures, even those inoculated with only one spore (Fig. 2a). The identifications of the melting peaks and DNA fragments in the electrophoresis were verified by comparison with the peaks obtained from purified DNA (Fig. 2b,c).

This nested PCR and the real-time PCR systems were then used to test nine field soil samples from Mongolia. In one sample (no. 3), a DNA fragment of the correct size was amplified using the *cap* and *sap* primer sets (Fig. 3a,c), and in two samples (nos 3 and 6), a DNA fragment of the correct size was amplified using the *pag* primer set (Fig. 3b), by nested PCR. Simultaneously, the amplification curves obtained by the real-time PCR were essentially identical to the ones obtained by the nested PCR, but, after about 30 cycles, the DNA fragments were amplified (Fig. 3d). However, those amplifications were not specific to the anthrax primer sets, given that only the melting points of the products detected in no. 3 using all the primers and no. 6 using the *pag* primers were identified by comparison with positive controls (Fig. 3e). Anthrax spores could not be isolated from both samples using PLET plates.

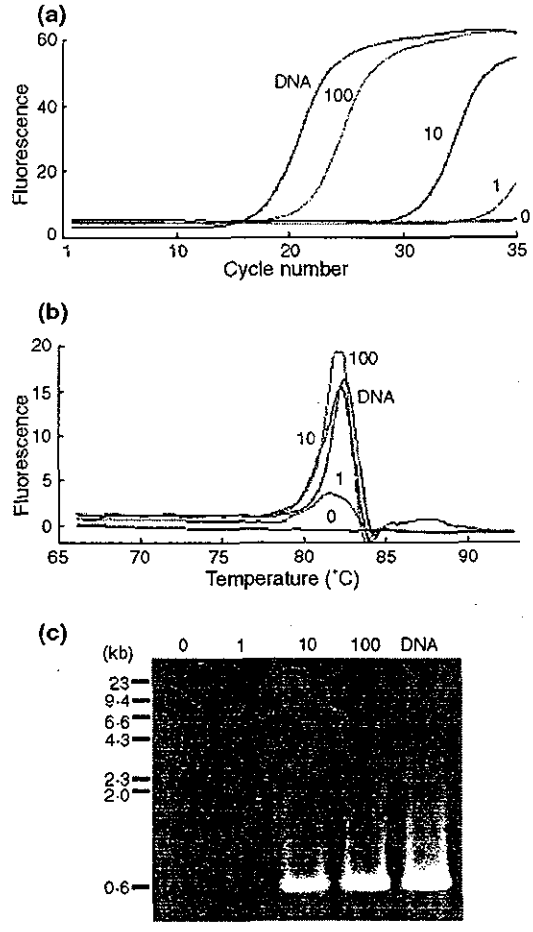

Fig. 2 Detection of anthrax DNA by monitoring the fluorescence levels during PCR amplification (a) and melting curve (b) using real-time PCR, and its agarose gel electrophoresis (c). As a representative experiment, the results using *cap* primers are shown

DISCUSSION

Anthrax is one of the most dangerous zoonotic infectious diseases and has been the first candidate for biological weaponry for over 80 years. Therefore, continuous surveillance for anthrax is essential to prevent its outbreak and minimize its threat. Rapid detection methods for *B. anthracis* from air and meat have already been reported (Cheun *et al.* 2001; Makino *et al.* 2001). However, it is very difficult to detect anthrax DNA from soil because of the presence of humic acid and many other nonsporulated and sporulated bacteria (Tebbe and Vahjen 1993). In this study, the soil samples were washed with ethanol to eliminate vegetative cells. Soil samples are usually heated at 65–80°C for 30 min because spores are highly resistant to heat, but we found that such heat treatment generated false-positive and unstable products in the PCR (Fig. 1b), and was therefore inappropriate for preparing template DNA suitable for PCR. The ethanol washing not only provided stable products but had the likely advantage of removing phospholipids, as well. Although Dragon and Rennie (2001) reported that there was no significant difference in heat and ethanol treatment to purify spore from soil, enrichment culturing of the ethanol-treated soil samples twice in nonselective rich medium would be essential for a PCR method suitable for detecting anthrax DNA (Fig. 1).

In addition, without enrichment culturing, anthrax DNA could be detected by the direct PCR of soil samples containing over 1000 spores, but not of those containing 100 spores or less (Fig. 1a). Furthermore, anthrax DNA could not be amplified even by nested PCR using template DNA from the first enrichment culture of the soil sample with less than 10 spores (Fig. 1c). Anthrax DNA in these samples could be detected sometimes by real-time PCR, but the results were unreliable (data not shown). These results suggested that two enrichments would remove inorganic and organic impurities that inhibit PCR, from the culture. The isolation frequency of enterohaemorrhagic *Escherichia coli* was improved by two enrichments of the faecal samples

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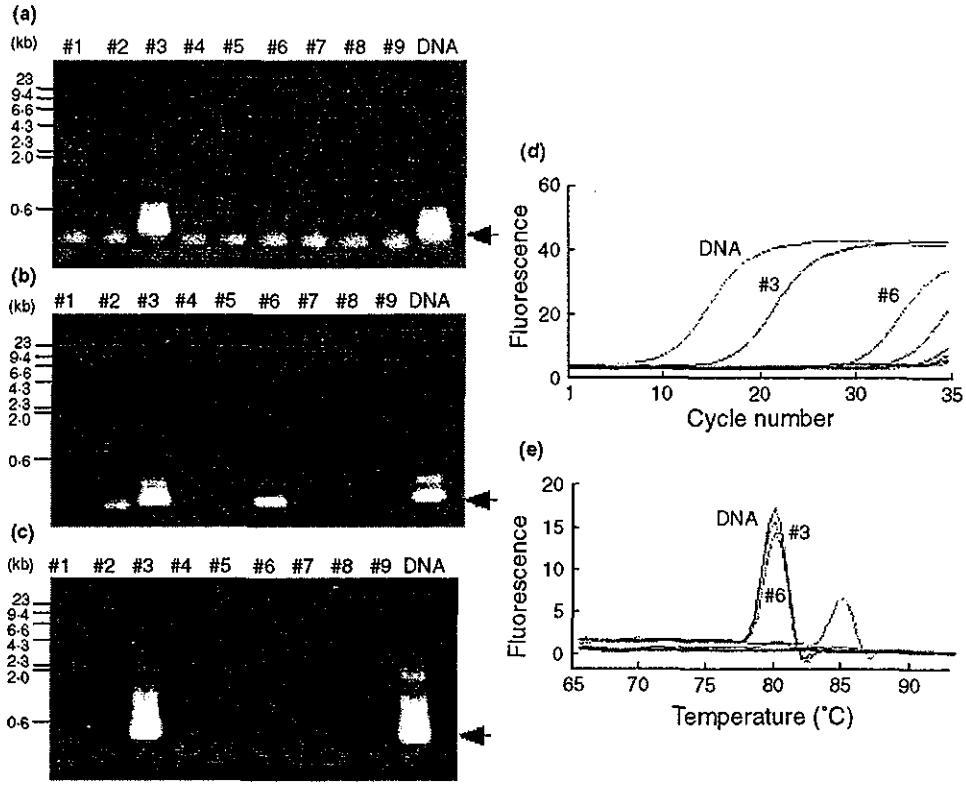

Fig. 3 *Detection of anthrax* DNA in soil samples from Mongolia using nested PCR and real-time PCR. The lane numbers are the sample designations and 'DNA' was a positive control. Arrows indicate the size that should be obtained by PCR. The molecular sizes of *λ HindIII* DNA are shown at the left. In this tests (a) *cap*, (b) *pag* and (c) *sap* primers were used. As a representative experiment, the results of the (d) PCR amplification and (e) melting curves by real-time PCR using *cap* primers are shown, respectively

(Asakura *et al.* 1998). Thus, it was recommend that samples with a small number of microbes should be taken through two enrichment culture steps in nonselective rich broth for their detection by PCR.

Nested PCR using samples that had been taken through two enrichment cultures enabled the highly sensitive detection of anthrax DNA from the soil, but the time required was about 4 h. However, real-time PCR revealed the result within 1 h, raised the detection sensitivity and, furthermore, enabled the detection of anthrax DNA using only one set of primers. These results indicate that real-time PCR is a powerful tool for epidemiological surveillance. Of the soil samples collected in Mongolia, one sample was shown to be positive for all three genes, indicating that the PCR method should be practicable. Another sample was only positive for the *cap* gene. This result does not indicate a weakness in our method, because DNA amplification using *pag* gene primers was rarely detected for some kinds of *B. cereus* (unpublished data) and the virulence genes are known to be lost occasionally in *B. anthracis* (Turnbull *et al.* 1992). Therefore, it is recommended that the three primer sets used in this study should be used for the detection of anthrax cells.

