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The role of wood-inhabiting bacteria in pine wilt disease

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Abstract: The pathogenicity of the pine wood nematode (PWN), *Bursaphelenchus xylophilus* together with the bacteria isolated from black pine (*Pinus thunbergii*) bark inoculated to axenic black pine seedlings, significantly exceeded that of the axenic PWNs alone, demonstrating that the bacteria play an important role in pine wilt disease. Inoculation of seedlings with bacteria-free culture filtrates of the seven isolates from the dead seedlings from the above experiment showed that all isolate filtrates killed the seedlings within 8 days. Identification of the bacteria using 16S rDNA sequencing showed that the isolates belonged to strains By253Ydz-fq, S209, 210-50 and 210-50 in *Bacillus* and the DN1.1 strain of *Stenotrophomonas maltophilia,* respectively. Completing Koch's postulates using the seven bacterial isolates to inoculate pine seedlings showed that all the seedlings that received aseptic PWNs mixed with the seven bacterial isolates died within 18 days post inoculation, while those inoculated with 'wild' PWNs died 16 days post inoculation. No disease symptoms developed on seedlings that received sterile water or aseptic PWNs. The horizontal transfer of the pathogenic bacteria may explain differences in bacterial species carried by PWN in different geographic areas.

Key words: Bursaphelenchus xylophilus, ecology, forest bacteria, pine wilt disease, *Pinus thunbergii.*

Pine wilt disease (PWD) causes serious damage to pine forests in Japan, China (Mamiya, 1983; Cao and Han, 2001) and Western Europe (Mota and Vieira, 2008). Earlier studies indicated that the disease is caused solely by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Mamiya, 1983; Mayer, 1988). Several studies have described bacteria associated with PWN (Oku *et al.*, 1980; Higgins *et al.*, 1999); and bacteria were observed, by using scanning electron microscopy, on the surface of the nematodes (Zhao *et al.*, 2000). Axenic PWNs reportedly lost their pathogenicity (Cao and Han, 2001; Kawazu and Kaneko, 1997). Based on field surveys in China and a series of greenhouse and forest inoculations, Zhao et al. (2003) hypothesized that PWNs carrying phytotoxin-producing bacteria are necessary to induce PWD. The above hypothesis has been supported by field tests in Korea with a bactericide and a nematicide to control PWD (Hyeok et al., 2010).

Field tests (Tamura, 1983) showed that inoculation of 10, 3-year-old Japanese black pine seedlings with 19,000 aseptic PWN per seedling resulted in 90% tree mortality 27 to 95 days after inoculation. Kawazu (1997) assumed that the above PWN inoculum became contaminated with toxin-producing bacteria during inoculation which was done under field conditions and thus, pathogenicity was attributable to these contaminants. However, Zhao and Lin (2005) reported a mutualistic symbiosis between PWN and its associated pathogenic bacteria which might explain how the mycotrophic PWN could gradually evolve as a pathogen on living pine hosts. The perpetuation of symbiosis through host generations relies on symbiont transmission (Bright and Bulgheresi, 2010). Microbial mutualism is the basis for the evolution of the eukaryotic cell and allows organisms to exploit inaccessible niches (Chaston and Goodrich, 2010). To understand this evolutionary process, it is important to know where and how PWN acquires its associated pathogenic bacteria. Since adult PWNs, and their offspring live in diseased trees, the offspring might acquire bacteria from their parents or the tree host.

The following questions need to be addressed: 1) what are the species of bacteria on the pine-wilt affected trees; 2) can these bacteria be carried by *B. xylophilus*; 3) what is the role the bacteria play in PWD inductions; 4) what is the bacteria identity and origin. To answer some of the above questions bacteria from a *P. thunbergii* forest in Jiangsu Province, China were isolated, grown in pure culture, and mixed with axenic PWNs and the PWN-bacterium inoculum was inoculated into axenic, black pine seedlings. Following inoculation, seven bacterial strains were isolated and purified from the dead seedlings. Additional seedling inoculations and identification of the bacterial isolates were done to determine the role of the bacteria in PWD.

