Prevention of aflatoxin contamination by a soil bacterium of *Stenotrophomonas* sp. that produces aflatoxin production inhibitors

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A soil bacterium, designated strain no. 27, was found to produce aflatoxin-production inhibitors. The strain was identified as a species of the genus *Stenotrophomonas*, and was found to be closely related to *Stenotrophomonas rhizophila*. Two diketopiperazines, cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro), were isolated from the bacterial culture filtrate as main active components. These compounds inhibited aflatoxin production of *Aspergillus parasiticus* and *Aspergillus flavus* in liquid medium at concentrations of several hundred μM without affecting fungal growth. Both inhibitors inhibited production of norsolorinic acid, a biosynthetic intermediate involved in an early step of the aflatoxin biosynthetic pathway, and reduced the mRNA level of *aflR*, which is a gene encoding a key regulatory protein necessary for the expression of aflatoxin-biosynthetic enzymes. These results indicated that the inhibitors targets are present in early regulatory steps leading to AflR expression. Co-culture of strain no. 27 with aflatoxigenic fungi in liquid medium effectively suppressed aflatoxin production of the fungus without affecting fungal growth. Furthermore, application of the bacterial cells to peanuts in laboratory experiments and at a farmer’s warehouse in Thailand by dipping peanuts in the bacterial cell suspension strongly inhibited aflatoxin accumulation. The inhibitory effect was dependent on bacterial cell numbers. These results indicated that strain no. 27 may be a practically effective biocontrol agent for aflatoxin control.

INTRODUCTION

Aflatoxins are fungal secondary metabolites with very strong toxicity and carcinogenicity. Aflatoxigenic fungi, such as *Aspergillus parasiticus* or *Aspergillus flavus*, infect crops and accumulate aflatoxins in agricultural products grown in tropical and subtropical areas. Aflatoxin contamination in food and feed seriously affects human and animal health (Cary et al., 2011). Outbreaks of aflatoxin poisoning frequently occur and a large percentage of hepatocellular carcinoma cases worldwide are estimated to be attributable to aflatoxin exposure (Wild & Turner, 2002; Liu & Wu, 2010). Aflatoxins also have a serious impact on the agricultural economy worldwide (Wu & Khlangwiset, 2010). However, to date, there are few practical methods for preventing aflatoxin contamination, and it is therefore critical to develop effective methods for prevention.

The use of antifungal agents is one possible method for controlling aflatoxin contamination. However, there are few fungicides that are practically effective for aflatoxigenic fungi on crops (Abbas et al., 2009), and the use of fungicides can produce resistant strains. Because aflatoxins are produced as secondary metabolites, their production is not necessary for the growth of aflatoxin-producing fungi. Therefore, specific aflatoxin-production inhibitors that do not affect fungal growth may be useful for aflatoxin control without incurring a rapid spread of resistant fungal strains. To date, pesticides, microbial metabolites and plant constituents (Ten et al., 1983; Norton, 1997; Jayashree & Subramanyam, 1999; Mahoney & Molyneux, 2004; Yoshinari et al., 2007; Holmes et al., 2008; Sakuda, 2010; Jermnak et al., 2012) have been shown to inhibit aflatoxin production. However, practical experimental data are necessary to examine their usefulness as aflatoxin control agents in fields.
Microbes have also been assessed as biocontrol agents for preventing aflatoxin contamination (Palumbo et al., 2008; Bianchini & Bullerman, 2010; Reddy et al., 2010). Atoxicogenic strains of Aspergillus that can competitively exclude toxigenic strains from crops have been used for aflatoxin control (Brown et al., 1991; Cotty, 1994; Dorner & Cole, 2002), but some of atoxicogenic strains produce a toxic metabolite, cyclopiazonic acid (Abbas et al., 2011). In addition to the atoxicogenic strains, many micro-organisms have been shown to be effective for aflatoxin control under laboratory conditions, but they have not been used practically. Several bacteria such as *Nannocystis exedens*, *Bacillus pumilus*, *Pseudomonas syringae*, *Ralstonia paucula* and *Burkholderia cepacia* have been reported to inhibit aflatoxin production as well as fungal growth (Cotty, 1992; Munimbazi & Bullerman, 1997; Taylor & Draughon, 2001; Palumbo et al., 2006). *Streptococcus lactis* inhibited aflatoxin production without affecting fungal growth (Haskard et al., 2001). *Achromobacter xylosoxidans* inhibited production of norsolorinic acid and cyclo(L-Leu–L-Pro) was isolated as an aflatoxin-production inhibitor from the culture filtrate of the bacterium (Yan et al., 2004). Some yeast species such as *Kluveromyces*, *Candida* and *Pichia* also inhibited aflatoxin production or production of norsolorinic acid and aflatoxin (Paster et al., 1993; Hua et al., 1999; Yin et al., 2008). Some bacteria, *Flavobacterium aurantiacum*, *Mycobacterium fluoranthenivorans*, *Rhodococcus erythropolis* and *Mycococcus fulvus*, have been found to degrade aflatoxin (Teniola et al., 2005; Reddy et al., 2009; Zhao et al., 2011). Lactic acid bacteria such as *Lactobacillus rhamnosus* have been shown to have the ability of binding aflatoxin (Haskard et al., 2000; Peltonen et al., 2001; Wu et al., 2009).