The use of PCR as a highly sensitive detection system has been reported here. However, after PCR screening, it is necessary to isolate the *B. anthracis* cells from the soil. Although *B. anthracis* cells could be isolated from soil containing 10^3–10^4 spores per gram, it was very difficult to isolate the cells when the soil contained a smaller number of cells. The use of a special selective medium may be necessary to isolate cells under these conditions. PLET medium is reported to be selective for *B. anthracis* (Knisely 1966), but it also strongly inhibits the growth of the anthrax cells. In general, 1 g of soil contains about 10^3–10^6 spores of different microbes, meaning that it would be difficult to isolate *B. anthracis* from soil containing one spore per gram, even if the enrichment culture were performed. Future studies to determine some tricks for specifically growing *B. anthracis* should be performed to address this issue.

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NOTES

Enzyme-Linked Immunosorbent Assay To Differentiate the Antibody Responses of Animals Infected with *Brucella* Species from Those of Animals Infected with *Yersinia enterocolitica* O9

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Enzyme-linked immunosorbent assays using antigens extracted from *Brucella abortus* with *n*-lauroylsarcosine differentiated natural *Brucella*-infected animals from *Brucella*-vaccinated or *Yersinia enterocolitica* O9-infected animals. A field trial in Mongolia showed cattle, sheep, goat, reindeer, camel, and human sera without infection could be distinguished from *Brucella*-infected animals by conventional serological tests.

Brucellosis is a worldwide infectious disease of domestic animals, and the causative agent, *Brucella* spp., is transmitted to humans by contact with infected animals or by contaminated dairy products (4). Serodiagnosis of acute and recent infections with *Brucella* and *Yersinia enterocolitica* O9 by using the commonly used microagglutination assay is seriously impaired by the well-documented and strong serological cross-reactivity between these bacteria (2, 6–11, 13, 14). The Rose Bengal test and complement fixation test are the most accepted tests worldwide (5). These tests are based on a reaction between a *Brucella* whole-cell antigen and antibodies produced in response to the infection. Differentiating between animals infected with *Brucella* and animals vaccinated against *Brucella* is too difficult by conventional serological tests, such as the Rose Bengal test, tube agglutination test, and complement fixation test (13), because vaccinated animals have a high titer against *Brucella* antigens. Therefore, we tried to find an easy serological method to differentiate *Brucella*-infected animals from vaccinated or *Y. enterocolitica* O9-infected animals.

To differentiate natural *Brucella*-infected animals from *Y. enterocolitica* O9-infected animals, antigens extracted from the virulent *Brucella abortus* strain 544 (15) with *n*-lauroylsarcosine were used for an enzyme-linked immunosorbent assay (ELISA), and the specificity of the ELISA was tested. The antigens were extracted as follows. *B. abortus* strain 544 cells were grown to A_{600} = 3.0 in brucella broth (Becton Dickinson, Sparks, Md.), and bacterial cells were harvested by centrifugation and washed once with distilled water (DW). For whole bacterial cell antigens, bacteria were inactivated by formalin (0.5% final concentration) and were concentrated to 1.5 (the optical density at 600 nm [OD_{600}]) in DW at this step. For *n*-lauroylsarcosine-extracted antigens, *n*-lauroylsarcosine (0.5% final concentration) was added to the bacterial suspension and the cells were incubated at room temperature for 30 min with shaking. The bacterial suspension was centrifuged and filtrated, and then the supernatant was transferred to a new centrifuge tube for use with the antigens. The protein concentration of antigen was checked by Bio-Rad protein assay, and the antigen was also checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Western blotting was done with anti-*B. abortus* or anti-*Y. enterocolitica* O9 rabbit serum for each preparation. To coat the antigens on Immuno plates for the ELISA, 50 μl of the antigens (sarcosine extracts; 4 μg/ml in DW) was added onto a 96-well Immuno plate (Nunc, Rochester, N.Y.) and left overnight. Then, the wells were blocked by 0.5% bovine serum albumin (BSA) for 30 min. Sera diluted 1/50 to 1/3,200 were applied to the wells. The wells were incubated at 37°C for 1 h and washed, and then horseradish peroxidase-labeled protein G was added. The wells were incubated at 37°C for 1 h and washed, and the substrate *o*-phenylenediamine was added. The absorbance was measured at 492 nm by using an ELISA reader (model 450; Bio-Rad, Hercules, Calif.).

Rabbit serum immunized with *B. abortus* 544 or *Y. enterocolitica* O9 had a strong positive reaction with whole bacterial cell antigens inactivated with formalin, as shown by ELISA. The OD_{492} values were 1.676 ± 0.12 or 1.38 ± 0.14 at 200-fold dilution, respectively (Fig. 1A). Serum immunized with *B. abortus* was also positive with sarcosine extracts, as shown by ELISA (OD_{492} = 1.344 ± 0.12 at 200-fold dilution) (Fig. 1B). In contrast, serum immunized with *Y. enterocolitica O9* was negative with sarcosine extracts as shown by ELISA (OD_{492} = 0.210 ± 0.04 at 200-fold dilution) (Fig. 1B). The competitive indices of 1/200 and 1/400 dilutions of anti-*B. abortus* sera were significantly different (Fig. 1B). Therefore, the 1/200 dilution was used as a single dilution.

To further investigate the serological reactivity of the sarcosine extracts, sera from unvaccinated *Brucella*-infected or noninfected animals, which were judged by conventional sero-

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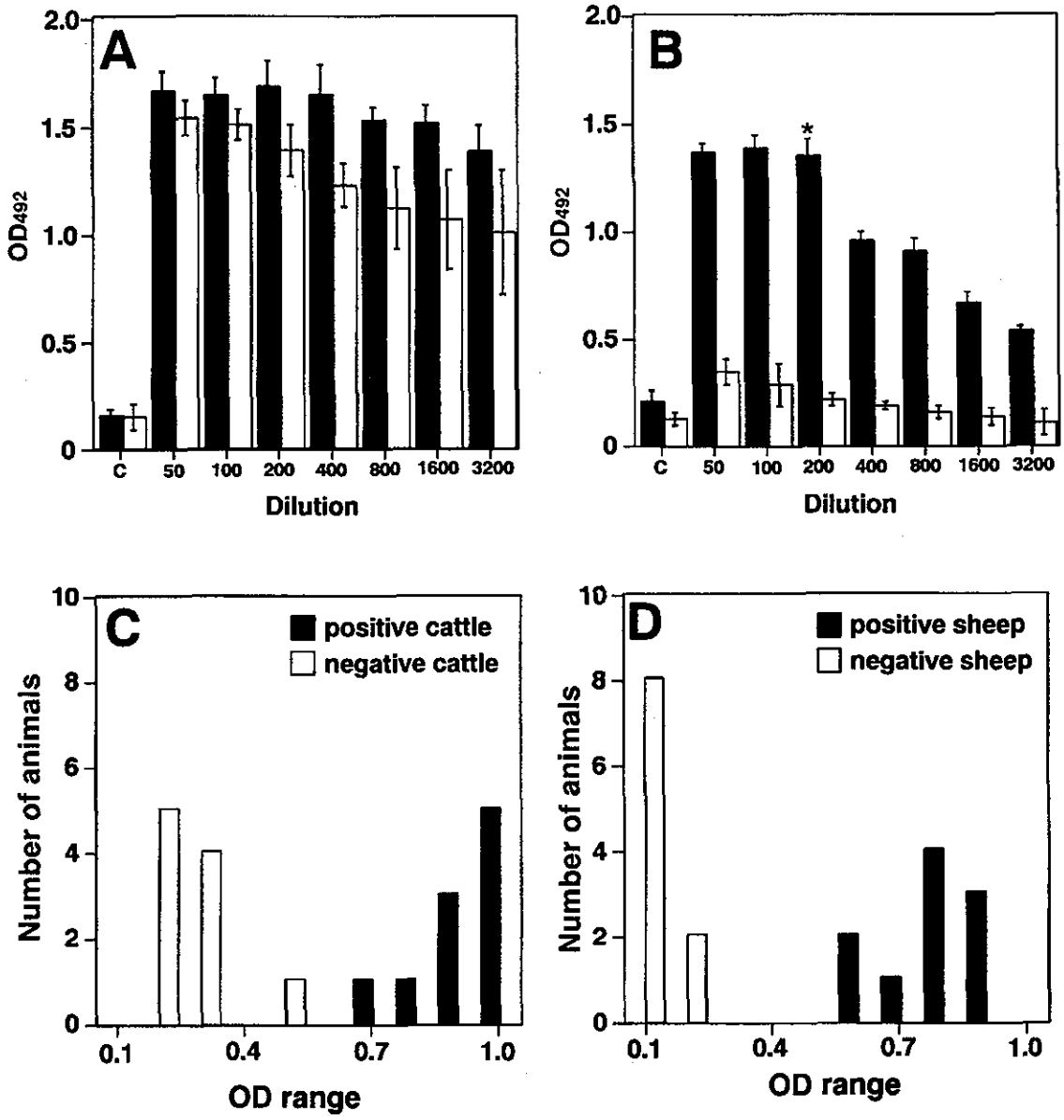

FIG. 1. ELISA absorbance values of sera by using whole bacterial cell antigens or *n*-lauroylsarcosine-extracted antigens. Whole bacterial cell antigens (A) or *n*-lauroylsarcosine-extracted antigens (B) were reacted with rabbit serum immunized with *B. abortus* (black columns) or *Y. enterocolitica* O9 (white columns) at the indicated dilutions. The BSA-coated control well results are in the columns marked "c." Values are averages and standard deviations of triplicate wells from three identical experiments. Three rabbits were immunized with *B. abortus* or *Y. enterocolitica* O9, and their sera showed similar results. Typical data of one rabbit serum are shown. Significant differences between competitive indices of 1/200 and 1/400 dilutions were compared by using the Student *t* test. *, $P < 0.001$. *n*-Lauroylsarcosine-extracted antigens were reacted with sera from 10 *Brucella*-positive (black columns) cattle (C) or sheep (D) and 10 negative (white columns) cattle (C) or sheep (D) at a 1/200 dilution.

logical tests, were tested by using an ELISA. The sarcosine extracts strongly reacted with sera from positive cattle and sheep, but not with sera from negative cattle, except for one serum, or sheep at a single serum dilution (1/200) (Fig. 1C and D). Anti-*B. abortus* or anti-*Y. enterocolitica* O9 rabbit serum at a 1/200 dilution was used as a standard for each assay. These results suggested that the ELISA with sarcosine extracts would be useful for specific detection of *Brucella*-infected animals.

