Isolation of *Bacillus sp.* as a Volatile Sulfur-degrading Bacterium and Its Application to Reduce the Fecal Odor of Pig

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ABSTRACT : Fecal malodor is an acute environmental issue to be solved for the intensive animal agriculture in Japan. Among these substances volatile sulfur such as hydrogen sulfide (HS), methanethiol, and dimethyl sulfide, and dimethyl disulfide are the ones most strictly controlled in the Japanese national regulations. In this experiment, we have screened a range of standard strains of chemoheterotrophic bacteria and of the presently isolated soil bacteria for their capacity to decompose HS. We have demonstrated that *Comamonas testosteroni* JCM5832^T and our isolate *Bacillus* sp. had a potential to reduce malodor when applied to the pig feces. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 12 : 1795-1798)***

Key Words : *Bacillus* sp. *Comamonas testosteroni*, Hydrogen Sulfide, Malodor, Pig Feces

INTRODUCTION

Fecal malodor is an acute environmental problem to be resolved in intensive animal agriculture. Its major causes are volatile fatty acids (VFA), ammonia, and volatile sulfurs (VS). Among these substances, volatile sulfurs (VS) such as hydrogen sulfide (HS), methanethiol (MT), dimethyl sulfide (DMS), and dimethyl disulfide (DMDS) are the ones most strictly controlled by Japanese national regulations. Fecal VS is produced from the bacterial metabolism of sulfate and/or sulfur-containing amino acids; the former is categorized as a dissimilatory sulfate reduction accomplished by sulfate-reducing bacteria such as *Desulfovibrio* spp., and the latter is the desulfhydrolation of sulfur amino acid accomplished by a range of anaerobic fermentative bacteria such as *Clostridium* spp. and *Fusobacterium* spp. (Arakawa et al., 2000; Ushida et al., 2001).

VS could be removed from the air with the use of chemoautotrophic bacteria such as *Thiobacillus thioparus* (Kanagawa and Mikami, 1989) and T. denitrificans (Ongcharit et al., 1991), chemoorganotrophic bacteria such as *Hyphomicrobium* sp. (Zhang et al., 1991) and *Xanthomonas* sp. (Cho et al., 1992), and phototrophs such as *Chlorobium* spp. (Kusai et al., 1973) and *Chlomatium* sp. (Fukumori et al., 1979). However, these autotrophic bacteria require various conditions for growth, such as low CO_2 and light, which are difficult to guarantee when the bacteria are mixed with pig manure. Therefore, these bacteria are rather difficult to apply directly to manure. Heterotrophic bacteria, on the other hand, are a potential alternative. However, few studies have dealt with chemoheterotrophic bacteria capable of oxidizing VS. Recently, Nakada and Ohta (1997) and Sato et al. (1999) isolated soil bacteria capable of decomposing VS in a batch culture system. They suggested that many chemoheterotrophic bacteria could remove VS from the air. Accordingly, we have tried to isolate heterotrophic bacteria that remove VS from the feces when applied directly to pig feces.

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MATERIALS AND METHODS

Soil (10 g) was sampled from a pig shed on an experimental farm at Kyoto Prefectural University. It was mixed with 750 ml of a phosphate buffer (0.01 M, pH 7.5) in a blender. After sedimentation of heavy particles by standing 10 min, a portion (5 ml) of the supernatants was inoculated to a 50 ml DMS enrichment medium. The medium was composed of $Na_2HPO_4 2H_2O$ (2 g/l), K_2HPO_4 (2 g/l), NH_4Cl (0.4 g/l), $CaCl_2$ (0.2 g/l), $MgCl_2$ (0.2 g/l), glucose (2 g/l), and DMS (0.74 ml/l). Incubation was carried out in serum bottles of 100 ml volume at 25°C for 14 days with shaking. The bottles were closed with butyl rubber septa to keep the DMS from evaporating. Additional DMS (0.37 ml) was introduced to the cultures on days 4 and 11. After incubation was completed, 0.1 ml of culture was applied to a Tryptic soy (TS) agar plate and incubated for 24 h at 25°C. The colonies that developed were transferred to fresh TS agar plates. A TS broth medium was used in further culture experiments. TSA and TS broth were obtained from Difco Laboratories (Sparks, MD, USA).