Materials and Methods

Isolation of bacteria from the bark of forest-grown, black pine: Bacteria used in our experiments were isolated from healthy, black pine trees, *P. thunbergii,* growing in a 10,000 ha suburban forest in Nanjing, Jiangsu Province, China. Twenty, 1x1cm, blocks of bark were collected from 10 random trees, 8-20 years old, DBH: 6-16 cm, and washed with 100 ml of sterilized water and filtered through a filter paper (qualitative filter

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paper, Grade 114v, 125 mm, Whatman Inc.) and centrifuged (CS150GXL, HITACHI, Japan) at 40,000g for 15 min. The supernatant was discarded and sterile water was added to the sediment to bring the volume to 1 ml.

PWN collection and production of axenic cultures: Bursaphelenchus xylophilus individuals were obtained from a naturally PWD-affected black pine from a forest in Conghua, Guangdong Province, China, and a xylem sample was cut into 2×2×10 mm chips. Twenty grams fresh weight of those chips were used to extract the nematodes using a Baermann funnel (Han et al., 2003). The nematodes were propagated on *Botrytis cinerea* fungus growing on potato-dextrose-agar (PDA) medium in Petri plates. The nematodes were washed off the PDA with sterile water and a single female nematode with eggs was obtained using a dissecting microscope. Specimens identified as *B. xylophilus* were propagated on *B. cinerea* growing on PDA in individual slant cultures in 2x19 cm test tubes. Two PWN juveniles were picked up by a micro-syringe under a microscope and transferred to each tube and the tubes incubated at 28 ^{0}C for 7-10 days to let juveniles develop and reproduce; PWN reproduction occurred in about half of the tubes. Culturing was repeated until sufficient nematodes were available for subsequent use. Twenty thousand PWNs in 2 ml of 0.3% sodium carboxyl methylcellulose were slowly pipetted into an acid burette containing 50 ml of 0.3% sodium carboxyl methylcellulose. Then the burette was kept in 28 ^{0}C for 12 hr when the liquid containing the PWNs was drawn down and 5 ml aliquots were pipetted into each of 10 test tubes. The liquid in six such tubes, microscope observation showed to contain only PWN juveniles, were combined and centrifuged at 800 g for 5 min. The 5 ml of precipitate with nematodes was washed five times with 5 ml sterilized 0.9% NaCl. The juveniles were sterilized with 3% H_2O_2 at 4 °C for 1 hr followed by five washings with sterilized 0.9% NaCl. The aseptic nematodes were transferred to cultures of *B. cinerea* and kept for 3-4 days before use. To verify that the nematodes were aseptic, a few nematodes were put onto NB medium for 24 hr at 30°C and subsequently checked for bacterial growth; any non-aseptic nematode cultures were discarded.

Axenic black pine seedlings: The axenic *P. thunbergii* seedlings were obtained according to the procedures of Zhao et al. (2003).

Inoculation experiment with axenic PWNs (ABX) + bacteria from black pine bark: Bacteria isolated from the pine bark plus axenic PWN (ABX) were inoculated into 3 month old axenic black pine seedlings. Forty thousand axenic nematodes in 1 ml of water were mixed with 1 ml of the bacteria and 5 µl of the mixture (ca. 100 nematodes and 5 x 10^4 bacterial cells) were inoculated into each wound made with a sterilized scalpel, on top of the seedling which was grown under aseptic conditions at 28 °C. The other treatment was 5 µl sterilized water containing 100 axenic PWNs. Five µl of sterilized water was used as the control inoculum. Symptoms developed on the inoculated seedlings were recorded every day using the scale: 0: no needles showing color change; 1: any needle on the seedling showing yellowing or browning for 1/4 its length; and 2: as for 1, but any needle on a seedling showing these symptoms for 1/4 -1/2 its length; 3: as for 1, but any needle on a seedling showing these symptoms for 1/2 -2/3 its length; 4: any needle of the seedling shows yellowish or brown more than 2/3 its length. The mean disease ranking = total disease level in the seedlings of each treatment / the number of seedlings in the treatment.

