

Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against Mucoraceae and *Aspergillus* species: preliminary report

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A compound produced by *Bacillus pumilus* (MSH) that inhibits Mucoraceae and *Aspergillus* species is described. Fungicidal activity was demonstrated by lawn-spotting and by diffusion through 0.45 µm Millipore membranes placed on 5% sheep-blood agar, nutrient agar, trypticase soy agar and Mueller–Hinton agar, followed by spore inoculation of the bacterium-free underlying agar surface. With either technique, zones of fungal inhibition correlated with the zone of haemolysis produced by *B. pumilus* (MSH). The active compound inhibited *Mucor* and *Aspergillus* spore germination and aborted elongating hyphae, presumably by inducing a cell-wall lesion. Antifungal activity was stable in agar for a minimum of 8 days, resistant to Pronase degradation, and partially inactivated by chloroform exposure and at pH 5–6. Its molecular mass was determined by diffusion through dialysis membrane to be 500–3000 Da. Attempts at further isolation of the compound have proven unsuccessful to date.

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INTRODUCTION

In the search for anti-infectives produced by *Bacillus* species, especially *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*, several antifungal compounds, mainly peptides, have been described (Katz & Demain, 1977; Kugler *et al.*, 1990; Lebbadi *et al.*, 1994; Shosi, 1978; Silo-Suh *et al.*, 1994). While investigating the microbial flora of a dry, used, synthetic sponge by touch inoculation to 5% sheep-blood agar, the concomitant growth of *Bacillus pumilus* and a *Mucor* species occurred after 24 h incubation. In areas where *B. pumilus* colonies had serendipitously developed either adjacent to, or in the centre of, a *Mucor* colony, a zone of growth inhibition of the fungus was visible. This preliminary report describes some of the biological characteristics of the *B. pumilus* antifungal compound, including its activity against Mucoraceae and *Aspergillus* species, partial physiochemical characteristics and a suggested mode of action.

METHODS

The producer strain was identified as *Bacillus pumilus* (MSH) by Gram stain, cultural characteristics and by using API 20E and API CHB strips (bioMerieux-ViTek). The isolate that served as the initial indicator strain was characterized as a *Mucor* species by examining slide-culture preparations, demonstrating sporangia borne on sporangiophores that arose directly from a hyphal stem cell (Richardson & Shankland, 1995). Other bacterial and mycotic strains used to assess the spectrum of the *B. pumilus* (MSH) compound, as well as selected bacterial strains tested for potential antifungal activity, were isolated from clinical specimens. *B.*

pumilus (MSH) and other bacterial test organisms were grown on 5% sheep-blood agar for 24–48 h at 35 °C prior to testing, while mycotic isolates were grown on Sabouraud's agar for 48–72 h.

Assay for antifungal activity. The lawn-spotting technique was used to determine the antimicrobial spectrum of the *B. pumilus* (MSH) inhibitory substance (Raubitschek & Dostrovsky, 1950). The producer *B. pumilus* (MSH) strain was grown for 18–24 h on 5% sheep-blood agar (BBL-Microbiology Systems), after which several haemolytic colonies were selected and superimposed individually onto lawns of test mycotic and bacterial isolates. Spore suspensions of Mucoraceae and *Aspergillus* species were prepared by washing the growth from 48–72-h-old Sabouraud agar slants with sterile distilled water. Spores were dislodged by gentle rubbing of the mycelial growth with a sterile cotton-tipped swab. Prior to preparing a fungal lawn, the spore suspension was examined by phase-microscopy to ensure a predominance (> 99%) of spores. Lawns were prepared by swabbing the surface of sheep-blood agar and spotting with *B. pumilus* (MSH). Plates were incubated at 35 °C and examined after 24 h. Plates showing a zone of growth inhibition were observed for 2 weeks for evidence of breakthrough growth.

To determine whether the compound could diffuse through a sterile membrane filter and remain active, 0.45 µm Millipore membrane filter was placed onto 5% sheep-blood agar and layered with an agar suspension of *B. pumilus* (MSH) prepared by inoculating a loopful of 24-h-old *B. pumilus* growth into melted and cooled TSA. The plates were incubated upright at 35 °C for 18–24 h, after which the membrane containing the agar overlay was aseptically removed and the underlying cell-free agar showing a zone of beta-haemolysis was flooded with a *Mucor* or *Aspergillus* spore suspension and examined for growth inhibition after an additional 24 h incubation.