To confirm the serological reactivity of the sarcosine extracts, antigens were tested by silver staining and Western blotting with anti-*B. abortus* or anti-*Y. enterocolitica* O9 rabbit serum at a 1/200 dilution. The 0.5, 1.0, and 1.5% sarcosine extracts reacted strongly with anti-*B. abortus* rabbit serum. The sarcosine extracts also reacted with anti-*Y. enterocolitica* O9 serum, but 0.5% sarcosine extracts showed much less reactivity (Fig. 2B). As the 0.5, 1.0, and 1.5% sarcosine extracts showed similar protein band patterns by silver staining and smear band patterns by Western blotting, the polysaccharide would have reacted with the antiserum (Fig. 2). Presumably, the differences in reactivity against anti-*B. abortus* or anti-*Y. enterocolitica* O9 serum depend on the concentration of polysaccharide in the sarcosine extracts.

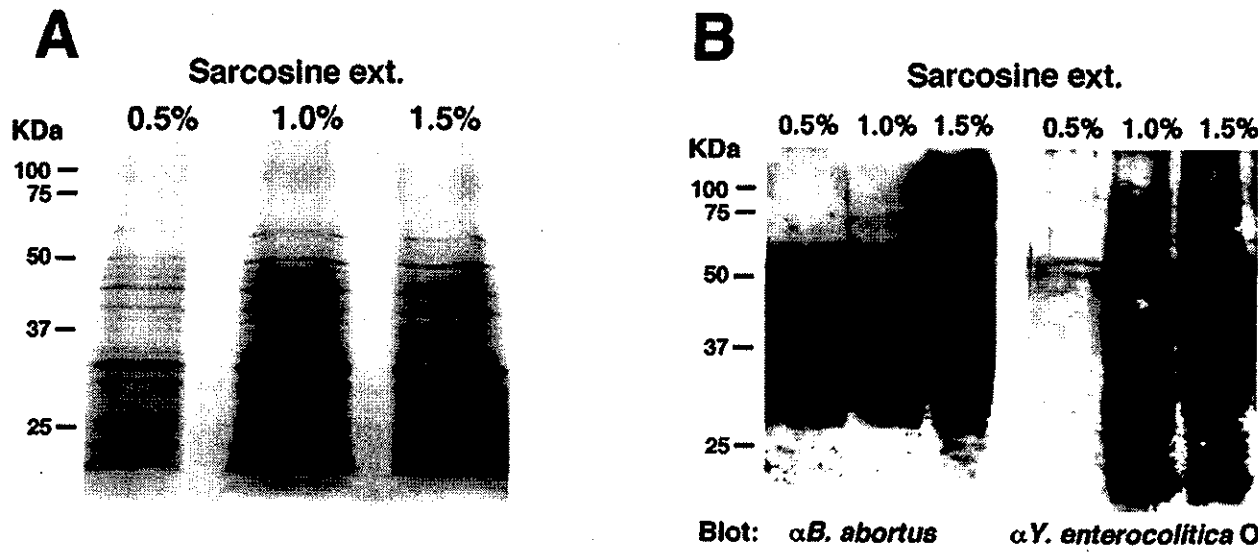

FIG. 2. Analysis of sarcosine extracts of *B. abortus*. Antigens were extracted by 0.5, 1.0, and 1.5% sarcosine and were analyzed by 12% SDS-PAGE and silver staining (A) or by Western blotting with anti-*B. abortus* or anti-*Y. enterocolitica* O9 serum (B).

To investigate if an ELISA with sarcosine extracts can differentiate vaccinated animals from naturally infected animals, sera from vaccinated and infected animals in Mongolia were tested by using an ELISA. Vaccinated animals were usually positive by the Rose Bengal test with 1/40 diluted serum. Forty-fold-diluted sera from vaccinated animals did not exceed an OD_{492} of 0.5, and 1/50- to 1/800-diluted sera from infected animals had OD_{492} values higher than 0.5 (Fig. 3A and B). Therefore, when the absorbance exceeded an OD_{492} of 0.5, the serum sample was judged as positive. Sera from cattle vaccinated with strain S-19 (1), which were positive by conventional serological tests, were negative with sarcosine extracts as shown by ELISA (Fig. 3C). Similar results were obtained with sheep sera vaccinated with strain Rev-1 (1) (Fig. 3D). For both strains, one serum from a vaccinated animal was judged as positive because the absorbance exceeded an OD_{492} of 0.5. But both animals were suspected of having brucellosis, because these animals were kept in *Brucella*-contaminated farms. These results showed that an ELISA with sarcosine extracts is useful for differentiating vaccinated animals from naturally infected animals.

To investigate if the ELISA with sarcosine extracts can be used to diagnose brucellosis, a field trial of the assay was done in Mongolia. Fifty-nine sera from unvaccinated cattle were collected from various places in Mongolia and were tested by using ELISA. Ten of the 59 sera were positive by conventional serological test, and 2 of 59 sera (sample numbers 4 and 26) were negative with sarcosine extracts as shown by ELISA (20% discrimination), suggesting that both cattle might be infected with *Y. enterocolitica* O9 (Fig. 4). Although four sera (serum numbers 18, 23, 40, and 43) were negative by conventional serological tests but were positive using sarcosine extracts by ELISA, they were not infected with *Brucella* and would have an unknown antibody that cross-reacted with sarcosine extracts. Other sera were negative by both ELISA and conventional serological tests (Fig. 4). Serological cross-reactions between *Brucella* species and organisms of other genera have been reported (3), including cross-reactions with *Pasteurella* species, *Salmonella* serotypes including *Salmonella enterica* serotype Urbana and *S. enterica* serotype Pullorum, *Francisella tularensis*, and *EsAcherichia coli* O:157. In Mongolia, *Y. enterocolitica* O9, *F. tularensis*, and *Salmonella* contaminations are a problem. The strongest cross-reaction is with *Y. enterocolitica* O9, which is the most important problem. As *F. tularensis* and *Salmonella* are partially cross-reactive with *Brucella*, sera of agglutination tests that are negative and of ELISA that are positive may react with these pathogens. In this study, by using an ELISA with sarcosine extracts, the cutoff absorbance value of 0.5 (OD_{492}) for brucellosis produced a sensitivity and specificity (serodiagnostic indices) of 66.7 and 92.2%, respectively. However, when sera were checked by the Rose Bengal test and then positive sera were assayed by ELISA, the sensitivity and specificity increased to 80 and 100%, respectively. Thus, ELISA with sarcosine extracts will be better when used together with the conventional serological tests.

We also investigated 162 sheep, 95 goat, 20 reindeer, 17 camel, and 29 human sera in Mongolia (Table 1). To eliminate

TABLE 1. Serological tests of domestic animals and humans in Mongolia

Animal	No. of animals tested	% of RBT-positive animals[a]	% ELISA positive in RBT-positive animals[b]	% Elimination[c]
Sheep	162	35.2	82.5	17.5
Goat	95	34.7	72.7	27.3
Reindeer	20	15.0	100	0
Camel	17	23.5	100	0
Human	29	44.8	53.9	46.1

[a] RBT, Rose Bengal test.
[b] RBT-positive sera were tested by ELISA with sarcosine extracts.
[c] Percent elimination represents percentage of animals that were negative for *Brucella* infection in RBT-positive animals by using ELISA. The percentage of elimination was determined as the percentage of ELISA positives in RBT-positive animals subtracted from 100% (RBT-positive animals).

