RESEARCH ARTICLE

Citrinin, a mycotoxin from *Penicillium citrinum*, plays a role in inducing motility of *Paenibacillus polymyxa*

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Abstract

Paenibacillus polymyxa, a Gram-positive low-G+C spore-forming soil bacterium, belongs to the plant growth-promoting rhizobacteria. The swarming motility of *P. polymyxa* strain E681 was greatly induced by a secondary metabolite, citrinin, produced by *Penicillium citrinum* KCTC6549 in a dose-dependent manner at concentrations of 2.5–15.0 $\mu g\,mL^{-1}$ on tryptic soy agar plates containing 1.0% (w/v) agar. Flagellum staining showed that citrinin activated the production of flagella by *P. polymyxa*. This result was supported by reverse transcriptase-PCR analysis of gene expression, which showed increased transcriptional levels of *sigD* and *hag* homologues of *P. polymyxa* E681 in the presence of citrinin. The results presented here show that a mycotoxin, citrinin, has a newly identified function of inducing bacterial motility by transcriptional activation of related genes. This finding contributes to our understanding of the interactions between bacteria and fungal strains in nature.

Introduction

Agricultural ecosystems contain a wide variety of microorganisms that play an integral role in plant growth, health, and productivity. In the past two decades, significant progress has been made in understanding the mechanisms of plant growth promotion and disease suppression by introduced bacteria, but the bacterial traits involved in the process of rhizosphere competence remain poorly understood. To achieve successful and reproducible performance, knowledge of the ecological interactions taking place in soil and root environments is required to predict the conditions under which biocontrol can be achieved (Whipps, 2001; Duffy *et al*., 2003).

In the rhizosphere, interactions between bacteria and fungi are common (Whipps, 2001; Boer *et al*., 2005) and diverse, ranging from synergistic to antagonistic. Many factors, such as siderophores, antibiotics, and extracellular enzymes, affect the physiological properties of microorganisms, ultimately leading to changes in their viability and behaviour. Some bacterial antagonists can control fungal pathogens directly by producing antibiotics and cell-wall-degrading enzymes, such as β-1,3-glucanases, cellulases, proteases, and chitinases (Chernin *et al*., 1995; Nielsen & Sorensen, 1997; Jijakli & Lepoivre, 1998; Budi *et al*., 2000; Dunne *et al*., 2000), and antagonism can be more effective when bacteria are attached to and colonizing the fungal surface. Chemotaxis and colonization of fungal hyphae by antagonistic bacteria is considered to be a first step in suppression of a fungal pathogen (Dijksterhuis *et al*., 1999; Hogan & Kolter, 2002; de Weert *et al*., 2004), and Dijksterhuis *et al*. (1999) suggested an important role for polar attachment of bacteria to fungal hyphae in the interaction between *Paenibacillus polymyxa* and *Fusarium oxysporum*,

and that clustering of bacterial cells around hyphae enhanced antagonism against the pathogen. However, despite many reports of fungal–bacterial interactions, signal molecules involved in interactions are rare. *Paenibacillus polymyxa*, the type species of genus *Paenibacillus* (Ash *et al.*, 1993), belongs to the group of plant growth-promoting rhizobacteria (PGPR), and is considered to be a promising biocontrol agent for the suppression of plant pathogens, due to its ability to produce plant growth-enhancing substances, such as auxin (Lebuhn *et al.*, 1997) and cytokinin (Timmusk *et al.*, 1999), nitrogen fixation (Grau & Wilson, 1962), phosphate solubilization and antagonistic activity against bacterial and fungal pathogens through production of antimicrobial substances such as polymyxin, fusaricidin, and chitinase (Storm *et al.*, 1977; Mavingui & Heulin, 1994; Pichard *et al.*, 1995; Beatty & Jensen, 2002; Choi *et al.*, 2008).

In a previous study, *P. polymyxa* strain E681, isolated from the rhizosphere of winter barley grown in the southern province of Korea, was found to be capable of suppressing plant diseases and promoting plant growth (Choi *et al.*, 2004; Ryu *et al.*, 2005, 2006). Recently, whole genome sequencing of E681 (unpublished data) and analysis of the genome of the type strain *P. polymyxa* ATCC 842^{TM} showed that both strains contain a high proportion of genes necessary for antibiotic biosynthesis, carbohydrate metabolism, nitrogenous compound metabolism, and production of hydrolytic enzymes such as glucanase, xylanase, inulinase, and polyphosphatase (Jeong *et al.*, 2006). These genes may favour growth on plant-derived compounds and may affect competition with other microorganisms. During a comprehensive study of the interactions between *P. polymyxa* E681 and fungi, we found that *Penicillium citrinum* could induce swarming motility of *P. polymyxa*. This study aimed to identify a fungal metabolite involved in induction of *P. polymyxa* motility and to analyse the mechanisms involved.