We screened soil bacteria in search of inhibitors and microbes useful for aflatoxin control. We isolated a soil bacterium, which was designated strain no. 27 and found to be a species of the genus *Stenotrophomonas* sp., as a strain that produces aflatoxin-production inhibitors. Here, we describe the isolation and identification of aflatoxin-production inhibitors produced by strain no. 27, as well as the effects of the inhibitors and the bacterial cells on aflatoxin production, including the effects of the cells on aflatoxin contamination of peanuts during storage in a tropical area where aflatoxin contamination occurs naturally.

**METHODS**

**Fungal strains and culture conditions.** *A. parasiticus* NRRL 2999, *A. parasiticus* ATCC 24690 and *A. flavus* IFO 47798 were used as producers of aflatoxins B₁ and G₁, norsolorinic acid and aflatoxin B₁, respectively. Aflatoxins B₁ and G₁ are the predominant aflatoxins produced by strain NRRL 2999. These strains were maintained on potato dextrose agar (PDA; Difco) medium and subcultured monthly. A spore suspension was prepared from a 2-week-old culture at a concentration of 2.5 × 10⁷ c.f.u. ml⁻¹ and used as the inoculum.

**Culture conditions for strain no. 27.** Bacterial strain no. 27 was isolated from a soil sample from the Yayoi campus at the University of Tokyo (Yayoi 1-1-1, Bunkyo-ku, Tokyo, Japan); see Supplementary Methods available with the online version of this paper. The bacterium was maintained on Bennett agar medium containing of 1% glucose, 0.2% peptone, 0.1% meat extract and 0.1% yeast extract, pH 7.2, at 27 °C and subcultured at about monthly intervals. Cells of the bacterium were inoculated into Bennett medium (100 ml) in a 500 ml Erlenmeyer flask, and the flask was incubated at 27 °C for 2 days statically. This preculture (5 ml) was transferred into Bennett medium (100 ml) in a 500 ml Erlenmeyer flask, and the flask was statically incubated at 27 °C for an additional 5 days. The number of bacterial cells per ml was determined from the number of c.f.u. Identification of the bacterium was performed by TechnoSuruga Laboratory (Shizuoka, Japan).

**Isolation of the active components from the culture broth of strain no. 27.** After 5 days of cultivation in Bennett medium, two litres of culture broth of strain no. 27 were centrifuged at 5000 g for 30 min and the culture supernatant was applied to a charcoal column (Activated Charcoal, 100 g; Wako) packed with water and eluted stepwise with 500 ml 10, 25, 50 and 100% (v/v) ethanol. The 25% ethanol fraction was lyophilized and the residue (160 mg) was further purified by HPLC on a 250 mm × 10 mm (i.d.) Capcell pak C₁₈ column (Shiseido) with a gradient of 10–90% CH₃CN in water containing 0.1% trifluoroacetic acid over 30 min at a flow rate of 3 ml min⁻¹ by detection at 220 nm to obtain the active fraction (52 mg). The active fraction was further purified with a 250 mm × 4.6 mm i.d. Capcell pak C₁₈ column with a gradient of 0–30% CH₃CN in water containing 10 mM CH₃COONH₄ (pH 8.9) over 25 min at a flow rate of 0.8 ml min⁻¹ with detection at 220 nm to obtain the active fraction (28 mg), which was finally purified by reverse-phase HPLC on the same column used for the second HPLC purification with a gradient of 0–20% CH₃CN in water containing 10 mM CH₃COONH₄ (pH 8.9) over 20 min at a flow rate of 0.8 ml min⁻¹ with detection at 220 nm to obtain the active component 1 (retention time: 13.8 min; yield: 2.8 mg). Component 1; electrospray ionization time of flight mass spectrometry (ESI-TOF MS) m/z 169 (M + H)⁺; [z]D¹₈ = −140 (c = 0.19, ethanol); ¹H (D₂O, 500 MHz): 4.38 (1H, m), 4.33 (1H, m), 3.50–3.61 (2H, m), 2.31–2.39 (1H, m), 1.94–2.11 (3H, m), 1.43 (3H, d, J = 7 Hz).