Isolation and purification of the bacterial isolates from diseased seedlings: Each dead pine seedling from the above experiment "Inoculation experiment with axenic PWNs (ABX) + bacteria from black pine bark" was cut into 3 mm-long segments and these were put onto NB medium in Petri plates and incubated at 28 °C. The bacterial colonies that grew along the trails of the nematodes that emerged from the segments on the NB medium were selected according to colony shape, color and diameter and re-cultured at 28 °C for 2-3 days on NB medium for subsequent purification and identification.

Toxicity of the cell-free filtrate from the cultures of bacteria isolated from black pine seedlings: Ten ml of the purified bacterial isolates, 1 x 10^6 cells/ml, were pipetted into 200 ml of NB liquid medium which was then shaken at 110 rpm at 28 °C for 6 days. Next, sterilized water was added to the culture medium to adjust the bacterial concentration to 1x10^7 cells/ml which was then passed through a bacterial filter (ME25STL, Whatman Inc). Two ml of the medium filtrates were added to a 0.75x4.0 cm test tube and an aseptic seedling with a cut root was inserted into the tube and kept under aseptic conditions. Liquid NB medium alone and sterilized water were used as the two control treatments. The inoculated seedlings were then incubated at 28 ^{0}C under aseptic conditions. Nine seedlings were used for each treatment. The symptoms were recorded every day according to the above symptom classification in "Inoculation experiment with axenic PWNs (ABX) + bacteria from black pine bark".

Identification of the bacterial isolates using 16S rDNA sequences: The CTAB (hexadecyltrimethy ammonium bromide) method was used to extract bacterial DNA (Tel-Zur *et al.*, 1999). This was done after a bacterial isolate was cultured in LB liquid medium for 3 days at 30 ^{0}C followed by 1.5 ml of the bacterial culture being centrifuged at 12,000 rpm. Next, 567 µl TE buffer (10 mM tris; 1 mM EDTA, pH 8.0), and 10 µl lysozyme (1mg/ml) were added to the deposit and incubated at 37 °C for 30 min with agitation. Next 30 µl of 10% SDS and 12 µl proteinase K (5mg/ml) were added and incubated at 65 ^{0}C for 10 min, and then 100 µl 5 M NaCl and 80 µl CTAB/NaCl were added and incubated at 65 ^{0}C for 10 min. Next, chloroform and phenol (1:1 vol/vol) and chloroform, phenol and isoamyl alcohol (1:1:1, vol/vol/vol) were used to extract the sample. A mixture of 3 M

sodium acetate and absolute ethanol (0.1 : 2 vol/vol, pH 5.2) was added to the supernatants to obtain the DNA deposit which was then washed with 500 µl 70% ethanol and then the mixture was centrifuged at 12,000 rpm at 4 ^{0}C for 10 min. Extraction and DNA quality were assessed by electrophoresis using a 1% agarose gel at 80 V for 30 min and staining with ethidium bromide. The fragment of 16S rDNA was amplified from the individual strain using the primers of 27f (5'-AGAGTTTGATCCT GGCTCA G-3') and 1492r (5'-GGTTACCTTGTTACGA CTT-3') by the PCR amplifer (BIO-RAD, ITALY). The PCR reactions were done in 200 µl volume containing 5 µl reaction buffer, 4 µl$MgCl_2$ (25 mM),4 µl dNTP (2.5 mM), 5 µl primer 27f and 5 µl primer 1492r, 5 µl DNA template, 0.5 µl Taq DNA polymerase (5 U/micro liter), 24 µlddH2O. Amplification was done as follows: an initial denaturation at 94 °C for 5 min; denaturing at 94 ^{0}C for 1 min; 30 cycles of denaturing at 94 ^{0}C for 1 min, at 55 ^{0}C for 1 min, at 72 ^{0}C for 2 min, and extension at 72 ^{0}C for 5 min. The amplification products were examined on a 1% agarose gel at 80 V for 30 min followed by staining with ethidium bromide. The products were purified using DNA Gel Extraction Kit (Beyotime Institute of biotechnology, China) following the instructions of the kit. DNA sequencing of the purified products was done by the Shanghai Sangon Biological Engineering Technology and Services co. Ltd, China. The sequences were blasted to databases using BlastN and the bacterial species identified on the basis of at least 99% similarity to NCBI database 16S rDNA sequences.