Materials and methods

Microorganisms and culture conditions

Paenibacillus polymyxa E681 was isolated from the roots of winter barley (Ryu *et al.*, 2005) and maintained at $-80\,^{\circ}C$ as frozen endospore suspensions containing 20% (v/v) glycerol. *Paenibacillus polymyxa* ATCC 842^{TM}, the type strain, was purchased from the American Type Culture Collection (ATCC), and *P. citrinum* KCTC6549 from the Korean Collection for Type Cultures (KCTC). *Paenibacillus* spp. were grown in Tryptic soy broth (TSB, BD Co.) or on Tryptic soy agar (TSA) solidified with 1.5% (w/v) agar at $30\,^{\circ}C$ unless otherwise specified. *Penicillium citrinum* was grown on potato dextrose agar (PDA, BD Co., Sparks) at $25\,^{\circ}C$ over 3 days, and was maintained at $4\,^{\circ}C$. To prepare fungal extracts, *P. citrinum* was grown in Czapek–Dox yeast extract broth (CYB; 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, $FeSO_4$ 0.01 g, and Bacto-yeast extract 1 g, glucose 30 g, and adjusted to pH 6.5 in 1 L). Citrinin was purchased from Sigma-Aldrich Inc. (St Louis, MO), dissolved in dimethyl sulphoxide (DMSO), and added to TSB at concentrations of 2.5–20 $\mu g\,mL^{-1}$, if necessary.

Preparation of fungal metabolites

Penicillium citrinum KCTC6549 was cultivated in 100 mL CYB at $25\,^{\circ}C$ for 5 days, centrifuged to remove mycelia and the supernatant was assayed for the inducing activity of bacterial motility by a disk-diffusion assay (Pandya *et al.*, 1999). The supernatant was then passed through a column (3 cm × 11 cm) filled with 10 g of Amberlite XAD-16 resin for 30 min. Fungal metabolites were extracted by passing through 50 mL of aqueous acetone solution at various concentrations (20%, 50%, 80%, and 100%), and the inducing activity of each extract was assayed subsequently. The extract was re-extracted with butanol (100%), and its inducing activity was confirmed. Finally, a yellowish residue was obtained and dissolved in 1 mL water for reverse-phase HPLC.

Analysis of fungal metabolites

HPLC analysis was performed using a YMC–Pack Pro C18 column (5 μm, 250 mm × 10 mm). A 0.4-mL sample was analysed using a binary gradient mobile phase at a flow rate of 3 $mL\,min^{-1}$. Mobile phase A was water and mobile phase B was acetonitrile. Gradient elution at a flow rate of 3 $mL\,min^{-1}$ was performed as follows: 0–30 min, 15–100% B (linear gradient), and 30–50 min, 100% B (isocratic). After evaporation of the solvent, each fraction was assayed to determine its activity. Active fractions were then pooled, evaporated, and redissolved in water for liquid chromatography (LC)-electrospray ionization (ESI)-MS. ESI-MS was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co.) equipped with a Finnigan electrospray source. Fractionation was performed by HPLC on the Finnigan Surveyor^{TM} Modular HPLC System (Thermo Electron Co.), using a YMC–Pack Pro C18 (5 μm, 2.0 mm × 50 mm) operated under the XCALIBUR software system (version 1.3 SP2, Thermo Electron). Mobile phase A was water and mobile phase B was acetonitrile, both of which contained 0.1% formic acid. Gradient elution at a flow rate of 0.2 $mL\,min^{-1}$ was performed as follows: 0–30 min, 30–100% B (linear gradient), 30–50, min 100% B (isocratic), and 50–70 min, 30% B (isocratic). Full-scan mass spectra were obtained in the range *m/z* 100–1000, with three microscans and a maximum ion injection time of 200 ms. Data-dependent tandem MS (MS/MS) experiments were controlled by the menu-driven software provided with

the system. All experiments were performed under automatic gain control conditions.