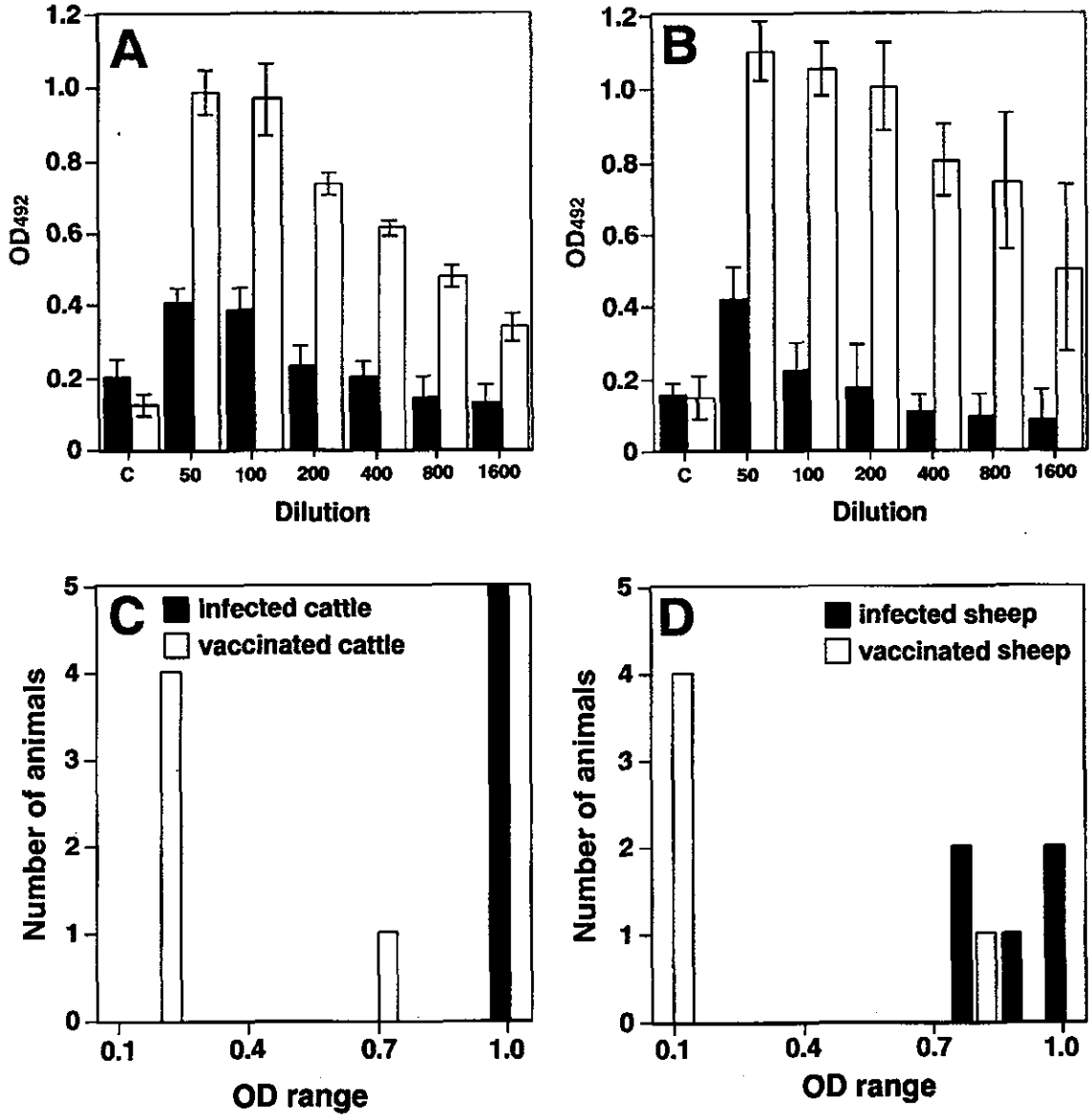

FIG. 3. ELISA absorbance values of sera from vaccinated or infected animals in Mongolia by using *n*-lauroylsarcosine-extracted antigens. Sera from vaccinated (black columns) cattle (A) or sheep (B) and infected (white columns) cattle (A) or sheep (B) were tested at the indicated dilution. The BSA-coated control well results are in the columns marked "c." Sera from three vaccinated or infected animals showed similar results. Typical data of one animal serum are shown. Values are averages and standard deviations of triplicate wells from three identical experiments. Sera from five infected (black columns) cattle (C) or sheep (D) and vaccinated (white columns) cattle (C) or sheep (D) were tested at a 1/200 dilution.

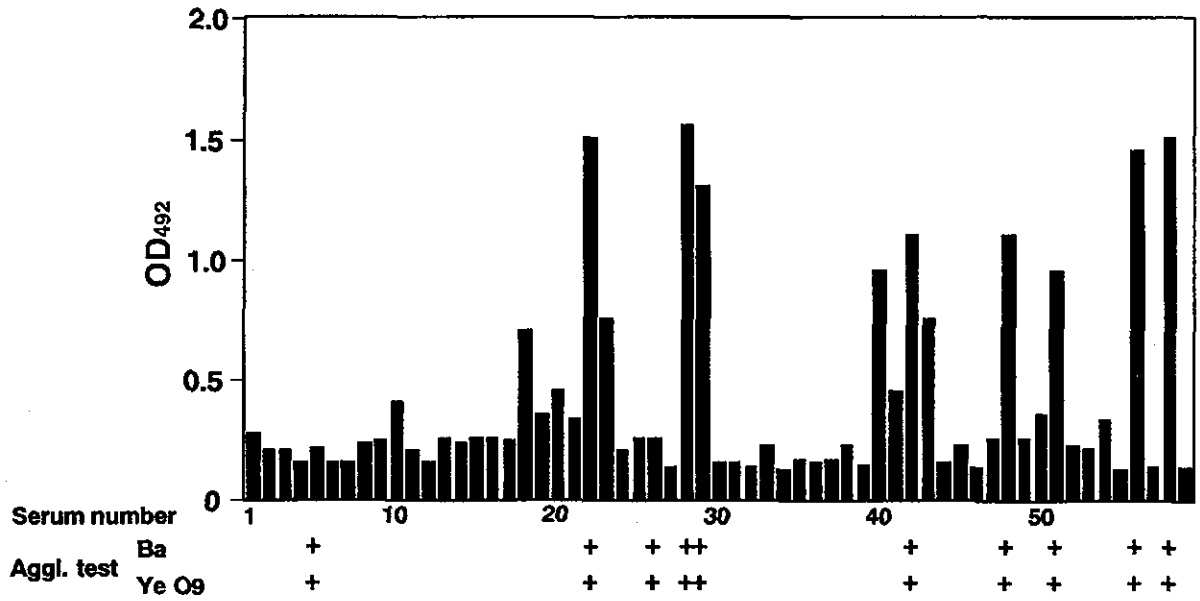

FIG. 4. ELISA absorbance values of sera from nonvaccinated cattle in Mongolia by using *n*-lauroylsarcosine-extracted antigens. Fifty-nine sera from nonvaccinated cattle were tested at a 1/200 dilution. Positive reactions by the tube agglutination test with *B. abortus* antigen (Ba) or *Y. enterocolitica* O9 antigen (Ye O9) are shown in columns marked with a "+."

Y. enterocolitica O9-infected animals from those with suspected brucellosis, the Rose Bengal test-positive sera were tested by ELISA with sarcosine extracts. The results showed that 17.5% of sheep, 27.3% of goat, 0% of reindeer, 0% of camel, and 46.1% of human sera were differentiated from suspected *Brucella*-infected animals by conventional serological tests (Table 1).

The agar gel immunodiffusion test that uses polysaccharide antigen differentiates infected and vaccinated cattle (12), but it does not differentiate *Brucella*-infected animals from *Y. enterocolitica* O9-infected animals. We believe our study is the first that uses an ELISA with sarcosine extracts to differentiate *Brucella*-infected animals from *Y. enterocolitica* O9-infected animals. The ELISA with sarcosine extracts in this study is an easier method than other conventional serological tests, and the ELISA can be done within 2 h after coating the antigen. Therefore, the ELISA with sarcosine extracts can be used to diagnose brucellosis and to identify *Brucella*-infected animals by using it together with other conventional serological tests.