The stability of the compound in agar was determined by preparing six

membrane-diffusion plates as above. After removal of the membranes, the agar surfaces were flooded with *Mucor* spores at 24 h intervals for 96 h, and at 144 and 168 h, incubated and examined 24 h later for growth inhibition. Zone diameters were measured and recorded. While awaiting inoculation intervals, the diffusion plates were kept at 4 °C.

The morphological effect of the compound on *Mucor* and *Aspergillus* was determined by microscopic ($\times 400$, $\times 1000$) examination of the inhibition zone on 5% sheep-blood agar or on TSA surrounding a spot-inoculum of *B. pumilus* (MSH). The zone of clearing beneath membrane filters on TSA and 5% sheep-blood agar were also examined 24–48 h after spore inoculation of the fungal species. Punch biopsies of these zones were also obtained for electron microscopy.

Susceptibility of the compound to Pronase K (10 mg ml⁻¹) and to chloroform was determined by flooding individual haemolytic zones of clearing beneath removed membrane filters on TSA and 5% sheep-blood agar with Pronase for 1 h, and exposing a separate set of zones to chloroform-saturated gauze for 1 h. Afterward, the gauze was removed and the agar was aerated for 1 h and then both agar surfaces were flooded with *Mucor* spores and examined after 24 h for growth. Controls consisted of treating 5% sheep-blood agar with Pronase and chloroform, followed by flooding with *Mucor* spores.

To estimate the compound's molecular mass, sterile dialysis tubing strips with a molecular mass cut-off of 6–8 kDa and 12–14 kDa were placed on the surface of 5% sheep-blood agar. These were inoculated with *B. pumilus* (MSH) in agar. After 48 h incubation, the dialysis tubing was removed and the bacterium-free agar surface was flooded with *Mucor* spores, incubated for 24 h, and examined for inhibition of *Mucor*.

Because the compound was not detected in overnight broth filtrates, two concentration methods were used. Initially, overnight nutrient broth and trypticase soy broth filtrates were individually dialysed against 0.15 M ammonium acetate using dialysis tubing with a 500 Da pore size. The dialysate was then dried by lyophilization and reconstituted in fresh sterile broth and assayed for activity by performing two-fold dilutions in nutrient broth in microtitre wells and adding 0.1 ml of a *Mucor* spore suspension to each well. After overnight incubation at 35 °C, wells were examined microscopically for growth inhibition and hyphal aberration. Controls consisted of spore-inoculation of nutrient broth alone, and using a nutrient broth filtrate of *B. subtilis* treated as above.

The second attempt at concentration included extracting overnight nutrient broth or BHIB culture filtrates by vigorous mixing with either chloroform or ethyl acetate. The resulting organic phase was then dried and the residue was dissolved and tested for activity as above. As it was strongly suspected that the antifungal activity resided in the haemolysin of the *B. pumilus* (MSH) producing strain, *Mucor* and *Aspergillus* species were tested against a leaky, weakly haemolytic spontaneous mutant of the parent strain as described above.

RESULTS

The *B. pumilus* (MSH) antifungal-producing strain grew on 5% sheep-blood agar, producing circular, adherent and sticky pearlescent colonies surrounded by a zone of beta-haemolysis (Fig. 1). Colonies, which measured 3 mm after 48 h, were further distinguished by their rough striated texture and three concentric rings (Fig. 1). Gram-stained smears prepared from colonies showed slender, elongated Gram-positive rods with a centrally situated ellipsoidal spore that bulged the bacillary wall. The highly motile bacterium was identified as *B. pumilus* (MSH) by the reactions listed in Table 1. When spot-inoculated onto sheep-blood agar



Fig. 1. Pearlescent, rough colonies of *B. pumilus* (MSH) showing concentric rings of growth and surrounded by a zone of beta-haemolysis after 48 h incubation on 5% sheep-blood agar.

seeded with *Mucor* or *Aspergillus* spores, a well-circumscribed clear zone of fungal growth inhibition was evident after 24 and 48 h, respectively. Zone diameters around a 1.5 cm spot-inoculum of *B. pumilus* (MSH) averaged 2.2 cm for Mucoraceae (*Mucor*, *Rhizopus*, *Cunninghamella*) and 2 cm for *Aspergillus* species (*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus glaucus*) and coincided with the zone of beta-haemolysis produced by *B. pumilus* (MSH) (Fig. 2). Antifungal activity could also be demonstrated by lawn-spotting on TSA, brain-heart infusion agar (BHIA), nutrient agar and Mueller–Hinton (M–H) agar, but not on Sabouraud's glucose agar. On the latter medium, although *B. pumilus* (MSH) grew readily, antifungal activity was absent, perhaps due to the low pH (5.6) of the medium. The *B. pumilus* (MSH) inhibitory principle was fungicidal, as shown by the failure of *Mucor* and *Aspergillus* spore germination on subculture of the inhibitory zone immediately surrounding the *Bacillus* spot-inoculum.