Motility assays

For the swarming motility assay, Tryptic soy swarm agar (1.0% agar) plates were prepared and air-dried for 30 min in a laminar flow hood. Inocula of fresh *P. polymyxa* E681 colonies were obtained by germinating spores preserved at − 80 °C followed by overnight growth in TSB and 100-fold dilution into fresh 20 mL TSB. Cells in the logarithmic growth phase ($OD_{600\,nm}$ of 0.5) were resuspended in 2 mL phosphate buffer (50 mM) after gentle spinning at 600 ***g*** and 4 °C for 3 min. Five microliters of the bacterial suspension was drop inoculated at the centre of a TSA (1.0%, w/v, agar) plate containing citrinin. For a simple swarming assay, paper disks (8 mm diameter), loaded with sample solutions, were placed at 0.5-cm distances from bacterial colonies. A disk plug of fungal mycelium was prepared by punching out a potato dextrose agar (PDA) culture with an 8-mm cork-bore.

The chemotaxis assay was performed in two different ways. For the first, a 0.8% Bacto-agar disk (8 mm) containing citrinin (50 μg mL^{-1}) was placed on a TSA plate (1%, w/v, agar), and 5 μL of a motile bacterial cell suspension was drop inoculated at distances of 0.5, 0.7, 0.9, and 1.2 cm from the disk. The plate was then incubated at 30 °C for 24 h. In the second assay, Tryptic soy swim agar (0.3%, w/v, agar) plates were made by mixing motile bacterial cells with premelted TSA (0.3% agar) at 45 °C; the mixture was then solidified by drying at 25 °C for 30 min in a laminar flow hood. Fifty microlitres of citrinin solutions of various concentrations (up to 100 μg mL^{-1}) were loaded on paper disks, dried, and placed on the centre of the swim plate, which was then incubated at room temperature for several hours to observe chemotaxis.

Light microscopic detection of flagella

Bacterial flagella were detected using the procedure described by Heimbrook *et al.* (1989). The bacterial cells grown on a swarm plate (with or without citrinin) for 12 h were mounted on a slide glass by touching a loopful of water to the colony margin, allowing cells to swim into this loopful of water, and then touching the loopful of motile cells to a drop of water on a slide. The slide was then covered with a coverslip, and 10 μL staining solution was applied to the vertical edge of the coverslip. After 5 min, the stained bacterial flagella were observed by phase-contrast microscopy.

Reverse transcriptase (RT)-PCR amplification

Total RNA was extracted from bacterial cells grown in TSB using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Bacterial cells, grown in 20 mL TSB at 30 °C with shaking at 250 r.p.m., were harvested after growth for 10 h and 16 h by centrifugation at 1000 ***g*** and 4 °C for 5 min and resuspended in 10 mL TE buffer. One hundred microlitres of cell suspension was treated with lysozyme at room temperature for 5 min, and 1 mL of TRIzol Reagent was then added. Extracted total RNA was cleaned by passing through a RNeasy mini kit with on-column DNAse I treatment following the manufacturer's instructions (Qiagen Inc., Hilden, Germany). RT-PCR was performed in two-step reactions using SuperScript transcriptase III (Invitrogen). Approximately 150 μg of DNAse-treated total RNA was used for synthesis of first-strand DNA in a 20-μL reaction mixture with 10 pmol primer following the manufacturer's instructions (Invitrogen). The secondary PCR reaction was performed in a mixture of 2 μL first-strand reaction and 20 pmol specific primers with 1 U Platinum *Pfx* DNA polymerase (Invitrogen), 0.3 mM dNTP mixture, and 1 mM $MgSO_4$ in 50 μL amplification buffer. The primers used in this study were designed based on the nucleotide sequences of *sigD* and *hag* homologues of strain E681 (unpublished data); sigD-F (5′-ATATTGTGGAATAT GTGTCAGGCC-3′) and sigD-R (5′-GAAACTCTTTCTCA GAAACGTCC-3′) for amplification of the *sigD* gene, and hag-F (5′-TGCTGGTCTCTCCATTTCCG-3′) and hag-R (5′-TCAATTGCTGCTGTCGTAGC-3′) for the *hag* gene.