Inoculation of identified bacterial isolates + axenic PWN (ABX) into axenic black pine seedlings: The seven purified bacterial isolates were mixed with the ABX and used to inoculate 3 month-old aseptic, black pine seedlings as follows: 5 µl of a mixture of ca. 100 nematodes and the bacterial isolate, $5x10^4$ cells/ml, being tested were inoculated into the wound, made with a sterilized needle, on the top of each aseptic seedling. The inoculated seedlings were kept in an incubator at 28 ^{0}C under sterile conditions. Seedlings in the three control treatments received 5 µl sterilized water, 5 µl sterilized water containing 100 ABX nematodes or 5 µl sterile water with 100 wild type PWNs. There were nine seedlings in each treatment. Seedling symptoms were recorded every other day using the disease symptom classification given in "Inoculation experiment with axenic PWNs (ABX) + bacteria from black pine bark" above.

Results

Results of inoculation with axenic PWN (ABX) + bacteria from the black pine bark: The results of the inoculation experiment with ABX + the bacteria isolated from the bark of the black pine seedlings are shown in Table 1. The mean disease level for the ABX + bacteria (from pine bark) treatment reached 4.0 within 12 days post-inoculation. Within the same time frame no disease symptoms appeared on any of the seedlings in the two controls.

Toxicity of cell-free culture filtrate of bacteria to seedlings: The cell-free culture filtrates of all seven bacteria resulted in severe disease symptoms (Table 2). Among the seven filtrates, filtrate from *Stenotrophomonas maltophilia* (isolate No. 4) was the most toxic to pine seedlings reaching a disease rating of 4.0 within 3 days post-inoculation. Filtrate from the *Bacillus sp.* isolate (No. 2) was the least toxic to the pine seedlings of all the treatments, reaching a disease level of 4.0 at 8 days post-inoculation. Toxicity to the pine seedlings of the remaining isolates was: isolate No. 6 > isolate No. 3 > isolate No. 7 > isolate No. 4 and No. 1 > isolate No. 5.

Identification of the bacterial isolates by 16S rDNA sequences: Based on the NCBI database comparisons isolates No. 1, No. 2, No. 3, No. 5 belonged to the strains By253Ydz-fq, S209, 210-50 and 210-50 in the *Bacillus* genus, respectively, and isolate 4, No. 6, No. 7 belonged to the DN1.1 strain of *Stenotrophomonas maltophilia* (Table 3).

Inoculation of bacterial isolates and axenic PWN (ABX) into aseptically-grown, black pine seedlings: The results showed that all pine seedlings inoculated with ABX mixed with the seven isolates were dead within 18 days post-inoculation while all of seedlings inoculated with wild PWNs were dead within 16 days (Table 4). No disease symptoms developed on pine seedlings inoculated with either sterile water or ABX.

Discussion

Our results show that pathogenic bacteria exist in the forest ecosystem and they can induce PWD in association with the PWN. These results could explain why

Table 1. Mean disease level in days after inoculation of aseptic *Pinus thunbergii* seedlings with Axenic *Bursaphelenchus xylophilus* (ABX) and bacteria from black pine bark

	Mean disease level in days after inoculation of aseptic seedlings ± S. D.				
Inoculate	Day 4	Day 6	Day 8	Day 10	Day 12
Sterilized water	0.00±0.00a*	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
ABX**	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
ABX+B	1.40±0.16b	2.95±0.3b	3.6±0.16b	3.85±0.19b	4±0.00b

* Means in the same column followed by the same letter are not significantly different at α=0.05 according to Duncan's multiple range test.