The *B. pumilus* (MSH) inhibitory principle was inactive against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Clostridium perfringens*, *B. subtilis*, *B. cereus*, *Bacillus thuringiensis*, *Escherichia coli*, *Erwinia herbicola* and *Klebsiella pneumoniae*. Conversely, several beta haemolysin producing bacterial species including *B. subtilis*, *B. thuringiensis*, *B. cereus*, as well as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Streptococcus agalactiae* failed to inhibit *Mucor* as tested by lawn-spotting. Furthermore, an American Type Culture Collec-

Table 1. Characteristics of the *Bacillus pumilus* (MSH) isolate

+, Positive; –, negative. Non-physiological characteristics: Gram-positive, slender rods and filaments, motile with ellipsoidal central spores.

API 50 CHB tests	
Glycerol	+
Erythritol	–
D-Arabinose	–
L-Arabinose, ribose, D-xylose	+
L-Xylose	–
Adonitol	–
β-Methyl xyloside	–
Galactose, glucose, fructose, mannose	+
L-Sorbose, rhamnose	–
Dulcitol, inositol, sorbitol	–
Mannitol	+
L-Methyl-D-mannoside, D-methyl-D-glucoside, N-acetylglucosamine	+
Amygdalin, arbutin, aesculin, salicin, cellobiose	+
Maltose, lactose, sucrose, trehalose	+
Gentiobiose, melibiose, raffinose	–
Melezitose	–
Starch, glycogen, inulin	–
D-Turanose, D-tagatose	+
D-Fucose, L-fucose, D-lyxose	–
D-Arabitol, L-arabitol, xylitol	–
Gluconate, 2-ketogluconate, 5-ketogluconate	–
API 20E tests	
ONPG	+
ADH, LDC, ODC	–
Citrate, H ₂ S, urea, TDA, indole	–
Voges–Proskauer	+
Gelatin	+
Nitrate	–
Oxidase	+
Additional tests	
Catalase	+
Growth 6.5 % NaCl	+
Casein hydrolysis	+

tion (ATCC 7061) strain of the type species of *B. pumilus*, which was non-haemolytic, did not inhibit five tested isolates each of Mucoraceae and *Aspergillus*.

The antifungal inhibitory activity passed through 0.45 µm Millipore filters placed on agar surfaces and layered with *B. pumilus* (MSH) in TSA. Removal of the filter pad after 24 h incubation and flooding the underlying agar surface with a *Mucor* or *Aspergillus* spore suspension resulted in a zone of fungal inhibition corresponding to the area immediately beneath the *Bacillus*-agar overlay. On sheep-blood agar this zone coincided with the zone of beta-haemolysis beneath the membrane (Fig. 3), whereas on blood-free media (TSA, BHIA or M–H) the zone of inhibition was colourless but equivalent in diameter to that on sheep-blood agar.

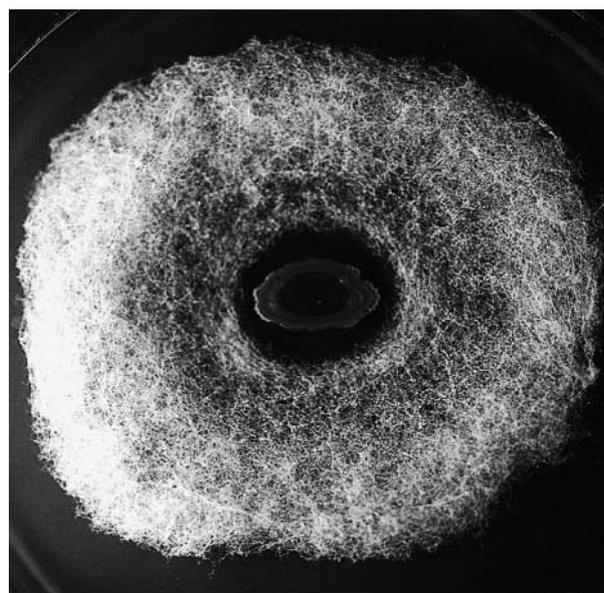


Fig. 2. Growth inhibition of *Mucor* species by spot-inoculation of *B. pumilus* (MSH) onto lawn of mucor spores on 5% sheep-blood agar. Zone of inhibition coincides with zone of beta-haemolysis. Similar results were obtained for *Aspergillus* species.

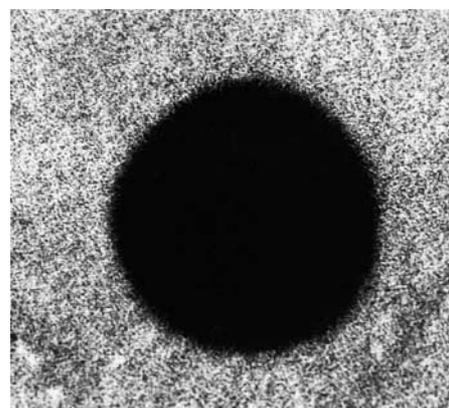


Fig. 3. Inhibition of *Mucor* species after diffusion of *B. pumilus* (MSH) antifungal through 0.45 µm membrane filter on 5% sheep-blood agar. Note zone of growth inhibition coincides with zone of beta-haemolysis.

Activity was stable in agar for a minimum of 8 days as shown by only a 1 mm decrease in the *Mucor* or *Aspergillus* zone-of-inhibition diameter after daily flooding of individually prepared plates with *Mucor* or *Aspergillus* spores. At no time did breakthrough growth of either fungus occur.

Microscopic examination of the *Mucor* zone of inhibition adjacent to the *B. pumilus* (MSH) spot-inoculum showed a gradation in *Mucor* hyphal germination and elongation across the growth-inhibition zone. Immediately adjacent to

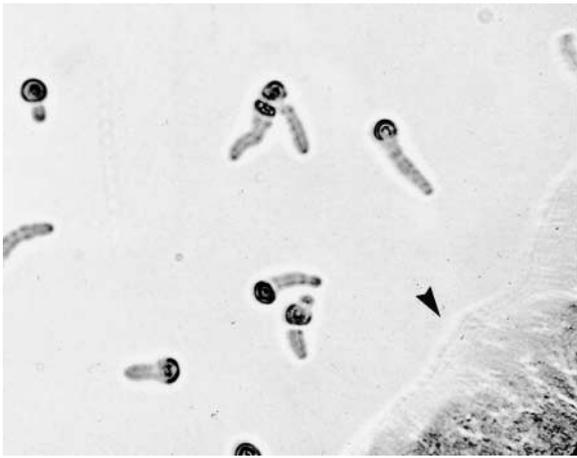


Fig. 4. Rudimentary *Mucor* spore germination and inhibition of hyphal elongation by *B. pumilus* (MSH) antifungal diffusing from spot inoculum (arrowhead) after 24 h incubation on Mueller-Hinton agar.

the spot-inoculum, rudimentary spore germination occurred with inhibition of hyphal elongation (Fig. 4). Further from the spot-inoculum, germinated hyphae were longer but were still inhibited and never traversed the inhibition zone. At the zone-of-inhibition perimeter, germinating spores oriented away from the spot-inoculum formed mycelial growth which spread away from the inhibition zone, whereas hyphae germinating from spores at the zone-of-inhibition border, but oriented so that the hyphae entered the inhibition zone, aborted as they approached the *B. pumilus* (MSH) spot-inoculum (Fig. 5). This same morphology was also noted on inhibition zones beneath membrane filters. *Aspergillus* spore germination was also rudimentary and nascent

hyphae became swollen with bulbous outpocketings. Electron microscopic analysis of punch biopsies of this zone showed hyphae with apparent breaks in their chitin wall.

By growing *B. pumilus* (MSH) in agar on dialysis tubing, the molecular mass of the antifungal compound was estimated to be greater than 500 Da but less than 3000 Da.

Concentrated supernatants of *B. pumilus* (MSH) inhibited *Mucor* sp. spore germination and hyphal elongation to a 1 : 16 dilution. However, as concentrated supernatants from *B. subtilis*, which were inactive against *Mucor* by lawn-spotting, were also inhibitory at a 1 : 8 dilution, non-specific inhibition of *Mucor* was suspected. Similar results were obtained after ammonium acetate elution and concentration of BHIB and nutrient-broth filtrates.

Treatment of haemolytic zones beneath Millipore membranes with chloroform, but not Pronase, resulted in partial inactivation of the *B. pumilus* (MSH) antifungal activity. Germination of inoculated *Mucor* spores onto the chloroform-treated zone, however, required 48 h of incubation, whereas *Mucor* growth occurred within 24 h on chloroform-treated control plates. When the leaky *B. pumilus* mutant was spot-inoculated onto lawns of *Mucor* or *Aspergillus* species, zone-of-inhibition diameters were distinctly smaller (≤ 1.5 cm) as contrasted to the parent *B. pumilus* (MSH) (~ 2.5 cm) strain.

DISCUSSION

Bacillus species produce 167 biological compounds active against bacteria, fungi, protozoa and viruses (Katz & Demain, 1977; Cordovilla *et al.*, 1993; Karuse *et al.*, 1990; Berdy, 1974). Most of the antibacterials are peptides and are active against Gram-positive species while compounds such as polymyxin and colistin are active against Gram-negative



Fig. 5. Inhibition of *Mucor* hyphal elongation advancing toward *B. pumilus* (MSH) spot inoculum (arrowhead) and those in area of graded concentration of antifungal compound. Mueller-Hinton agar 24 h incubation.

species. Several antifungal compounds synthesized by *Bacillus* species are active against filamentous fungi and yeasts. Recently, Munimbazi & Bullerman (1998a) described antifungal metabolites produced by *B. pumilus* grown in potato glucose broth, which inhibited mycelial growth (spore germination) of *Aspergillus*, *Penicillium* and *Fusarium* species, and aflatoxin production by *Aspergillus parasiticus* (Munimbazi & Bullerman, 1998b). There are, however, several differences between our *B. pumilus* (MSH) antifungal compound and that described by Munimbazi & Bullerman (1998a). *B. pumilus* (MSH) produces an antifungal metabolite that inhibits the growth of Mucoraceae and *Aspergillus* species including *A. flavus*, *A. fumigatus* and *A. terreus*. While no data were given by Munimbazi & Bullerman (1998a) regarding Mucoraceae, their *B. pumilus* compound was inactive against *A. flavus* and *A. terreus*, and was not tested against *A. fumigatus*. Additionally, in contrast to the *B. pumilus* metabolite found by Munimbazi & Bullerman (1998a), the *B. pumilus* (MSH) product does not inhibit *Fusarium* species.

Mechanistically, the *B. pumilus* (MSH) metabolite inhibits both spore germination and hyphal elongation, as well as newly germinated advancing hyphae. Preliminary electron microscopy of inhibited germinating hyphae suggests a cell-wall lesion that could lead to leakage of intracellular components and hyphal death. Such a mechanism of action could also account for inhibition of advancing hyphae upon contact with the *B. pumilus* (MSH) antifungal compound. The *B. pumilus* strain described by Munimbazi & Bullerman (1998a) inhibits spore germination without mention of hyphal inhibition.

The *B. pumilus* (MSH) antifungal principle appears to equate to the haemolysin produced by this strain, which is corroborated by a reduction in the zone of antifungal activity by the isogenic haemolysin-deficient *B. pumilus* and the complete absence of antifungal activity by the type strain of *B. pumilus*, ATCC 7061^T, which is non-haemolytic.

Our metabolite was inactive when tested on Sabouraud's glucose agar, which has a pH of 5-6, in contrast to that of Munimbazi & Bullerman (1998a) whose metabolite was active over a pH range of 2-10. Pronase treatment of haemolytic zones beneath membrane filters did not inactivate activity whereas chloroform exposure resulted in partial inactivation as manifested by breakthrough germination of *Mucor* spores 48 h after chloroform treatment. Taken in concert with an estimated molecular mass of between 500 and 3000 Da, our compound resembles other peptide antifungals produced by *Bacillus* species (Katz & Demain, 1977; Lebbadi *et al.*, 1994; Wakayama *et al.*, 1984), but it may also contain a lipid moiety.

Although our compound is stable in agar for a minimum of 8 days, we have been unable to isolate it in sufficient concentrations to perform activity, cytotoxicity and more definitive physicochemical studies. Perhaps the active molecule (haemolysin) requires a stabilizing carrier molecule, e.g. erythro-

cyte, spore/hyphae or agar, to maintain activity. When dissociated, activity is lost. The ability of the compound to remain active (stable) for more than 8 days in agar supports this concept.

Conclusions

We have described a compound produced by *B. pumilus* (MSH) that has fungicidal activity against Mucoraceae and *Aspergillus* spp. The compound is of low molecular mass and inhibits spore germination and hyphal elongation, rendering it versatile in potential prophylaxis against, and treatment of, opportunistic infections caused by the above angioinvasive fungal pathogens. Purification and molecular characterization of the compound could lead to development of an innovative antifungal in an area of clinical need. The *B. pumilus* (MSH) strain was deposited in the American Type Culture Collection as '*B. sinai*' ATCC 55692 prior to our obtaining a species designation, and has been accorded United States Patent Number 6,090,613.

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