Results

Induction of swarming motility of *P. polymyxa* by a fungal metabolite

Paenibacillus polymyxa strain E681 formed round, convex, mucoid, and opaque colonies on TSA medium containing 1.5% agar, without showing any distinctive motility (Cheong *et al.*, 2005). Interestingly, E681 colonies exhibited robust swarming motility when grown in the close vicinity of a fungal strain, *P. citrinum* KCTC 6549, on a TSA plate (Fig. 1a). Swarming motility was not observed when grown at positions distant from the fungus (Fig. 1a, lower colonies). The inoculum for this experiment was an agar plug containing fungal hyphae (and maybe some excreted metabolites) excised from a PDA plate culture. In a similar swarming assay, using agar plugs excised from the same plate but at positions distant from fungal growth, to exclude fungal hyphae, the same results were obtained within 12 h of transferring the agar plugs (data not shown). When E681 swarm cells taken from a swarm plate were transferred to a fresh TSA (1.0% agar) plate, no swarming expansion was observed (data not shown). These results strongly imply that induction of swarming motility of the E681 strain might be mediated by a metabolite(s) secreted from *P. citrinum*.

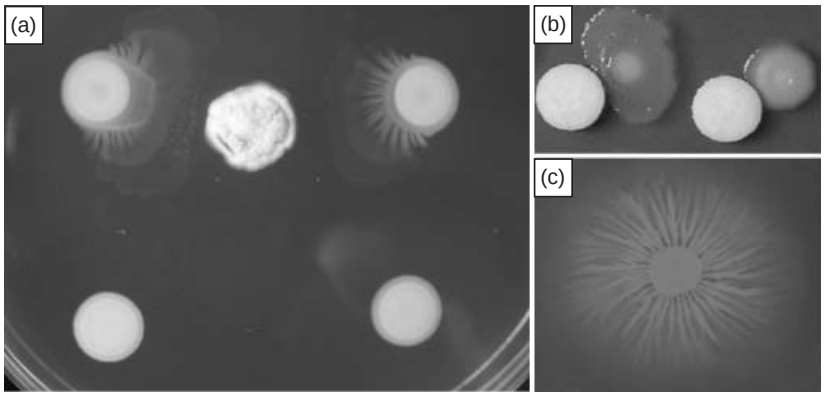

Fig. 1. Swarming motility of *Paenibacillus polymyxa* E681 induced by adjacent fungal growth (a) and fungal metabolites (b and c) on TSA plates. (a) An agar plug containing *Penicillium citrinum* hyphae was placed at the centre of a TSA (1.0% agar) plate and a strain E681 cell suspension was drop inoculated. After 24 h of incubation at 30 °C, swarming motility was observed. (b) Active prep-LC fractions were screened by a disk-diffusion assay. Disks on the left and right represent the active and inactive fractions, respectively. (c) Swarming expansion of *P. polymyxa* colony induced by addition of 10 μg mL^{-1} of fraction 12 to TSA medium.