The 50% ethanol fraction was lyophilized and the residue (145 mg) was partitioned with ethyl acetate (100 ml) and aqueous 5% NaHCO₃ (30 ml). The ethyl acetate layer was washed with aqueous 5% NaHCO₃ (30 ml × 2), dried with anhydrous Na₂SO₄ and evaporated to dryness. The residue (18 mg) was further purified by reverse-phase HPLC on a 250 mm × 4.6 mm i.d. Capcell pak C₁₈ column with a gradient of 5–30% CH₃CN in water containing 0.1% trifluoroacetic acid over 25 min at a flow rate of 0.8 ml min⁻¹ with detection at 220 nm to obtain the active component 2 (retention time: 17.8 min; yield: 3.2 mg). Component 2; ESI-TOFMS m/z 197 (M + H)⁺; [z]D¹₈ = −150 (c = 0.065, ethanol); ¹H (D₂O, 500 MHz): 3.32 (1H, m), 4.17 (1H, br.s), 3.56–3.66 (2H, m), 2.44 (1H, m), 2.37 (1H, m), 1.90–2.13 (3H, m), 1.09 (3H, d, J = 7 Hz), 0.88 (3H, d, J = 7 Hz).

**Analysis of the concentrations of cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro) in a culture filtrate of strain no. 27.** The culture broth of strain no. 27 was filtered by being passed through a 0.25 μm sterile filter to obtain the culture filtrate. The culture filtrate (0.2 ml) was subjected to reverse phase HPLC on a 250 mm × 4.6 mm i.d. Capcell pak C₁₈ column with a gradient of 0–30% CH₃CN in water containing 10 mM CH₃COONH₄ (pH 8.9) over 25 min at a flow rate of 0.8 ml min⁻¹ by detection at 220 nm to obtain two fractions having retention times from 11.2 to 12.2 min.

**Identification of the bacterium.** Identification of the bacterium was performed by TechnoSuruga Laboratory (Shizuoka, Japan).
containing 0.1% trifluoroacetic acid over 25 min at a flow rate of 0.8 ml min\(^{-1}\) and detection at 220 nm [retention time of cyclo(- Ala–I-Pro) and cyclo(-Val–I-Pro): 11.8 and 18.2 min, respectively].

**Analysis of mycelial weight and production of aflatoxin and norsolorinic acid.** 
An aqueous solution of each sample was added to 1 ml potato dextrose liquid medium in a microplate well at the indicated concentrations. The spore suspension described above for strain NRRL 2999 or IFM 47798 was inoculated into the medium and statically incubated for 3 days at 27 °C. The culture broth of each well was then filtered through miracloth to obtain the mycelia and culture filtrate. The mycelia were washed with 5 ml distilled water and collected in a 1.5 ml microtube. After drying the mycelia at 100 °C for 3 h, the mycelial weight was calculated by subtracting the weight of a 1.5 ml microtube without mycelia from the total weight. To analyze the concentration of aflatoxins B\(_1\) and G\(_1\) in the culture filtrate, the filtrate (1 ml) was extracted with 200 μl chloroform, and the chloroform solution was evaporated to dryness using a centrifugal evaporator. The residue obtained was dissolved in 100 μl 90% acetonitrile in water and subjected to LC/MS analysis using a 2695 HPLC system (Waters) equipped with a Capcell-Pak C18 column, 4.6 mm i.d.

**Inhibition of aflatoxin production by co-culture of strain no. 27 with an aflatoxigenic fungus and that of the culture filtrate of the co-culture broth.** 
Each culture broth in a microplate well obtained by the co-culture of strain no. 27 with an aflatoxigenic fungus and that of the culture filtrate of strain no. 27 was filtered by being passed through a 0.25 μm filter to obtain a culture filtrate. The culture filtrate (0.5 ml) of each well was transferred into a new well containing 0.5 ml potato dextrose liquid medium in a microplate well. A spore suspension of strain NRRL 2999 or IFM 47798 was inoculated into the medium and incubated for 3 days at 27 °C. Mycelial weight and aflatoxins involved in each well were examined using the same methods as described above.