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Need to Differentiate Lethal Toxin-Producing Strains of *Burkholderia gladioli*, Which Cause Severe Food Poisoning: Description of *B. gladioli* Pathovar *cocovenenans* and an Emended Description of *B. gladioli*

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***Abstract*: *Burkholderia cocovenenans* produces a lethal toxin (Bongkrekic acid) that leads to high fatality in food poisoning cases. However, *B. cocovenenans* was combined in *Burkholderia gladioli* in 1999. *B. gladioli* was originally described as a phytopathogenic bacteria that sometimes causes pneumonia in humans and that acts as an opportunistic pathogen. We thought that it was clinically dangerous to describe these two species without considering their pathogenicities. From our data of 16S rRNA sequence analysis, DNA-DNA hybridization, and fatty acid analysis, we could confirm that *B. cocovenenans* and *B. gladioli* should be categorized as a single species. However the species really weaved lethal toxin-producing strains with non-lethal strains. To emphasize that *B. gladioli* contains two different pathogens, we describe a new pathovar, *B. gladioli* pathovar *cocovenenans*, for the lethal toxin-producing strains. We provide characteristics that differentiate this lethal toxin-producing pathovar from other phytopathogenic pathovars within *B. gladioli*, together with an emended description of *B. gladioli*.**

***Key words*: *Burkholderia cocovenenans*, *Burkholderia gladioli*, Food poisoning, Bongkrekic acid**

Burkholderia gladioli, formerly known as *Pseudomonas gladioli* (18) and *Pseudomonas marginata* (14), was first described as a phytopathogenic bacteria. In 1970, Ballard et al. (1) studied DNA-DNA hybridization and phenotypic characteristics of *Pseudomonas alliicola* and *P. marginata* and proposed that the former should be regarded as a junior-synonym. Hildebrand et al. (8) corrected the previous description of *P. marginata* and found it was identical to the original description of *P. gladioli*; thus, they proposed that the two names be synonymous with *P. gladioli*. Yabuuchi et al. (23) then proposed that the seven species of Pseudomonas RNA homology group 2, which included *P. gladioli*, be assigned to a new genus, *Burkholderia*, on the basis of a polyphasic taxonomic study (summarized taxonomic changes are shown in Fig. 1).

Burkholderia gladioli has three pathovars and all pathovars are recognized as mainly phytopathogenic bacteria: *B. gladioli* pathovar *gladioli*, which causes gladiolus rot (8, 23); *B. gladioli* pathovar *alliicola*, which causes onion bulb rot (24); and *B. gladioli* pathovar *agaricicola*, which causes rapid soft rot of cultivated mushrooms (10).

Pseudomonas cocovenenans was first described by vanDamme et al. (21) as a food-poisoning bacterium. It produces two highly toxic compounds: toxoflavin and bongkrek acid (BA). In the 1970's, a high fatality rate (more than 40%) of food poisoning cases caused by the

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Abbreviations: BA, Bongkrekic acid; FAME, fatty acid methyl ester; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; 16S rRNA, 16S ribosomal RNA; SSC, saline sodium citrate solution.

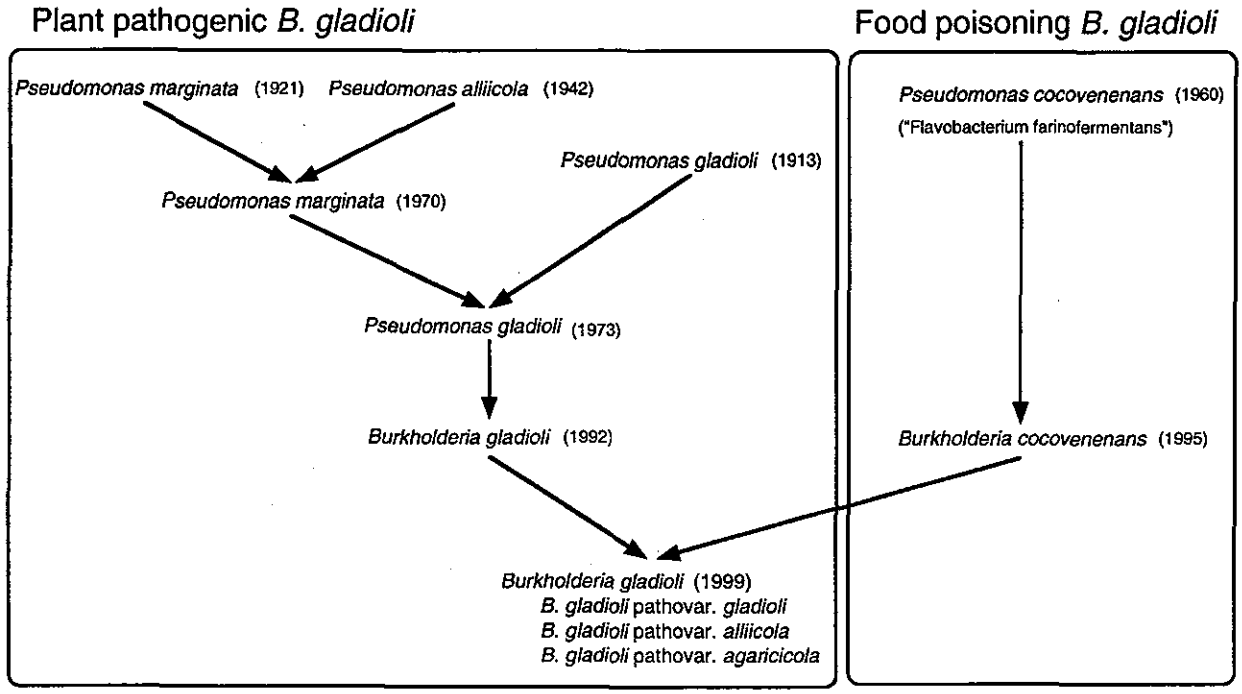

Fig. 1. Taxonomic changes of *Burkholderia gladioli* and related species. The numbers in parentheses are the valid publication year.

ingestion of fermented corn or deteriorated *Tremella faciformis* (white fungi) was occasionally reported in the northeastern and southwestern regions of the People's Republic of China (15). Meng et al. demonstrated that the food poisoning was caused by a yellow pigmented gram-negative rod-shaped bacteria, temporarily termed "*Flavobacterium farinofermentans*" (16). It was later shown that "*F. farinofermentans*" was identical to *Pseudomonas cocovenenans* (25). In 1995, Gillis et al. (7) transferred *P. cocovenenans* to the genus *Burkholderia* as *B. cocovenenans*. This assignment was then verified by Zhao et al. (26) on the basis of partial 16S rRNA sequences.

In 1999, Coenye et al. (3) investigated the relationship between *B. gladioli* and *B. cocovenenans* with whole-cell protein assays and DNA-DNA hybridization and concluded that the latter was a junior synonym of the former. However, in that study, they used only one *B. gladioli* pathovar and failed to consider the pathogenicity.

In the present study, a polyphasic taxonomic study, including a genetic, chemotaxonomic, and phenotypic approach, was performed and the relationship between *B. gladioli* and *B. cocovenenans* was reevaluated using the reference strains for all of the pathovars together with our isolates, which were collected from Chinese food-poisoning cases. Here, we describe a new pathovar, *B. gladioli* pathovar *cocovenenans*, for the lethal toxin-producing strains and provide differential characteristics of the food-poisoning pathovar from phytopathogenic pathovars. We also provide an emended description of *B. gladioli*.

Materials and Methods

Bacterial strains. Twenty-two strains were tested, including 7 strains of *B. cocovenenans* (one reference strain and six isolates from Chinese food-poisoning cases), the reference strains for each of the three pathovars of *B. gladioli*, and the type strains of 12 other species in the genus *Burkholderia* (Table 1).

All strains were maintained in 25% (v/v) glycerol at −80 C, and cultivated on agar plates or in broth (Luria Broth or trypticase soy medium) unless indicated otherwise.

Biochemical characteristics. The BiOLOG GN2 plate was used according to the manufacturer's recommendations. Results were read automatically with a spectrophotometer after 24 hr incubation at 35 C under aerobic conditions. The test was run in duplicate to confirm reproducibility.

BA toxin detection and toxicity tests were done by the methods of Hu et al. (9). Briefly, the crude BA toxin was produced on a semisolid potato-dextrose agar plate at 26 C for 5 days. The plate was heated at 100 C for 30 min, and then placed in a freezer (−20 C) overnight. After thawing, the liquid fraction was collected and concentrated 5-fold with a rotary evaporator. This crude BA toxin solution was orally administered to mice (0.4 ml/mouse). The animals were observed for up to 2 hr.

MIDI-FAME. The MIDI-FAME technique is based on

Table 1. Bacterial strains

Burkholderia	Source	Isolated	Accession No.
B. gladioli pathovar *gladioli* NCPPB 1891^T	Gladioli sp.	1921	X67038
B. gladioli pathovar *alliicola* NCPPB 947	Onion		
B. gladioli pathovar *agaricicola* NCPPB 3580	Mushroom		
B. cocovenenans ATCC 33664^T	Fermented coconut	1960	U96934
B. cepacia ATCC 25416^T	Sour onion skin	1981	U96927
B. andropogonis ATCC 23061^T	Sorghum	1911	X67037
B. cariophylli ATCC 25418^T	Carnation	1942	X67039
B. plantarii JCM 5492^T	Rice pathogen	1987	U96933
B. vandii JCM 7957^T	Orchid rhizosphere	1994	U96932
B. vienamiensis LMG 10929^T	Rice rhizosphere	1995	U96928
B. pyrrocinia ATCC 15958^T	Soil	1965	U96930
B. mallei ATCC 23344^T	Human	1885	AF110188
B. pseudomallei ATCC 23343^T	Human	1913	U91839
B. thailandensis BSU 91838^T	Soil	1988	U91838
B. glathei ATCC 29195^T	Soil	1975	Y17052
B. phenazinium LMG 2247^T	Soil	1973	U96936
B. cocovenenans			
HN2y (=GTC 1080)	Tremella	1989	AB012916
Co14 (=GTC 1081)	Fermented corn	1977	AB013111
Sx8801(=GTC 1087)	Bean-starch noodles	1987	AB023647
1A (=GTC 1085)	Tremella	1988	
Co18 (=GTC 1084)	Fermented corn	1979	
90-3 (=GTC 1086)	Fermented corn	1990	AB023646

T, type strain.