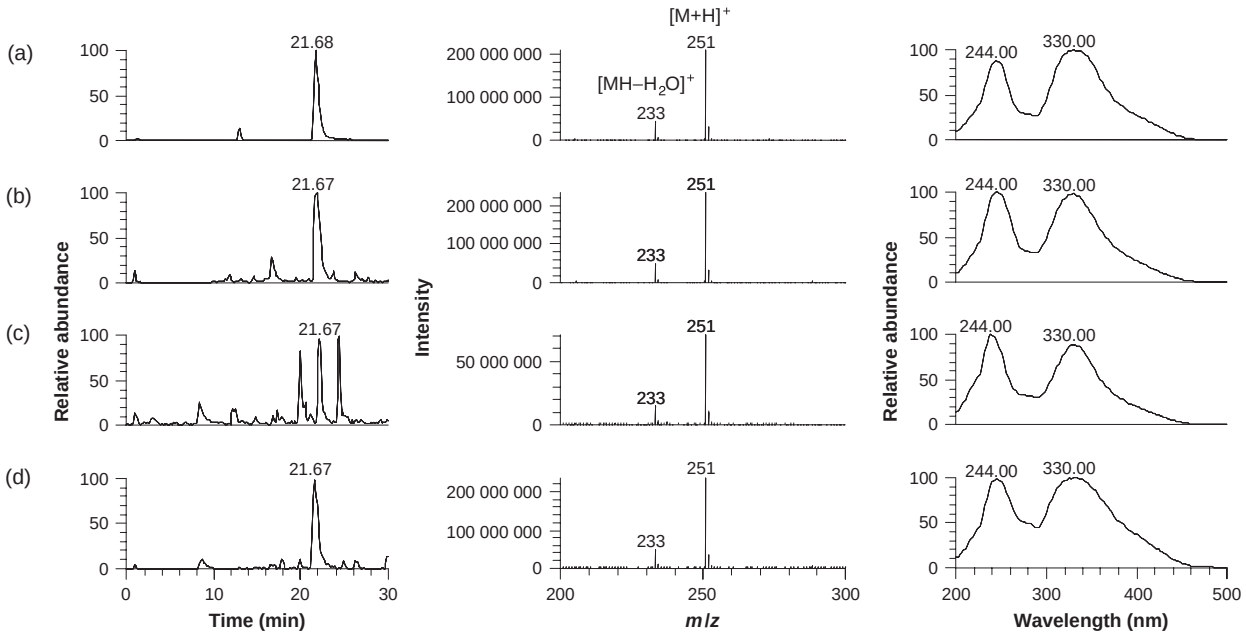

Fig. 2. LC chromatograms (left), full-scan mass spectra generated in positive ionization mode (middle), and UV absorption (right) of citrinin standard (a) and active fractions 10 (b), 11 (c), and 12 (d) of *Penicillium citrinum* extract separated with Sephadex LH 20.

Identification of the fungal metabolite responsible for the induction of swarming motility of *P. polymyxa*

To analyse the active compound(s) of the fungal strain, *P. citrinum* KCTC 6549, the strain was grown in CYB at 25 °C for 5 days. The culture supernatant was harvested and its ability to induce swarming motility was confirmed by a disk-diffusion assay (data not shown). Crude acetone and butanol-extracted fungal metabolites (100 mg) were dissolved in 1 mL distilled water and fractionated into 50 fractions using a prep-LC. To identify the active fractions, each fraction was checked for its swarming induction capacity by a disk-diffusion assay. Among the 50 fractions, only three (10, 11, and 12), obtained at a retention time of 10–12 min, showed induction activity (Fig. 1b), and addition of fraction 12 to TSA (1.0% agar) at a final concentration of 10 μg mL^{-1} led to induction of robust swarming motility of *P. polymyxa* strain E681 cells (Fig. 1c).

Analysis of the three positive fractions by LC-MS/MS generated a common peak with a retention time of 21.67 min (Fig. 2, left). Full-scan mass spectra were generated in both the positive and negative ionization mode and the three fractions yielded common ion peaks at *m/z* 251 (Fig. 2, middle) and *m/z* 267 (data not shown) in the positive and the negative ionization modes, respectively. The patterns of MS spectra and the *m/z* values of 251 and 267 identified the active compound in the three fractions of fungal extracts as a fungal mycotoxin, citrinin, following

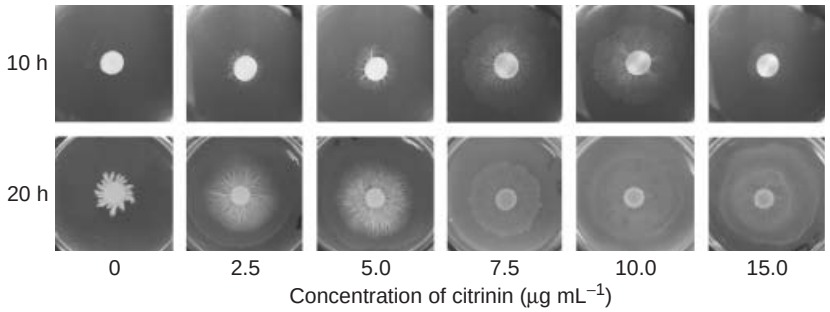

Fig. 3. Swarming motility of *Paenibacillus polymyxa* E681 on TSA plates amended with commercial citrinin at final concentrations of 2.5–15 μg mL^{-1} centrally inoculated with 5 μL of E681 cells freshly grown in TSB and incubated for 10 h (upper line) and 20 h (lower line) at 30 °C.

database searching (http://www.microbank.re.kr/, Lee *et al*., 2005) and a previously published report (Devi *et al*., 2006). The above data and the UV absorption patterns (between 200 and 460 nm) of the three fungal fractions coincided exactly with those of standard citrinin (Fig. 2, right). The results therefore indicate that citrinin was the active compound that was secreted from *P. citrinum* and induced the swarming motility of *P. polymyxa*.