TABLE 2. Mean disease level in days after seedling inoculation of cell-free filtrates of bacterial isolates into aseptic black pine seedlings

	Mean disease level in days after seedling inoculation ± S. D.						
Treatment	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Medium	0.00±0.00a*	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
Filtrate from isolate 1**	0.80±0.16bc	2.20±0.43b	3.70±0.35cd	4.00±0.00d	4.00±0.00c	4.00±0.00c	4.00±0.00b
Filtrate from isolate 2	0.20±0.16ab	0.40±0.23a	1.00±0.23b	1.90±0.12b	2.70±0.26b	3.40±0.33b	4.00±0.00b
Filtrate from isolate 3	1.10±0.42cd	2.85±0.66c	3.60±0.33c	4.00±0.00d	4.00±0.00c	4.00±0.00c	4.00±0.00b
Filtrate from isolate 4	2.10±0.48e	4.00±0.00d	4.00±0.00d	4.00±0.00d	4.00±0.00c	4.00±0.00c	4.00±0.00b
Filtrate from isolate 5	1.50±0.42de	2.50±0.20bc	3.40±0.33c	3.70±0.35c	4.00±0.00c	4.00±0.00c	4.00±0.00b
Filtrate from isolate 6	1.80±0.52e	2.65±0.44bc	4.00±0.00d	4.00±0.00d	4.00±0.00c	4.00±0.00c	4.00±0.00b
Filtrate from isolate 7	0.80±0.59bc	2.20±0.52b	3.70±0.12cd	4.00±0.00d	4.00±0.00c	4.00±0.00c	4.00±0.00b
water	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a

* Means in the same column with the same letter were not significantly different at α=0.05 according to Duncan's multiple range test.

** Isolates 1, 2, 3, and 5 have been identified as strains By253Ydz-fq, S209, 210-50 and 210-50 in the *Bacillus* genus, respectively and isolates 4, 6, and 7 as DN1.1 strains of *Stenotrophomonas maltophilia*.

aseptic PWNs can induce PWD under field conditions (Tamura, 1983). PWNs might acquire pathogenic bacteria from their parents or from the pine trees; the vertical transfer of the bacteria to the offspring from the parents is likely because adults and juveniles co-inhabit in the same host trees. Our results show that horizontal transfer of the bacteria from the forest ecosystem to PWNs as being another possible mode for PWNs to acquire bacteria. The finding of the possible horizontal transfer explains why the bacterial species carried by the PWN in China, Korea, Japan and Portugal are not the same (Zhao et al., 2003; Han et al., 2003; Kawazu and Kaneko, 1997; Hyeok et al., 2010; Kawazu et al., 1996a; Kawazu et al., 1996b; Proença et al., 2010). Differences in bacteria species associated with the PWN in certain area likely result from differences in the bacterial community structure in different forest ecosystem types. The differences in bacteria species associated with PWN could also explain the variation in the pathogenicity of the PWNs from different areas. For example, *Cedrus deodara* is susceptible in both the USA (Wingfield and Blanchette, 1982) and Japan (Mamiya, 1983) to local PWN, but not in China (Ge et al., 1992). Inoculation of PWN strains from both Japan and China into 2-year-old *C. deodara* trees produced in a nursery in LianYungan, Jiangsu Provice, China, resulted in 70% mortality of trees inoculated with PWNs from Japan compared to less than 10% mortality for trees inoculated with Chinese PWNs. However, inoculation of Chinese or Japanese PWN strains to 2-year-old *P. thunbergii* trees produced in the same nursery as the above *C. deodara* induced almost the same mortality, i.e. about 90% (Ju et al., 2007). Horizontal transfer of bacteria for PWN could allow PWN to exploit otherwise inaccessible new areas and host species or both, e.g. the local pine in China, *P. massoniana*. Mutualistic symbiosis between PWN and its associated bacteria and horizontal bacterial transfer might explain why PWN is one of the only two tree-pathogenic species in the genus *Bursaphelenchus* consisting over 75 species (Ryss et al., 2005).

The method used here to obtain sterile PWNs was based on our earlier findings that it is easier to sterilize PWN juveniles than adults (Zhang and Zhao 2010). We also found that the specific gravity of the juveniles is less than that of the adults, so we were able to use a new method that employed an acid burette containing 0.3% sodium carboxyl methylcellulose for separating juvenile from adult PWNs. The method to get axenic PWN in large scale by separating PWN adults from juveniles based on specific gravity difference is reported for the first time.

The fact that inoculation of pathogenic bacteria alone into black pine seedlings resulted in no host symptoms and no bacteria were recovered in inoculated seedlings (Han et al., 2003; Zhao et al., 2003; Kawazu et al., 1996a; and Kawazu et al., 1996b) demonstrates that the bacteria could not invade pine trees without PWN. Consequently,

TABLE 3. Identity of bacterial isolates using 16S rDNA sequence

Isolate No.	NCBI accession No.	Length of 16S rDNA gene sequence/bp	16S rDNA-based identification	Strain	Similarity%
1	EU070406.1	1453	*Bacillus sp.*	By253Ydz-fq	100
2	AB425363.1	1452	*Bacillus sp.*	S209	99
3	GQ199752.1	1451	*Bacillus sp.*	210_50	99
4	EU034540.1	1444	*Stenotrophomonas maltophilia*	DN1.1	99
5	GQ199752.1	1452	*Bacillus sp.*	210_50	99
6	EU034540.1	1447	*Stenotrophomonas maltophilia*	DN1.1	99
7	EU034540.1	1445	*Stenotrophomonas maltophilia*	DN1.1	99

TABLE 4. Mean disease level in number of days post inoculation with axenic *Bursaphelenchus xylophilus* (ABX) + the identified bacterial isolates into aseptic black pine seedlings

Treatment	The mean disease level in the days after inoculation to the axenic seedlings ± S. D.							
	Day 4	Day	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
Water	0.00±0.00a*	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
ABX**	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a
Wild PWN	0.50±0.10b	1.10±0.25c	1.90±0.10e	2.55±0.16c	3.00±0.16c	3.500±0.19d	4.00±0.28c	4.00±0.00b
ABX+isolate 1***	0.00±0.00a	0.10±0.12a	1.00±0.28c	1.45±0.53b	2.10±0.73b	2.70±0.62b	3.25±0.38b	4.00±0.00b
ABX+isolate 2	0.00±0.00a	0.20±0.00a	0.55±0.10b	1.25±0.10b	2.00±0.28b	2.80±0.23bc	3.55±0.10c	4.00±0.00b
ABX+isolate 3	0.15±0.10b	0.85±0.19bc	1.70±0.35de	2.00±0.23c	2.80±0.33c	3.25±0.38cd	3.70±0.19c	4.00±0.00b
ABX+isolate 4	0.20±0.00b	0.90±0.26bc	1.70±0.26de	2.35±0.34c	2.75±0.19c	3.30±0.26d	3.75±0.19c	4.00±0.00b
ABX+isolate 5	0.10±0.12ab	0.70±0.35b	1.50±0.35d	2.20±0.59c	2.90±0.38c	3.35±0.19d	3.80±0.16c	4.00±0.00b
ABX+isolate 6	0.40±0.16c	0.90±0.26bc	1.60±0.28de	2.20±0.16c	2.60±0.16c	3.10±0.26bcd	3.65±0.10c	4.00±0.00b
ABX+isolate 7	0.10±0.12ab	1.05±0.25c	1.85±0.34e	2.45±0.30c	2.95±0.19c	3.20±0.16cd	3.70±0.12c	4.00±0.00b

* Results in the same column with the same letter were not significantly different at the level of α=0.05 by Duncan tests.

** Abbreviation of aseptic *Bursaphelenchus xylophilus*.

*** Isolates 1, 2, 3, and 5 have been identified as strains By253Ydz-fq, S209, 210-50 and 210-50 in the *Bacillus* genus, respectively and isolates 4, 6, and as DN1.1 strains of *Stenotrophomonas maltophilia*.

in the present study we did not use the bacterial isolates alone as control for the inoculation experiments. Our future research will examine whether symbiotic relationship exists between the PWN and the pathogenic bacteria from the forest ecosystem to understand the role of symbiosis in PWD. Full genome sequences of PWN and its associated pathogenic bacteria will be necessary to fully understand the role of various nematode and bacterial genes in symbiosis and pathogenicity.

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