ATCC: American Type Culture Collection, Rockville, Md., U.S.A. NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, U.K. NCPPB: National Collection of Plant Pathogenic Bacteria, Haependen, U.K. JCM: Japan Collection of Microbiology, Tokyo, Japan. GTC: Gifu Type Culture Collection, Gifu, Japan. LMG: Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, Gent, Belgium.

the conversion of fatty acids to their methyl esters by mild alkaline methanolysis followed by GLC analysis.

Isolates were cultured on trypticase soy agar overnight at 37 C. Cells were carefully scraped from the plate with a plastic inoculating loop to avoid including media in the samples. Cells were transferred to 8 ml glass tubes, mixed with 1 ml methanol base (45 g NaOH, 150 ml methanol, 150 ml distilled water), vortexed for 5–10 sec, and then heated to 100 C for 5 min. After vortexing again, tubes were heated at 100 C for 25 min. Cells were then methylated as follows: after cooling in water to room temperature, 2 ml of methylation reagent (325 ml 6 M hydrochloric acid, 275 ml methanol) was added and the sample was vortexed for 5–10 sec, heated at 80 C for 10 min, and then rapidly cooled in ice water. Fatty acid methyl esters were extracted by the addition of 1.25 ml of a mixture of hexane and diethyl ether (2:1 (v/v), HPLC grade), followed by mixing up and down for 10 min, and then discarding the aqueous phase. Three ml of NaOH (0.3 M) was added, and the combination was mixed prior to centrifugation. The upper phase was carefully removed and used for analysis.

Analysis was carried out with a Hewlett Packard HP 6980 Gas Chromatograph equipped with a phenyl methyl silicone fused silica capillary column (HP Ultra 25 m-0.2 mm-0.33 mm film thickness) and a flame ionization detector. Hydrogen was used as the carrier gas. The temperature program was initiated at 170 C and increased at 5 C/min to 260 C and then increased at 4 C/min to 310 C.

DNA base composition and DNA hybridization. Genomic DNA was extracted as described previously (11) with a minor modification.

The G+C mol% content of the DNA was determined by the thermal denaturation method of Marmur and Doty (12) with Beckman's nuclease and protein analyzer (Beckman Instruments Inc., Calif., U.S.A.). *Escherichia coli* K12 strain was used as the reference.

Quantitative microplate DNA-DNA hybridization was carried out according to previously described methods (5). Hybridization experiments were carried out at 35 C (optimal condition) and 45 C (stringent condition) using 2×SSC and 50% formamide.

16S rRNA gene sequencing. The 16S rRNA gene was amplified by PCR with universal primers. Sequences were determined with the dye terminator method and an automatic sequencer (Model 373A, Applied Biosystems, Inc., Foster City, Calif., U.S.A.). The 16S rRNA

Table 2. Differential characteristics among pathovars of *B. gladioli*

Characteristics	*B. gladioli*			
	Pathovar *gladioli* NCPPB 1891	Pathovar *agaricicola* NCPPB 3580	Pathovar *alliicola* NCPPB 947	Pathovar *cocovenenans* ATCC 33664 and 6 strains
Source	Gladiolus	Mushroom	Onion	Coconut, fermented corn
Bangkrekic acid production	−	−	−	100% (7/7)*
Growth at 41 C	+	−	+	100% (7/7)
Growth at 4 C	+	+	−	0% (0/7)
Nitrate reduction	−	−	+	100% (7/7)
Assimilation				
Xylitol	−	−	−	100% (7/7)
Gentiobiose	+	+	+	0% (0/7)
N-Acetyl-D-galactosamine	+	+	+	0% (0/7)
Acetic acid	+	−	+	86% (6/7)
L-Pyroglutamic acid	−	+	+	100% (7/7)
Maltose	−	+	−	0% (0/7)
D-Raffinose	−	−	+	0% (0/7)
Sucrose	−	−	+	0% (0/7)
Glycyl-L-aspartic acid	−	−	+	0% (0/7)

* % of positive strains.
+: Positive or weak positive reaction, −: negative reaction.

sequence from position 145 to position 1435 (*E. coli* numbering) was determined for each organism.

Phylogenetic analyses of 16S rRNA sequences. Sequences of 16S rRNA genes for comparative analyses were obtained from DDBJ, GenBank, and EMBL databases. The CLUSTAL-W software originally described by Thompson et al. (19) was used to align the sequences, and the phylogenetic distances were calculated using the neighbor-joining (NJ) method. The phylogenetic tree was drawn using TreeView software (17). The stability of the relation was estimated by bootstrap analysis (6).

Nucleotide sequence accession numbers. Nucleotide sequences that we determined have been deposited in the DDBJ Data library under the following accession numbers: AB012916 for HN2y strains, AB013111 for Co14 strain, AB023646 for 90-3 strain, and AB023647 for Sx8801 strain.

Results

Phenotype Characteristics

Phenotypic comparisons between our isolates, the reference strain for *B. cocovenenans*, and the reference strains of the three pathovars of *B. gladioli* were based on the oxidation of 95 carbon substrates. The reproducibility of the assays was confirmed because results were identical each time the assays were performed. The biochemical reaction patterns of our six isolates and of the reference strain of *B. cocovenenans* were similar to those of the reference strains for *B. gladioli*. Because *B. gladioli* was not included in the BiOLOG database (version 4.01C), all strains were actually identified as *B. cocovenenans*. However, the results of the assimilation tests of xylitol and *N*-acetyl-D-galactosamine differed between *B. cocovenenans* and *B. gladioli*. Some other tests also seemed to be useful for differentiating pathovars within *B. gladioli* (data summary is shown in Table 2).

Isolation of BA and toxicity tests were also performed. BA toxin from the reference strain and six Chinese isolates of *B. cocovenenans* was detected. The toxicity was confirmed by administration to mice. Within 45 min after oral administration of the *B. cocovenenans* culture supernatant, all mice died with a stiff tail and feet (Fig. 2). In contrast, we failed to detect BA toxin from the reference strains for three pathovars of *B. gladioli*. Mice orally given these culture supernatants survived.

Fatty Acid Composition

The cellular fatty acid compositions of *B. gladioli*, *B. cocovenenans*, and isolated strains are shown in Table 3. All contained C14:0, Mix3, Mix4, C16:0, C17:0 cyclo, C16:0-3OH, and Mix7. The major components were Mix4, C16:0, and Mix7, which accounted for 68.9% to 81.9% of the total fatty acids in *B. gladioli* and *B. cocovenenans*. All strains had similar fatty acid profiles and were identified as *B. gladioli*, except two isolates (strain HN2y and Sx8801), which were more similar to *B. cocovenenans* than to *B. gladioli*.

DNA Base Composition and DNA-DNA Hybridization

The G+C mol% of the strains analyzed in this study were from 66.8 to 68.2 (Table 4). The average G+C

Fig. 2. Mice toxicity of toxins produced by *B. gladioli* pathovar *cocovenenans*. The culture supernatant of *B. gladioli* pathovar *cocovenenans* GTC 1080 was administered orally to mice. The mice died within 45 min with stiff tails and feet.

mol% of the type strain and reference strains of the other two pathovars and *B. cocovenerans* were 67.5, 68.0, 68.2, and 67.1%, respectively. Our isolates showed 66.8 to 67.8 G+C mol%.

DNA-DNA hybridization similarity values between the *B. gladioli* type strain, other reference strains, and our isolates were 81 to 93% (Table 4). The similarity values among the three pathovars of *B. gladioli* were 81 to 94%, whereas the values between the type strain of *B. gladioli* or *B. cocovenenans* and of *B. cepacia* were 36 to 46%, and 28 to 34%, respectively.

Phylogenetic Analysis

The 16S rRNA sequences were determined for strains HN2y, Co14, Sx8801, and I-90-3. Sequences were aligned and compared with those of the type strain of *B. gladioli*, *B. cocovenenans*, and other species in the genus *Burkholderia*. Phylogenetic trees were constructed with the neighbor-joining method rooted with *E. coli* (Fig. 3).

The type strain of *B. gladioli* and the reference strain of *B. cocovenenans* appeared to be closely related and showed 99.8% sequence homology value (Table 5). Our isolates, HN2y, Co14, I-90-3, and Sx8801, were closely related to each other and to the reference strains of *B. gladioli* and *B. cocovenenans* (greater than 99.3%).

On the phylogenetic tree, the type strain of *B. gladioli*, the reference strain of *B. cocovenenans* and our isolates made one cluster. *B. plantarii* and *B. glumae* also showed high homology values with *B. gladioli* and *B. cocovenenans* (greater than 99.1%, Table 5), but the phylogenetic tree indicated that they formed another cluster (Fig. 3).