Validation of citrinin activity for inducing motility of *P. polymyxa*

To validate induction of swarming motility of *P. polymyxa* E681 by citrinin, TSA (1% agar) plates containing five different concentrations of citrinin, from 2.5 to 15 μg mL^{-1}, were prepared and inoculated with E681 cells, grown in TSB at 30 °C for 12 h with rapid shaking, by dropping 5 μL of the culture (10^8 cells mL^{-1}) at the centre of each TSA plate. Citrinin strongly induced swarming motility at all the citrinin concentrations tested (Fig. 3). After 10 h of incubation at 30 °C, swarming motility was induced in a dose-dependent manner. At a concentration of 15 μg mL^{-1}, however, swarm expansion of E681 cells was inhibited, possibly because such a high concentration of citrinin was toxic to E681 cells. After 20 h of incubation, robust swarming of E681 cells was observed at all the citrinin concentrations tested. The E681 cells grown on TSA plates without citrinin, however, showed no swarming motility at 10 h of incubation, and showed meagre swarming motility at 20 h of incubation. The same experiments were repeated with *P. polymyxa* ATCC842^{TM}, the type strain of the species, and the same results were obtained (data not shown).

Raistrick & Smith (1941) reported that citrinin has bactericidal activity. To examine whether citrinin is harmful to the growth of E681 cells, TSB was prepared with citrinin at concentrations of 2.5, 5.0, 10.0, and 20.0 μg mL^{-1} and the growth of E681 strain was monitored by measuring $OD_{600\,nm}$ and analysis of growth curves. Citrinin had no significant effect on growth at concentrations $\leq$ 10 μg mL^{-1}, but was significantly inhibited at a concentration of 20.0 μg mL^{-1} (data not shown).

Effect of citrinin on the development of flagella of *P. polymyxa*

To examine whether induction of swarming motility of strain E681 by citrinin was a direct effect of citrinin, flagella of E681 cells were examined by light microscopy after staining. E681 cells grown on TSA containing 5 or 7.5 μg mL^{-1} citrinin had multiple lateral flagella (Fig. 4a, II and III), but in the absence of citrinin had only one or a few flagella (Fig. 4a, I). The development of lateral flagella has also been reported in other bacterial cells with swarming motility (Merino *et al*., 2006). This indicates that induction of swarming motility in E681 by citrinin was a direct effect occurring directly through increased production of flagella. This was supported by analysing expression levels of a gene homologue of *sigD*, encoding a sigma factor for the *fla*/*che* cluster, and the homologue of *hag*, encoding flagellin, which may be linked to bacterial motility. The deduced amino acid sequence of the *hag* homologue of E681 was shown to have 52.3% identity with that of *Bacillus subtilis* 168 and its flanking region included many flagella-associated genes. The amino acid sequence of the *sigD* homologue was shown to have 43.3% identity with that of *B. subtilis* 168 and was located in the *fla*/*che* operon structure, as in *B. subtilis* 168. Expression levels of *sigD* and *hag* in E681 cells grown in TSB in the presence of 5 μg mL^{-1} citrinin were significantly greater than those of the control after growth for 16 h (Fig. 4b).

Chemotactic motility of *P. polymyxa* E681 induced by citrinin

To confirm whether migration of *P. polymyxa* E681 to *P. citrinum* was mediated by citrinin in a specific direction, a small agar plug containing 50 μg mL^{-1} citrinin was placed at the centre of a TSA swarm plate (1.0% agar), and motile bacterial cells (10^9 CFU mL^{-1}) were drop inoculated at distances of 0.5, 0.7, 0.9, and 1.2 cm from the citrinin plug. All colonies except one migrated toward the citrinin plug, the only exception being the colony closest to the plug, possibly due to citrinin toxicity (Fig. 5a). To confirm further chemotaxis toward citrinin, a chemotaxis assay was