**Quantitative real-time PCR (qRT-PCR) analysis of the genes encoding proteins responsible for aflatoxin biosynthesis.** 
Strain NRRL 2999 was cultured for 3 days under the culture conditions described above with or without an inhibitor at the indicated concentrations. The culture broth was filtered to obtain the mycelia. The mycelial cake was homogenized in liquid nitrogen and total RNA was obtained from the homogenates by using TRizol Reagent (Invitrogen). First-strand cDNA was prepared with the SuperScript III First Stand Synthesis System (Invitrogen), using random hexamer primers, according to the following protocol. A solution (11 μl) containing 1 μg extracted RNA, 50 ng random hexamer and 10 mM dNTP mixture in 200 mM Tris/HCl (pH 8.4) and 500 mM KCl was reacted at 63 °C for 5 min and cooled on ice for 1 min. After adding 1 μl 10 × RT buffer, 2 μl 0.1 M DTT, 4 μl 25 mM MgCl\(_2\) and 0.5 μl Super Script III RT (200 U μl\(^{-1}\)) (Invitrogen) to the reaction solution, the solution was incubated as follows: 25 °C for 10 min; 50 °C for 50 min; 85 °C for 5 min. The cDNA derived from 1 μg total RNA was used as a template. qRT-PCR was carried out using the SYBR Green Master Mix (Applied Biosystems), in a final volume of 25 μl for each reaction, and an ABI PRISM 7300 thermal cycler (Applied Biosystems). The two-step PCR conditions were as follows: after an initial incubation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. All primers were designed with Primer Express Software Version 3.0 (Applied Biosystems). The specificity of each primer was checked by analysing a melting curve of the PCR product. A clear single peak was observed for each PCR product. The PCR primers for each gene were as follows: aflR, 5'-GGCTGGTCTCAAGGCAAAAC-3' and 5'-CCCGGATATTCCGAATCGG-3'; pkas, 5'-TGCAATGCGATGTTAGT-3' and 5'-GTAAAGCGGCGGAAGAACG-3'; mnaT, 5'-GGCCATATCCGCGGACT-3' and 5'-CCATGACCACTACCTAAAT-3'; β-actin (control gene), 5'-AGAGGAATTTGCGTCCTTCG-3' and 5'-TTGAGACCACCTTGCGTGA-3'. The amount of each mRNA was normalized to the amount of β-actin mRNA in each sample. The mean mRNA level of expression relative to that of aflR, pkas and mnaT in the control medium was defined as a percentage.

**Aflatoxin degradation activity of the culture filtrate of strain no. 27.** 
The culture broth of strain no. 27 was filtered by being passed through a 0.25 μm filter to obtain a culture filtrate. The culture filtrate (0.5 ml) of each well was transferred into a new well containing 0.5 ml potato dextrose liquid medium in a microplate well. A spore suspension of strain NRRL 2999 or IFM 47798 was inoculated into the medium and incubated for 3 days at 27 °C. Mycelial weight and aflatoxins involved in each well were examined using the same methods as described above.

**Aflatoxin degradation activity of strain no. 27.** 
The culture broth of strain no. 27 was filtered by being passed through a 0.25 μm filter to obtain the culture filtrate. The culture filtrate (0.9 ml) was added to the wells of 24-well microplates, and PD liquid medium (0.1, 0.3, 0.5 or 0.9 ml per well) was added to the wells containing the culture filtrate (final volume was 1 ml per well). Aflatoxins B\(_1\) and G\(_1\) were added to the solution at a concentration of 0.5 μg ml\(^{-1}\) each. The microplates were kept in the dark at 37 °C for 72 h and 0.5 ml of the solution from each well was extracted with 200 μl chloroform. The chloroform solution (100 μl) was transferred into a 1.5 ml microtube and evaporated to dryness. The residue obtained was dissolved in 100 μl 90% acetonitrile in water and then subjected to LC/MS analysis.

**Peanut experiments in the laboratory.** 
Raw peanuts without shells were purchased from Qingdao Planter Import and Export (China). Each peanut weighed approximately 1 g. A 10 ml volume of distilled
water was added to a 100 ml Erlenmeyer flask together with 30 kernels of raw peanuts and the flask was autoclaved at 120 °C for 15 min. Each of the autoclaved peanuts was dipped in the sample solutions [control (no treatment) and cell suspension of strain no. 27 containing a various cell numbers]. Each peanut was transferred into one well of a 24-well microplate and inoculated with a spore suspension of A. parasiticus NRRL 2999 or A. flavus IFM 47798 (10 µl per kernel). The plates were then statically incubated at 27 °C for 3 days. After incubation, each peanut kernel was transferred to a vial (16.5 mm in diameter × 40 mm in height) and crushed well with a spatula after addition of 2.0 ml chloroform. The chloroform solution (0.1 ml) was transferred into a 1.5 ml microtube and air-dried, and then the residue obtained was dissolved in 100 µl 90 % acetonitrile in water. After being passed through a 0.25 µm sterile filter, the solution was subjected to LC/MS analysis.