Isolates were microscopically checked for purity and transferred to a TS broth medium. Isolates were incubated to obtain OD. 660=0.2, and a portion (1 ml) was inoculated to a 10 ml TS broth in a 30 ml serum vial closed with a

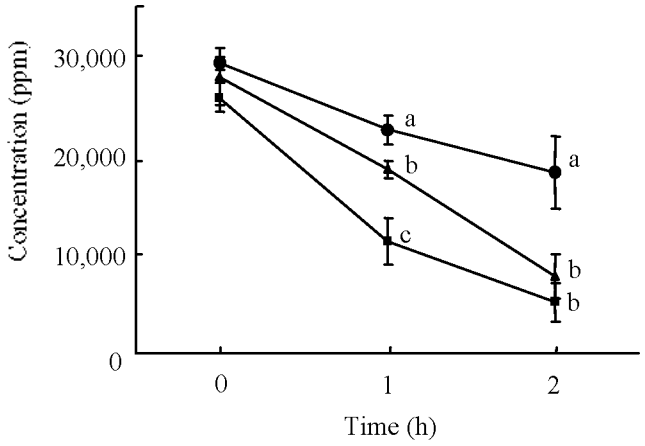

Figure 1. Hydrogen sulfide concentration in the headspace gas of the bacterial cultures. Negative control (●), *Bacillus* sp. KPU 0013 (▲), *C. testosteroni* JCM5832 (■). The values are means for three determinations with standard deviations.
a, b, c; Values at the same sampling time followed by different superscript differ significantly ($p<0.05$)

butyl rubber septum. HS gas (Sumitomo Seika, Osaka) was introduced into a vial. Vials were placed at 25°C and incubated for 2 h with shaking. Headspace gas (0.5 ml) was sampled at 0, 1 and 2 h of incubation by a gas-tight syringe after the addition of 1 ml HCl (6 N). Three vials were allotted to each sampling time; therefore, nine vials in total were used for one isolate. The gas was analyzed for VS using a flame photometric detector (FPD)-gaschromatograph (GC 14B, Shimadzu, Kyoto) as described by Ushida et al. (1998). The same test was also used for the following laboratory strains: *Alcaligenes faecalis* JCM5485^T, *Comamonas testosteroni* JCM5832^T, and *Pseudomonas putida* IFO14164^T. Bacteria that significantly decreased the hydrogen sulfide concentration in the headspace were obtained as VS-decomposing bacteria and subjected to further experiments. The growth of bacteria during incubation was estimated by protein determination. A portion (1 ml) of cultures was centrifuged at 15,000 rpm to precipitate bacteria, and cells were solubilized in 1 N NaOH for the protein assay (Lowry et al., 1951).

Pig feces were sampled just after defecation, and a portion (100 g) was poured in a plastic tray (10 cm×10 cm). Isolates and laboratory strains selected from the *in vitro* HS decomposition test were precultured to reach OD 660=0.2 in the TS broth medium. Three levels of bacterial cultures, 10, 20 and 30 ml, were inoculated into pig feces and mixed manually. Feces were thereafter incubated at 25°C for 23 h. Feces were placed in a 10 l Tedlar bag (Ohmi Odoair Service, Ohmihachiman, Shiga) and incubated for an additional 1 h. A portion (3 l) of gas in a Tedlar bag was sampled and used as an original odor source in the odor-ranking analysis by the triangle odor bag method. The odor-ranking analysis is based on an olfactory sensory test and was done according to the standard method defined by the Environmental Agency of the Japanese government to estimate an odor index (Ishiguro, 1997). Polyester bags (Flek-sampler®, Ohmi Odoair Service, Ohmihachiman, Shiga) with a 3 l volume were filled with odor-free air for the test. Three bags, one of which was injected with the original odor, were presented to a panelist. The odor-free air was prepared using an activated charcoal column. The amount of original odor introduced was reduced stepwise by 3/10 from 100 ml to 0.1 ml. Six female panelists were asked at each step of the dilution to attempt to detect the bag that smelled. The detection records of the six panelists were treated to estimate an odor index according to Ishiguro (1997). Rank odor analyses were done twice for each treatment.

Table 1. Effect of *Comamonas teststeroni* JCM 5832 and *Bacillus* sp. KPU 0013 of odar index of pig feces

Bacteria	Added culture volume[a]	Odor index[b]	
		1	2
Control[c]	20	26	27
Comamonas teststeroni	20	20	22
Bcillus sp.[d]	10	19	20
Comamonas teststeroni	20	20	19
Bcillus sp.[d]	40	25	26

[a] Bacterial culture was added to fresh pig feces (100 g) and mixed. Details, see text. [b] Odor ranking analyses were repeated twice. [c] TS broth medium was added to the feces as a control. [d] Isolate of the present study.

Isolates were microscopically observed after Gram-staining. The presence of spores was checked by heating cells to 80°C for 15 min. Isolates were cultured, and the cells were analyzed for SSU rDNA sequence. Primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGCTACCTTGTTACGACTT-3') and r-*Taq* polymerase (Toyobo, Tokyo) were used to amplify 16S rDNA in a thermal cycle consisting on an initial 3 min of denaturation at 94°C, 30 cycles of 94°C 1 min; 58°C 1 min; 72°C 1.5 min, and the final 6 min of elongation at 72°C. Amplicons were further subjected to TA-cloning using the pGEM-T Easy Vector system (Promega, Madison, WI, USA) and *Escherichia coli* JM109 (Toyobo, Tokyo) as indicated by the manufacturer. After blue white selection, purified plasmids were subjected to a DNA sequencing by a Shimadzu DNA autosequencer (DSQ 2000). The obtained 16S rDNA sequences were analyzed with the BLAST program.

HS concentration after incubation (Figure 1) was subjected to ANOVA with Dunn's post hoc comparison.

RESULTS AND DISCUSSION

C. testosteroni JCM5832 decomposed 75% of HS in a

headspace in 2 h in this experiment (Figure 1). Therefore, we have used this strain as a positive control to screen for soil bacteria that decompose HS. One strain (Strain KPU 0013) out of 197 was obtained as a bacterium that had the same potential as *C. testosteroni* JCM5832. This isolate decomposed 80% of the HS in 2 h (Figure 1). The protein assay of cultures indicated that *C. testosteroni* JCM5832 and the present isolate grew well in this culture condition with a relatively high initial hydrogen sulfide (data not shown). When these two types of bacteria were mixed with pig feces, both reduced the odor index of feces (Table 1).

The isolate, KPU 0013, was a Gram-positive stained rod. Heat resistance indicated that the bacterium possessed endospores, and not even microscopic observation can easily detect endospores. A 16S rDNA sequence of the bacterium showed high homologies with *Bacillus* thuringensis and *B. cereus*.

Sato et al. (1999) isolated chemoheterotrophic bacteria capable of decomposing MT and HS from various soil samples using a DMS enrichment culture. We used their method to enrich VS-decomposing bacteria. Sato et al. demonstrated that a range of heterotrophic bacteria could decompose MT and HS *in vitro*. Among IFO strains, they found that *C. testosteroni*, *A. faecalis*, and *P. putida* had the potential to decompose HS. In the present screening system, *C. testosteroni* JCM5832 also effectively decomposed HS in our in vitro system. They also demonstrated that soil isolates belonging to the genera *Bacillus*, *Pseudomonas*, *Alcaligenes*, and *Enterococcus* effectively decomposed HS. Among them, *Bacillus* spp. was the most frequently isolated (16 out of 28 VS-decomposing isolates). We have also isolated one *Bacillus* sp (Strain KPU 0013), capable of decomposing HS as efficiently as *C. testosteroni* JCM5832. In order to examine the practical values of VS-decomposing bacteria in decreasing fecal malodor, we have applied our isolate to rank odor analyses after inoculation to pig feces. The result of the rank odor analyses showed that our isolate had the ability to reduce malodor from pig feces in the aerobic condition. In particular, the pungent smell of feces disappeared. The decrease in the odor index from 26 to 20 corresponded to a 1.5 point decline in the rank of odor intensity from 5 (very strong smell) to 3.5 (easily noticeable) (Ishiguro, 1997). Such a decline in the rank of odor intensity corresponds to the decline in HS concentration from 8 ppm to 0.2 ppm (Ishiguro, 1997). Obviously, the smell of feces is composed of a range of chemicals, such as ammonia, volatile fatty acids, and VS, and the decline in the odor index was not completely attributable to the decline in the HS concentration. However, the malodor of feces consists mainly of VS (Hamaguchi and Kawahara, 1997). The relationship between an *in vitro* HS-decomposing test and an *in situ* rank odor analysis has not been demonstrated so far. The present experiment indicated that the *in vitro* HS-decomposing capacity of bacteria could correlate with an *in situ* reduction in malodor. Although several heterotrophic bacteria were isolated as VS decomposers (Zhang et al., 1991; Cho et al., 1992; Nakada and Ohta, 1997; Sato et al., 1999), only one isolate has been tested in a practical system. Our odor ranking analyses on pig feces suggested that, in practical situations, chemoheterotrophic bacteria have potential as a deodorant for feces that were screened by DMS enrichment followed by an *in vitro* HS-decomposition test.

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