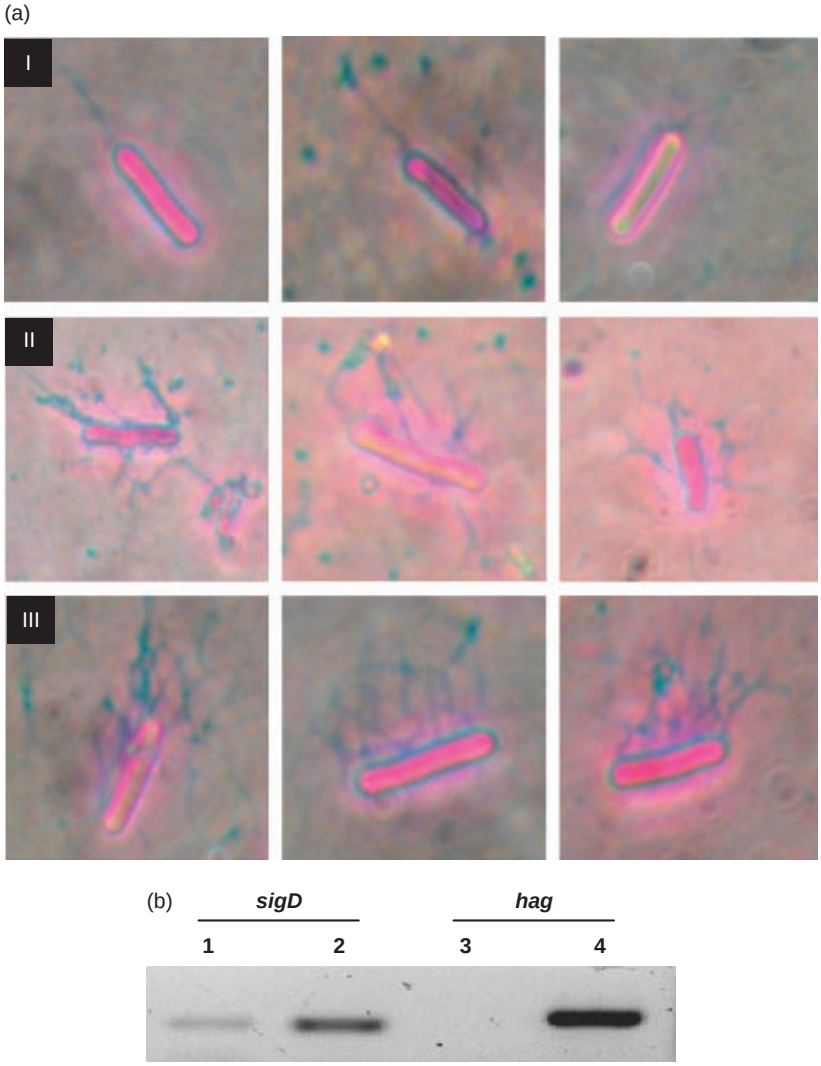

Fig. 4. Light microscopic detection of flagella (a) and analysis of expressions of *sigD* and *hag* genes by RT-PCR (b). (a) Flagella of the E681 strain were observed by phase-contrast microscopy after staining of the cells grown on TSA without citrinin (I), with 5 (II), or 7.5 μg mL^{-1} citrinin (III). (b) Transcription of *sigD* and *hag* genes of E681 strain grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of citrinin (5 μg mL^{-1}) was detected by RT-PCR.

conducted using a swim agar plate (0.3% agar). Motile E681 cells were mixed into swim agar, and paper disks containing 50 μg mL^{-1} citrinin and DMSO (as a negative control), respectively, were laid on the swim plate. *Paenibacillus polymyxa* cells clustered around the paper disk containing citrinin (Fig. 5b).

Discussion

Citrinin is a well-known mycotoxin, produced by fungi such as *Penicillium* spp., *Aspergillus* spp, and several saprophytic fungi (Pitt, 2002). The natural habitats of these citrinin-producing fungi are diverse, including air, soil, rhizosphere, and water. In the 1940s, citrinin was characterized as an antibiotic active against most Gram-positive bacteria (Raistrick & Smith, 1941; Wang *et al.*, 1947). Citrinin was known more widely as a nephrotoxin in animals and, during the past two or more decades, many functional studies have been performed to elucidate its cytotoxic mechanisms resulting from interference with energy generation causing a decreased cellular ATP level, facilitation of superoxide anion production in the respiratory chain, and perturbation of the iron redox cycle (Chagas *et al.*, 1992; Hoehler *et al.*, 1996; Stormer & Hoiby, 1996; Ribeiro *et al.*, 1997, 1998; Da Lozzo *et al.*, 1998, 2002). Ueno & Kubota (1976) and Martin *et al.* (1986) reported that citrinin has a DNA-attacking ability *in vitro* or *in vivo*. Initially, we suspected increased swarm motility of strain 681 by citrinin to be a mutational event. In this study, however, the swarm motility was confirmed not to originate from mutation by observing that cells taken from swarming colonies showed a normal growth pattern when inoculated on citrinin-free medium (data not