**Peanut experiment in a storage room in Thailand.** Peanuts were purchased from a local farmer in Nakorn Ratchasima Province, Thailand. The peanuts were harvested and dried during the middle of March 2012 by the farmer. The moisture content of the peanut seed was around 12–17 % (Duangpatra & Pumdeeying, 2002). The experimental schedule is shown in Fig. S1. The peanuts were divided into six experimental groups with 60 kg in each group. Each group was further divided into six subgroups with 10 kg in each group. Peanuts of each subgroup, except for the control 1 group, were dipped in 8 l water (control 2) or a cell suspension of strain no. 27 containing the indicated cell numbers using a bucket and basket. The peanuts of each subgroup of the control 1 group were collected immediately and used as a control sample at day zero. Peanuts of each subgroup from the other five groups were dried in the storage room overnight, packed in a sack and stored in the storage room for three weeks. The temperature and humidity of the storage room were approximately 34–39 °C and 40–50 %, respectively, during storage. Samplings were done twice, immediately after packing and after three weeks of storage. At each sampling, 1 kg of peanuts was collected from each sack and used for LC/MS analysis. A 1 kg sample of peanuts from each sack was shelled manually and ground to obtain peanut paste. A 50 g sample of the homogenized peanuts was then extracted with 100 ml of chloroform and the mixture was filtered to obtain a chloroform solution. This procedure was repeated five times with the homogenized peanuts from each sack. The solution (3 ml) was transferred into a 5 ml microtube and air-dried, and the residue obtained was dissolved in 100 µl of 90 % acetonitrile in water. After being passed through a 0.25 µm sterile filter, the solution was subjected to LC/MS analysis.

**Data analysis.** Data are presented as the mean± SD. Differences between groups were assessed by one-way ANOVA followed by Dunnett’s test. Values of P<0.05 were considered to be significant.

**RESULTS**

**Isolation and identification of strain no. 27**

Strain no. 27 was isolated from soil during our search for bacteria that produce an aflatoxin-production inhibitor. The culture filtrate of strain no. 27 inhibited aflatoxins B<sub>1</sub> and G<sub>1</sub> production of A. parasiticus and aflatoxin B<sub>1</sub> production of A. flavus in a dose-dependent manner (Fig. 1). Strain no. 27, a rod-shaped Gram-negative bacterium, was identified as Stenotrophomonas sp. by morphological and biochemical analysis as well as a comparison of its 16S rDNA sequence with those in a database (Fig. S2 and Table S1). The 16S rDNA sequence of strain no. 27 was most similar to that of Stenotrophomonas rhizophila (99.5 % identity).

**Isolation and identification of aflatoxin production inhibitors produced by strain no. 27**

The culture filtrate of strain no. 27 was purified by charcoal column chromatography. Aflatoxin production inhibitory activity was observed in the 25 and 50 % ethanol eluate fractions. The 25 % ethanol fraction was further purified by reverse-phase HPLC to obtain active component 1. The 50 % ethanol fraction was purified by partitioning with ethyl acetate and an aqueous 5 % NaHCO<sub>3</sub> solution. The ethyl acetate fraction was further purified by reverse-phase HPLC to obtain active component 2. Using the purification procedure, 2.8 and 3.2 mg of active components 1 and 2 were obtained from 2 l of the culture broth, respectively. The <sup>1</sup>H NMR spectrum of the active component 1 (Fig. S3) suggested the presence of one residue each of alanine and
proline in component 1. The \((M+H)^+\) ion peak at \(m/z\) 169 in the ESI-TOF mass spectrum of component 1 strongly indicated that it was a diketopiperazine, consisting of Ala and Pro [cyclo(Ala–Pro)]. Four possible stereoisomers of cyclo(Ala–Pro) were prepared (see Supplementary Methods) to identify the structure of active component 1. The \(^1\text{H}\) NMR spectrum and retention time on HPLC of component 1 were identical to those of cyclo(l-Ala–l-Pro) or cyclo(d-Ala–d-Pro) and were different from those of cyclo(l-Ala–d-Pro) or cyclo(d-Ala–l-Pro), indicating that active component 1 was cyclo(l-Ala–l-Pro) or cyclo(d-Ala–d-Pro). Based on the optical rotation value of component 1 and the results of the aflatoxin production inhibitory activity described below, active component 1 was identified as cyclo(l-Ala–l-Pro) (Fig. 2). Active component 2 was identified as cyclo(l-Val–l-Pro) (Fig. S4) based on the \(^1\text{HNMR}\), optical rotation data as well as comparison of the data, retention time on HPLC and aflatoxin production inhibitory activity described below with those of the authentic sample.

**Aflatoxin production inhibitory activity of the inhibitors and related compounds**

Cyclo(l-Ala–l-Pro) inhibited production of aflatoxins B\(_1\) and G\(_1\) by *A. parasiticus* with IC\(_{50}\) values of 0.75 mM and 0.68 mM, respectively, without affecting fungal mycelial weight (Fig. 3a). Cyclo(l-Ala–l-Pro) also inhibited aflatoxin B\(_1\) production of *A. flavus* with an IC\(_{50}\) value of 0.68 mM (Fig. 3b). None of the stereoisomers [cyclo(d-Ala–d-Pro), cyclo(l-Ala–d-Pro), or cyclo(d-Ala–l-Pro)] showed significant inhibitory activity (Figs S5 and S6). The inhibitory activity of cyclo(l-Val–l-Pro) was almost the same as that of cyclo(l-Ala–l-Pro) (IC\(_{50}\) values for aflatoxins B\(_1\) and G\(_1\) production of *A. parasiticus* and aflatoxin B\(_1\) production of *A. flavus*: 0.8, 0.7 and 0.75 mM, respectively) (Fig. S7a and b). Both cyclo(l-Ala–l-Pro) and cyclo(l-Val–l-Pro) inhibited norsolorinic acid production by *A. parasiticus* ATCC 24690 in a dose-dependent manner with IC\(_{50}\) values of 1.8 mM and 0.8 mM, respectively, without affecting fungal mycelial weight (Figs 3c and S7c). Cyclo(l-Ala–l-Pro) did not affect the conidiation of *A. parasiticus* (data not shown). Cyclo (Gly–l-Pro) did not inhibit aflatoxin production of *A. parasiticus* and *A. flavus* at any of the concentrations tested (Fig. S8).

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**Fig. 2.** Structures of cyclo(l-Ala–l-Pro) and cyclo(l-Val–l-Pro).

**Fig. 3.** Effects of cyclo(l-Ala–l-Pro) on growth and aflatoxins production of *A. parasiticus* (a) and *A. flavus* (b) and on growth and norsolorinic acid production of *A. parasiticus* ATCC 24690 (c): grey bars, aflatoxin B\(_1\) production; black bars, aflatoxin G\(_1\) production; white bars, norsolorinic acid production; ▲, mycelial weight. Data are represented as mean±SD (n=6). Differences between groups were assessed by one-way ANOVA followed by Dunnett’s test. *P* <0.05, vs control.

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**Co-culture of strain no. 27 with an aflatoxigenic fungus**

The effects of co-culturing strain no. 27 with an aflatoxigenic fungus on aflatoxin production were examined in liquid medium. *A. parasiticus* or *A. flavus* was cultured in 1 ml medium containing culture broth of strain...
no. 27 (0.25–2.0 µl containing 1.2 × 10⁸–9.6 × 10⁸ cells). By this co-culturing, strain no. 27 inhibited aflatoxin production of A. parasiticus and A. flavus in a bacterial-cell-number-dependent manner (Fig. 4). Aflatoxin production of A. flavus was completely inhibited by the addition of 2 µl bacterial culture broth (9.6 × 10⁸ cells) but the mycelial weight of the fungus was not affected significantly. The co-culture broth was filtered to obtain the culture filtrate without bacterial cells and fungal mycelia. Aflatoxin-production-inhibitory activity of the culture filtrate that was prepared from each culture broth obtained by co-culture was examined. The culture filtrate from the co-culture of 2 µl bacterial culture broth with A. flavus inhibited aflatoxin production of A. flavus as strongly as the culture filtrate of strain no. 27 (Figs 5 and 1b). The former and latter culture filtrates contained cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro) at almost the same concentrations (0.24 and 0.15 mM and 0.20 and 0.13 mM, respectively).

**Fig. 5.** Effects of culture filtrate (500 µl) from co-culture broth on growth and aflatoxin production of A. flavus: grey bars, aflatoxin B₁ production; ▲, mycelial weight. Data are represented as mean ± SD (n=5). Differences between groups were assessed by one-way ANOVA followed by Dunnett’s test. *P<0.05, vs control.

**Effects on the transcription of genes encoding proteins required for aflatoxin biosynthesis**

Biosynthetic genes for aflatoxins are clustered in a region of DNA of A. parasiticus and A. flavus. The cluster contains not only genes encoding biosynthetic enzymes, but also a regulatory gene, aflR, whose product regulates transcription of most genes encoding biosynthetic enzymes (Yu et al., 2004). Expression of AflR is absolutely necessary for initiation of aflatoxin biosynthesis and is regulated through environmental and nutritional factors as well as some key regulatory proteins (Woloshuk et al., 1994), but the exact molecular mechanism for the regulation remains unclear. The effects of co-culture of strain no. 27 on mRNA levels of aflR and two genes (omtA and pksA) encoding biosynthetic enzymes in A. parasiticus were examined. Their mRNA levels were suppressed by co-culture with strain no. 27 (Fig. 6). Cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro) also repressed mRNA levels of the three genes in a dose-dependent manner (Figs 7 and 8).

**Aflatoxin degradation activity of strain no. 27**

It has been previously reported that the culture supernatant of Stenotrophomonas maltophilia showed aflatoxin B₁-degrading activity (Guan et al., 2008). Therefore, aflatoxin-degradation activity of the culture filtrate of strain no. 27 was examined. When aflatoxins B₁ and G₁ were incubated in a solution containing the culture filtrate of strain no. 27, the concentration of the aflatoxins did not decrease (data not shown). When the aflatoxins were incubated with culture broth of strain no. 27 in liquid medium, no decrease in concentration was observed (Fig. S9). These results indicated that strain no. 27 does not possess aflatoxin-degradation activity.
Experiments with peanuts at a storage room in Thailand

The effects of strain no. 27 on aflatoxin contamination in peanuts were tested at a storage room in Thailand. In Thailand, peanuts are stored at farmers’ warehouses after harvest for several weeks (Duangpatra et al., 2005). Severe aflatoxin accumulation in the peanuts often occurs during storage (Sukharomana & Dobkuntod, 2003). A. flavus is predominantly present as an aflatoxigenic fungus in Thailand (Ehrlich et al., 2007). Before the experiments were conducted in the peanut storage room, a model
experiment with peanuts was performed in the laboratory. After autoclaving a raw peanut, the peanut was dipped in a cell suspension of strain no. 27 with various cell numbers. The peanut was then inoculated with spores of *A. flavus* and incubated for 3 days. The level of aflatoxin produced in the peanuts was significantly reduced by the presence of the strain in a bacterial-cell-number-dependent manner (Fig. 9). Similar experiments were conducted in a peanut storage room in Thailand. After harvesting and drying the peanut under sunlight, peanuts with shells were dipped in the bacterial cell suspension of various cell numbers, dried overnight, packed in a sack and kept in the storage room for three weeks. Fig. 10 shows the percentages of increased aflatoxin levels in the peanuts during storage. Cells of strain no. 27 strongly suppressed aflatoxin accumulation in the peanuts in a bacterial-cell-number-dependent manner compared with controls.

**DISCUSSION**

Strain no. 27, isolated in this study, was found to produce selective aflatoxin-production inhibitors. Analysis of the 16S rDNA sequence indicated that strain no. 27 is a member of the genus *Stenotrophomonas* and most closely related to *Stenotrophomonas rhizophila*. The *Stenotrophomonas* species are often found in association with plants, but are not phytopathogenic. They are promising candidates for biotechnological applications in agriculture due to their beneficial functions, such as enhancing plant growth and bioremediation (Ryan et al., 2009). In particular, *S. rhizophila* is suitable for application due to its non-pathogenic properties.

Two diketopiperazines, cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro), were isolated from the 25 % and 50 % ethanol fractions from charcoal column chromatography, respectively. Each compound was obtained as the main active component from each fraction. If the activity of the culture filtrate is due to the activity of the two inhibitors, the total amount of the two compounds in one litre of culture filtrate is estimated to be more than 100 mg. However, HPLC analysis showed that the culture filtrate contained cyclo(L-Ala–L-Pro) and cyclo(L-Val–L-Pro) at approximately 37 mg l$^{-1}$ (0.2 mM) and 25 mg l$^{-1}$ (0.13 mM), respectively. A synergistic effect of the two compounds was not observed (data not shown). Therefore, it may be suggested that other active components or

![Fig. 8. Effects of cyclo(L-Val–L-Pro) on the transcription of aflR (a), pksA (b) and omtA (c): the mean level of mRNA expression relative to that of aflR, pksA and omtA in the control medium was defined as a percentage. Data are presented as the mean±SEM (n=6). Differences between groups were assessed by one-way ANOVA followed by Dunnett’s test. *P<0.05, vs control.](image1)

![Fig. 9. Effects of strain no. 27 on aflatoxin B$_1$ production of *A. flavus*. Data are represented as the mean±SD (n=12 peanut kernels/group). Differences between groups were assessed by one-way ANOVA followed by Dunnett’s test. *P<0.05, vs control.](image2)
synergistic effects among active components are present. However, no other active fractions were identified in this study. The two diketopiperazines were partly sublimated during lyophilization, and the low yields of the compounds after isolation may be mainly due to repeated lyophilization during purification.

Cyclo(1-Ala–1-Pro) and cyclo(1-Val–1-Pro) are widely found as natural products in a number of organisms such as bacteria, fungi and plants as well as foods and drinks, including coffee and beer (Pickenhagen et al., 1975; van der Greef et al., 1987; Trigos et al., 1996; Holden et al., 1999; Wang et al., 2010). They have a variety of biological activities, such as antimicrobial, anti-germination, anti-weed, anti fouling and auxin-like activities (Li et al., 2006; Campbell et al., 2009; Oliveira et al., 2009; Qi et al., 2009; Ortiz-Castro et al., 2011). This study is the first, to our knowledge, to report the isolation of cyclo(1-Ala–1-Pro) from Stenotrophomonas sp. and demonstrate its aflatoxin-production-inhibitory activity. Cyclo(1-Leu–1-Pro) produced by A. xylosoxidans is known to inhibit aflatoxin production of A. parasiticus selectively in liquid medium (Yan et al., 2004). The aflatoxin-production-inhibitory activity of cyclo(1-Leu–1-Pro) was not stronger than that of cyclo(1-Ala–1-Pro) or cyclo(1-Val–1-Pro). It has been reported that cyclo(1-Leu–1-Pro) inhibited aflatoxin production as strongly as cyclo(1-Leu–1-Pro), whereas only cyclo(1-Ala–1-Pro) showed significant aflatoxin-production-inhibitory activity among stereoisomers of cyclo(Ala–Pro). Yan et al. (2004) showed that cyclo(1-Leu–1-Pro) suppressed norsolorinic acid production and aflR transcription and that synthetic cyclo(1-Val–1-Pro) inhibited the aflatoxin production of A. parasiticus. Therefore, these diketopiperazines showing selective aflatoxin-production-inhibitory activity may be good lead compounds for developing practical pesticides that prevent aflatoxin contamination due to the ease of preparation of these compounds as well as their derivatives.

The inhibition of norsolorinic acid production by cyclo(1-Ala–1-Pro) and cyclo(1-Val–1-Pro) indicated that both compounds inhibited an early step of the aflatoxin biosynthetic pathway. Furthermore, these compounds clearly reduced the mRNA levels of aflR, pksA and omtA. Therefore, they may inhibit aflatoxin production by affecting a pathway present upstream of AflR expression or by directly affecting expression of the aflR gene. Identification of their target molecule is very important not only for investigating the regulatory mechanisms of fungal secondary metabolism, but also for developing a more effective pesticide for aflatoxin control.

Strong aflatoxin production inhibition was observed when strain no. 27 was co-cultured with aflatoxigenic fungi in liquid medium. The culture filtrate from the co-culture broth also inhibited aflatoxin production and contained almost the same concentrations of cyclo(1-Ala–1-Pro) and cyclo(1-Val–1-Pro) as the culture filtrate from strain no. 27. Furthermore, we found that the strain did not have aflatoxin degradation or absorption capabilities. These results indicate that the diketopiperazines may play an important role in aflatoxin production inhibition by the bacterial cells.

In Thailand, peanuts and peanut products are the most severely aflatoxin-contaminated foods among all food and food products (Thasnakorn, 1976). Aflatoxin contamination in peanuts mainly occurs during the drying process and warehouse storage, particularly during the late rainy or early dry seasons (Wanenlor & Wiwanitkit, 2003). Temporary storage of peanuts that are not sufficiently dried for one to six weeks in farmers’ warehouses can cause severe contamination. Therefore, appropriate post-harvest handling is necessary to reduce aflatoxin contamination in peanuts. In this study, we evaluated the application of strain no. 27 to peanuts during storage in a farmer’s warehouse in Thailand in order to evaluate the strain’s ability to prevent aflatoxin contamination. The culture broth of strain no. 27 was very effective at reducing aflatoxin contamination of peanuts stored in their shells, indicating that the strain may be useful as a biocontrol agent for preventing aflatoxin contamination. This is the first report, to our knowledge, in which the practical effectiveness of a bacterium that produces aflatoxin-production inhibitors is shown. We are now planning experiments to test the effectiveness of not only strain no. 27 but also cyclo(1-Ala–1-Pro) and cyclo(1-Val–1-Pro) for preventing aflatoxin contamination in various crops.

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