Discussion

On the phylogenetic tree based on 16S rRNA gene sequences, our four isolates formed a single cluster together with the type strain of *B. gladioli* and the reference strain of *B. cocovenenans*. The branching order of our phylogenetic tree was similar to that of a previously reported tree (4). The sequence homology values among most of these strains were greater than 99.0%, indicating that these strains were genetically very close.

The level of DNA-DNA similarity values between the type strain of *B. gladioli* and the reference strain of *B. cocovenenans* were determined as 80 to 96% in our experiment. These findings support the proposal by Coenye et al. that *B. gladioli* and *B. cocovenenans* are

Table 3. The components of fatty acid and FAME identification results of the studied strains

FAME	*B. gladioli* pathovar:			*B. cocovenenans*					
	gladioli	*alliicola*	*agaricicola*	ATCC 33664	HN2y	Co14	Sx8801	90-3	Co18
C10:0, 3OH	ND	0.31	0.53	ND	ND	ND	ND	ND	ND
C14:0	3.94	3.7	3.82	4.32	4.25	4.05	3.88	3.92	3.92
Mix3	5.3	4.59	4.99	5.42	5.41	4.73	4.58	4.62	4.95
Mix4	18.68	18.49	10.91	14.44	18.21	8.8	11.1	12.64	15.5
C16:0	28.08	27.63	24.77	30.53	27.64	25.74	28.57	26.72	25.7
C17:0, cyclo	5.41	8.4	12.4	9.66	7.99	13.97	13.09	10.42	7.27
C16:1, 2OH	ND	1.18	1.45	ND	ND	1.57	1.27	1.23	1.11
C16:0, 2OH	ND	Tr	Tr	ND	ND	1.35	1.19	1.34	0.94
C16:0, 3OH	5.9	3.94	3.51	5.91	5.84	5.07	5.16	5.09	4.99
Mix7	29.74	24.66	24.18	25.42	27.36	20.47	19.37	24.7	28.3
C18:0	ND	1.31	1.19	ND	ND	1.24	1.27	1.35	1.27
C19:0, cyclo ω8c	2.95	3.63	8.36	4.3	3.3	8.61	8.56	6.14	4.51
C18:1, 2OH	ND	1.09	1.88	ND	ND	2.24	1.39	1.84	1.48
Result of identification (No. 1) and SIM Value	*B. gladioli* 0.717	*B. gladioli* 0.897	*B. gladioli* 0.805	*B. gladioli* 0.653	*B. cocovenenans* 0.823	*B. gladioli* 0.817	*B. cocovenenans* 0.787	*B. gladioli* 0.842	*B. gladioli* 0.886
Result of identification (No. 2) and SIM Value	*B. cepacia* 0.611	*B. cepacia* 0.701	*B. cepacia* 0.487	*B. cepacia* 0.352	*B. gladioli* 0.813	*B. cocovenenans* 0.611	*B. gladioli* 0.705	*B. cepacia* 0.585	*B. cepacia* 0.786

Percentages of total fatty acids.

ND: not detected, Tr: trace amount (<1%). Mix3: iso I 16:1 and 3OH-14:0 fatty acid, which can not be differentiated under the studied conditions; Mix4: 2OH-iso15:0 and 16:1 ω7c fatty acid, which can not be differentiated under the studied conditions; Mix7: 18:1 ω7c/ω9t/ω12t fatty acid, which can not be differentiated under the studied conditions.

Table 4. DNA base composition and DNA-DNA similarity values

Source of unlabeled DNA	G+C content[a]	DNA similarity values to labeled strain: *B. gladioli* pathovar *gladioli* NCPPB 1891	*B. gladioli* pathovar *alliicola* NCPPB 947	*B. gladioli* pathovar *agaricicola* NCPPB 3580	*B. cocovenenans* ATCC 33664	*B. cepacia* ATCC 25416
B. gladioli pathovar *gladioli* NCPPB 1891	**67.5**	**100**	94	84	81	46
B. gladioli pathovar *alliicola* NCPPB 947	**68.0**	89	**100**	85	90	35
B. gladioli pathovar *agaricicola* NCPPB 3580	**68.2**	92	94	**100**	89	37
*B. cocovenenans*ATCC 33664	**67.1**	93	92	83	**100**	34
B. cocovenenans HN2y	**66.8**	81	90	84	78	31
B. cocovenenans Co14	**67.6**	89	90	85	83	48
B. cocovenenans Co18	**67.2**	89	92	82	93	52
B. cocovenenans Sx8801	**67.8**	93	89	81	94	31
B. cocovenenans I-90-3	**67.2**	90	87	83	85	44
B. cepacia ATCC 25416	**67.5**	36	44	35	28	**100**
B. vandii JCM 7957	**68.5**[b]	42	32	25	46	36
B. plantarii JCM 5492	**68.7**[b]	38	33	40	41	34
B. pseudomallei ATCC 23343	**69.5**	31	24	32	35	30
B. mallei ATCC 23344	**69**	30	23	32	33	30
B. thailandensis BSU 91838	**ND**	26	26	25	32	30
B. vietnamiensis LMG 10929	**67.9**[b]	47	40	36	39	50
B. glathei ATCC 29195	**64.8**[c]	28	21	23	30	29
B. phenazinium LMG 2247	**ND**	25	24	41	32	18

[a] Results are the mean of three replicates.
[b] Reference, IJSB: 1994, **44**: 235–245.
[c] Reference, Bergey's manual 9th ed.

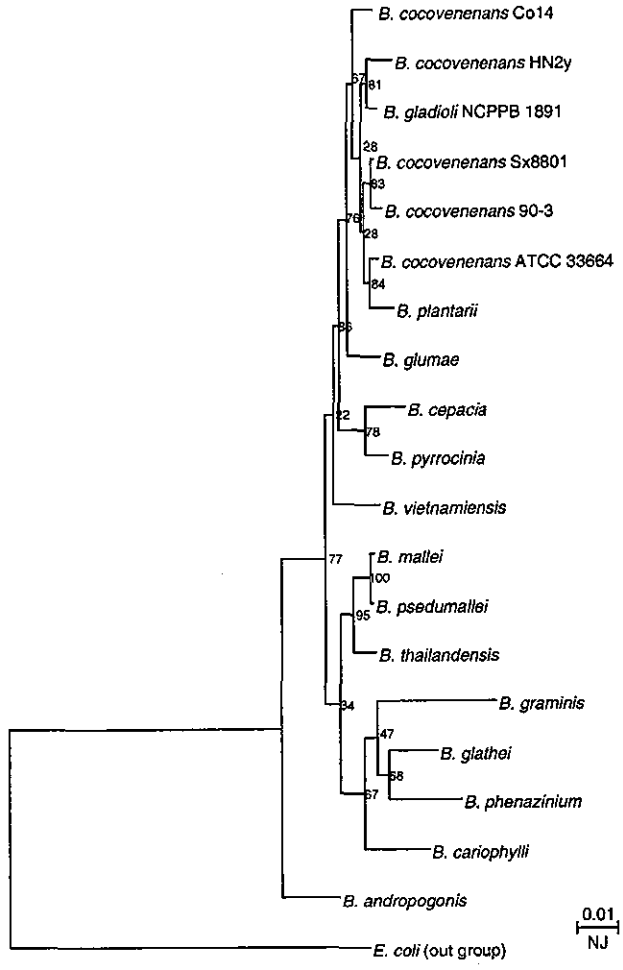

Fig. 3. Phylogenetic position of the representative isolates of *B. cocovenenans* among selective members of the genus *Burkholderia*. Distances were calculated by the neighbor-joining (NJ) method. The numbers at the branch points are bootstrap values. *E. coli* was used as the outgroup.

synonyms (3). Furthermore, we concluded that our Chinese isolates also belong to *B. gladioli* because they showed greater than 81% similarity values with the type strain of *B. gladioli*.

Fatty acid analysis did not reveal any useful characteristics to differentiate *B. cocovenenans* from *B. gladioli*. According to the MIDI Sherlock microbiology identification system, two of our Chinese isolates were more closely related to *B. cocovenenans* than to *B. gladioli*; however, there were no significant differences in fatty acid composition between the strains identified as *B. gladioli*.

In the present study, the amounts of some fatty acids differed from those reported by Urakami et al. (20). They reported amounts of 2.9 to 3.0% for C17:0 cyclo and 0.7 to 0.9% for C19:0 cyclo. However, we found more than twice the amount of these fatty acids. Increases in the relative amounts of cyclopropane acids were reported by Marr and Ingraham (13), and we hypothesized that one reason for this discrepancy was a difference in culture temperatures: Urakami et al. (20) cultivated bacterium at 30 C, whereas we cultivated at 37 C. Another possible reason was the difference in the method of sample preparation: Urakami et al. (20) liberated and methylated the fatty acids with HCl-methanol at once; however we first released the fatty acids with alkaline saponification before methylating them with HCl-methanol.

In the present study, we used the BiOLOG GN2 plate on which 95 different reactions can be carried out. This system seems to be useful for the differentiation of closely related species. Although most of the traits assessed on the BiOLOG GN2 plate were identical between strains with only minor differences, we could find useful differential characteristics between *B. cocovenenans* and *B. gladioli*. Those were differences of assimilation of xylitol, gentiobiose, and *N*-acetyl-D-galactosamine. *B. cocovenenans* was previously reported to be positive for the xylitol assimilation test by Zhao et al. (25), and actually the seven strains of *B. cocovenenans* analyzed in the present study showed positive reactions. However, Gillis et al. (7) reported that *B. gladioli* strains showed variable results with the xylitol assimilation test. In their study, 10 to 90% of the strains, including the type strain, were positive, whereas our three reference strains, including the type strain of *B. gladioli*, showed negative in this test. We repeated the analysis four times and obtained the same result. Therefore, we concluded that *B. gladioli* strains can not assimilate xylitol. Similarly, the assimilation tests for gentiobiose and *N*-acetyl-D-galactosamine were always positive for the three reference strains of *B. gladioli* and negative for all strains of *B. cocovenenans* used in this study. Therefore, these tests might be useful for differentiating *B. cocovenenans* from *B. gladioli*.

We could find some other characteristics that might be helpful for differentiating each pathovar of *B. gladioli*. However, we used only representative strains of each pathovar. The data for many strains should be accumulated.

B. gladioli was first described as a phytopathogenic bacterium in 1913 (18). Presently, this species comprises three pathovars, all of which are recognized as phytopathogenic bacteria. *B. gladioli* strains have been isolated from cystic fibrosis patients (2). However, there have been no reports that these isolates produce a toxin lethal to humans. *B. cocovenenans* strains isolated from patients with food poisoning after ingestion of fermented coconuts, fermented corn, or white fungi have been reported to produce lethal Bongkrekic acid (15). With such cases, the fatality rate was reported to be more

Table 5. Homology values among members of the genus *Burkholderia*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *B. gladioli* ATCC 10248^T	100.0																			
2. *B. cocovenenans* LMG 11626^T	99.6	100.0																		
3. *B. cocovenenans* 90-3	99.6	99.2	100.0																	
4. *B. cocovenenans* Co14	99.4	99.1	98.7	100.0																
5. *B. cocovenenans* HN2y	99.6	98.9	98.4	98.2	100.0															
6. *B. cocovenenans* Sx8801	99.7	99.3	99.6	98.9	98.9	100.0														
7. *B. plantarii* LMG 9035^T	99.4	99.2	98.9	98.8	98.6	99.0	100.0													
8. *B. glumae* LMG 2196^T	99.1	98.0	98.7	98.6	98.4	98.8	97.6	100.0												
9. *B. andropogonis* ATCC 23061^T	96.6	96.2	96.6	96.1	96.1	96.7	96.5	96.4	100.0											
10. *B. pyrrocinia* LMG 14191^T	98.6	98.0	98.2	98.1	97.9	98.3	96.1	97.6	95.9	100.0										
11. *B. cepacia* ATCC 25416^T	98.1	97.6	97.6	97.6	97.4	97.7	95.3	97.3	95.4	98.5	100.0									
12. *B. vietnamiensis* TVV75^T	98.4	97.9	97.9	97.8	97.7	98.0	96.5	97.8	95.7	96.8	97.4	100.0								
13. *B. thailandensis* E264^T	97.7	97.5	96.9	97.2	96.3	97.1	97.7	98.0	95.4	98.1	98.1	98.1	100.0							
14. *B. pseudomallei* 1026b^T	97.8	97.6	97.0	97.3	96.3	97.1	98.0	98.1	95.6	98.0	97.9	98.1	99.0	100.0						
15. *B. mallei* ATCC 23344^T	97.7	97.5	96.9	97.2	96.3	97.1	97.9	98.0	95.5	97.9	97.9	98.1	98.9	99.8	100.0					
16. *B. caryophylli* ATCC 25418^T	96.1	96.1	95.9	95.6	95.7	96.0	96.2	96.4	94.6	96.5	96.4	96.3	96.7	96.9	96.8	100.0				
17. *B. phenazinium* LMG 2247^T	96.1	95.7	95.4	95.6	95.4	95.6	95.7	94.9	94.8	94.3	94.0	94.1	96.2	96.2	96.1	96.3	100.0			
18. *B. glathei* ATCC 29195^T	96.4	96.2	95.5	96.0	95.2	95.8	96.5	96.4	95.7	96.4	96.0	96.0	96.2	96.3	96.3	95.7	97.0	100.0		
19. *B. graminis* C4D1M^T	95.3	94.8	94.6	94.8	94.6	94.7	92.8	94.4	94.0	93.6	90.5	94.4	96.0	96.1	96.0	95.7	95.1	95.6	100.0	
20. *E. coli* (out group)	82.1	81.8	81.6	82.3	81.2	81.9	81.8	81.8	83.0	81.5	81.7	81.8	81.6	81.8	81.8	81.2	80.9	81.2	81.4	100.0

than 40% in China (15). Although many taxonomic data suggest that these strains should be included in *B. gladioli*, we were reluctant to include these strains in *B. gladioli* because *B. gladioli* has been recognized mainly as a phytopathogenic bacteria, whereas *B. cocovenenans* produces a lethal toxin.

The actual mechanism of toxicity is not clear at the present time. However, autopsy finding in the patients of this food poisoning demonstrated that multiple organs were damaged, such as the liver, kidney, brain, lung, heart, stomach, intestine, and spleen (15). When culture supernatant of *B. cocovenenans* was administered orally to mice, 100% of the mice died within 45 min with a stiff tail and feet (Fig. 2). From this observation, the toxin may be associated with rigorous muscle paralysis or neurotoxicity, in addition to multiple organ and brain damage.

On the basis of the findings described above and those of other reports of genetic approaches to the taxonomy of *B. gladioli* and *B. cocovenenans*, we also concluded that *B. gladioli* and *B. cocovenenans* should be combined into a single species as *B. gladioli*. However, this species should be considered to contain two different groups in pathogenicity; an animal pathogen, which produces lethal toxins, and a plant pathogen, which is further divided into three pathovars.

To emphasize the recognition that *B. gladioli* involves two different pathogens, we propose a new pathovar, *B. gladioli* pathovar *cocovenenans*, for the lethal toxin-producing strains. To confirm this proposal, we are accumulating more data using a number of strains isolated from cystic fibrosis patients and environmental samples. We are also testing some practical techniques to detect BA-toxin in test strains, such as thin-layer chromatography or high-performance liquid chromatography, to avoid troublesome animal experiments to detect the BA-toxin.

Here, we present the emended description of *B. gladioli* and describe a new pathovar, *B. gladioli* pathovar *cocovenenans*, for the lethal toxin-producing strains.

Emended Description of B. gladioli *(Severini 1913) Yabuuchi et al. 1992 and Coenye et al. 1999*

The following description is based on the data in this study and on the results of previously published studies (3, 7, 26).

Description of B. gladioli *(Plant Pathogen, Including* B. gladioli *Pathovar* gladioli, B. gladioli *Pathovar* agaricicola, *and* B. gladioli *Pathovar* allicola*)*

The description of *B. gladioli* is the same as those given by Coenye et al. (3) and VanDamme et al. (22). Other investigators have also reported the detailed biochemical characteristics of this species (7, 20).

The strains are isolated primarily from plants, but are sometimes isolated from cystic fibrosis patients (2). The strains including those isolated from human patients have not been reported to produce a toxin lethal to humans or other animals (toxoflavin and bongkrekic acid).

This species has been divided into three pathovars: *B. gladioli* pathovar *gladioli*, which causes gladiolus rot (8, 24); *B. gladioli* pathovar *alliicola*, which causes onion bulb rot (24); *B. gladioli* pathovar *agaricicola*, which causes rapid soft rot of cultivated mushrooms (10).

The type strain of this species is NCPPB 1891 (=GTC 1038=ATCC 10248=DSM 4285=JCM 9311). The representative strains of *B. gladioli* pathovar *alliicola* and *B. gladioli* pathovar *agaricicola* are NCPPB 947 (=GTC-P3-431=ATCC 19302), and NCPPB 3580 (=GTC-P3-432), respectively.

The biochemical characteristics of these pathovars are almost identical, but some characteristics (Table 2) may be helpful for pathovar differentiation.

Description of B. gladioli *Pathovar* cocovenenans, *Which Is an Animal Pathogen*

B. gladioli pathovar *cocovenenans* is primarily isolated from food, which is processed from plants (coconut mould, fermented corn, and deteriorated white fungi). It produces two highly toxic compounds, toxoflavin and bongkrekic acid, and is therefore a dangerous pathogenic bacteria for humans and animals. The basic biochemical traits of the strain are almost identical to those of *B. gladioli*; however, this pathovar can be differentiated from plant pathogenic *B. gladioli* strains by its production of bongkrekic acid and by other phenotypic characteristics shown in Table 2. The representative strain is ATCC 33664 (=GTC 586=JCM 10561=NCIB 9450=DSM 11318).

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