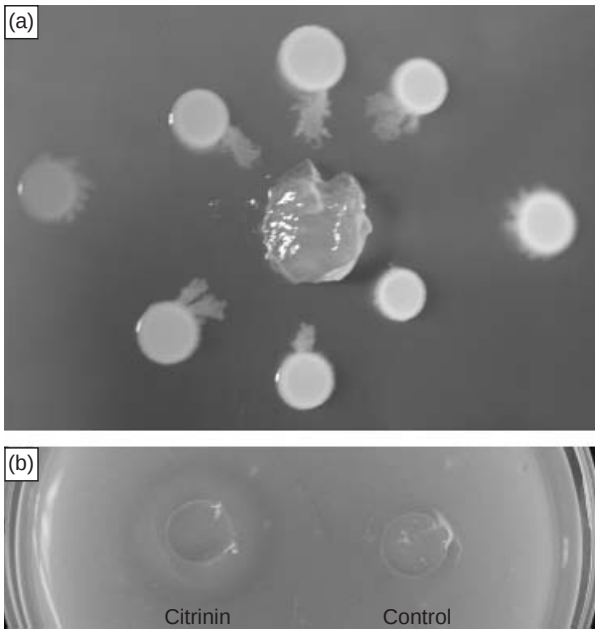

Fig. 5. Chemotactic response of *Paenibacillus polymyxa* E681 to citrinin on a swarm plate (1.0% agar) (a) and in a swim plate (0.3% agar) (b). (a) A small agar plug containing citrinin (50 μg mL^{-1}) was placed at the centre of each TSA plate (1.0% agar), and E681 cells were drop inoculated at different distances from the citrinin plug. (b) Motile E681 cells were mixed with swim agar before hardening, and the paper disk containing citrinin or DMSO as a control was laid on the swim plate.

shown). *Paenibacillus polymyxa* ATCC842™, the type strain, also showed the same responses to citrinin as those of E681 strain. Therefore, the phenomenon of swarm expansion by *P. citrinum* or by citrinin seemed to be a general cellular response of *P. polymyxa* species.

As shown in Fig. 4a, multiple lateral flagella were increased in E681 cells growing in the presence of citrinin, and the induced expression of homologues of *sigD* and *hag* supported this finding. These results suggested that the robust swarming of E681 in the presence of citrinin was caused by the increased number of flagella. The development of motility of *P. polymyxa* cells by citrinin seemed to be an interesting phenomenon because it will probably have an important role in *P. citrinum*–*P. polymyxa* interaction. We also found that the bacterium showed directional migration toward fungal or citrinin plugs (Figs 1a and 5a) and chemotaxis toward citrinin in an experiment using a swim plate (Fig. 5b). This supports the suggestion that citrinin, a fungal mycotoxin, will be an important factor mediating fungal–bacterial interactions. Interestingly, de Weert *et al.* (2004) reported that *Pseudomonas fluorescens* WCS365 showed chemotaxis toward a fungal plant pathogen, *F. oxysporum*, and the major chemotactic attractant was fusaric acid, a mycotoxin produced by *F. oxysporum*. It was also reported that fusaric acid decreased the production of polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) of *P. fluorescens* via repressed *phlA* expression, directly involved in DAPG production (Schnider-Keel *et al.*, 2000; Notz *et al.*, 2002; Schouten *et al.*, 2004), and thus played a role in self-defence of *F. oxysporum* against antagonizing bacterium (Duffy *et al.*, 2003). Although we know little about the regulation of *P. polymyxa* gene expression by citrinin, it is interesting that the two mycotoxins, fusaric acid and citrinin, produced by two different fungal species, *F. oxysporum* and *P. citrinum*, respectively, play a common role as chemotactic attractants for bacterial species.

In conclusion, this study showed that citrinin, a mycotoxin produced by *P. citrinum*, has a newly identified function of inducing bacterial motility by transcriptional activation of some genes related to the expression of flagella. This finding will provide an insight that will allow us to better understand the ecological roles of fungal mycotoxins beyond their well-known toxicities to other organisms.

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References

Ash C, Priest FG & Collins MD (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie Van Leeuwenhoek* **64**: 253–260.

Beatty PH & Jensen SE (2002) *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. *Can J Microbiol* **48**: 159–169.

Boer W, Folman LB, Summerbell RC & Boddy L (2005) Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol Rev* **29**: 795–811.

Budi SW, van Tuinen D, Arnould C, Dumas-Gaudot E, Gianinazzi-Pearson V & