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Amplification of Irrelevant Sequence from *Bacillus subtilis* Using a Primer Set Designed for Detection of the *pag* Gene of *Bacillus anthracis*

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Anthrax is one of the major bacterial zoonoses caused by spore-forming Gram-positive rods, *Bacillus anthracis*. After the bioterrorist attack in the USA in September 2001, the probability of anthrax attacks has increased worldwide, and the number of hoaxes involving "white powder" has increased in Japan as well. Since rapid and precise diagnosis is the most powerful measure to counter bioterrorism, it is of particularly importance to have a diagnostic tool to detect anthrax. Polymerase chain reaction (PCR) is regarded as one of such diagnostic method for the detection of anthrax, although definitive diagnosis can only be made when other methods support the PCR results. The PCR method recommended by the World Health Organization (WHO) is widely accepted in Japan as the standard approach. A presumptive diagnosis of anthrax is made if bands with predicted sizes are detected after PCR with primer sets targeted to *pag* and *cap* genes residing on pXO1 and pXO2 plasmids, respectively (1).

On March 27, 2002, "white powder" was found disseminated inside a public restroom located in Sapporo City. The powder was subjected to microbiologic tests and PCR at the Sapporo City Institute of Public Health directly and after cultivation in TSB medium. Although no bacterium was found when the sample was directly examined, Gram-positive, spore-forming bacilli were detected after cultivation in TSB medium (Table 1). Since the bacteria were motile and hemolytic, the possibility that isolated bacteria were *B. anthracis* was ruled out; however, the PCR with primers designed for detection of the *pag* gene resulted in amplification of a 596 bp fragment (Fig. 1). The presence of the PCR amplicon of this size usually indicates that the sample may contain bacteria harboring a pXO1 pathogenic plasmid. We have thus attempted to determine whether or not the bacteria were indeed *B. anthracis*. First, microplate hybridization (kindly provided by Dr. Matsunaga of Wakunaga Pharmaceutical Co., Ltd., Hiroshima) was employed for detection of the Ba813 chromosomal gene that was thought to be anthrax-specific (2). The results revealed no significant signals, suggesting that the bacteria did not have sequences homologous to Ba813. Second, real-time PCR was performed using a LightCycler-*Bacillus anthracis* Detection Kit (Roche Diagnostics, Tokyo). No anthrax-specific signals were detected when the sample was tested for the presence of *pag* and *cap* genes. We then sequenced the amplicon after cloning it into the pCR2.1 plasmid. The nucleotide sequence showed substantial homology with *B. subtilis* ATP-dependent deoxyribonuclease, but no homology with any known *B. anthracis* genes was determined. An increase in the annealing temperature had no effect on the production of

Table 1. Characteristics of bacteria isolated after cultivation in TSB media

Smear	Gram stain	Gram +, rods
	Spore	+
	Capsule	–
PCR	Pag	+
	Cap	–
Isolation	Blood Agar	hemolytic
	NGKG agar	lecithinase+, Medusa's head-
	BCA*	lecithinase+, Medusa's head-

**Bacillus cereus* Agar

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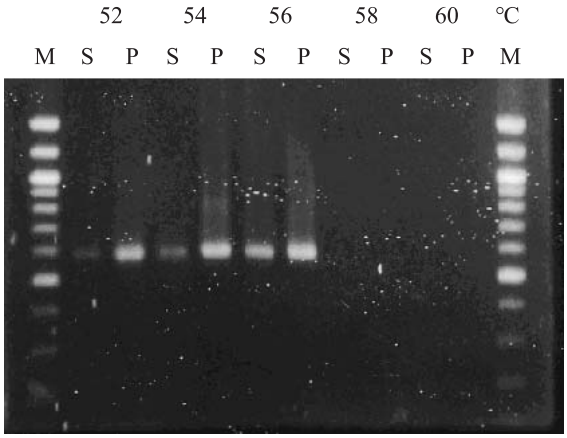

Fig. 1. Effect of annealing temperature on amplification of fragments from the Sapporo sample.
M: 100 bp Ladder
S: Sapporo Sample
P: Positive control (*B*. *anthracis* Pasteur II)

these bands when the sample was subjected to PCR using the same *pag* primer set (Fig. 1). The results of biochemical tests indicated that the bacteria isolated belonged to the *B*. *subtilis* group (data not shown). These findings indicate that the PCR protocol recommended by WHO is not sufficiently specific, unless the internal nucleotide sequence is determined, since there remains the possibility that a bacterium exists, the genome of which contains sequence(s) amplifiable by the primers. Moreover, the size of the amplified fragments is indistinguishable from those amplified from *B*. *anthracis*.

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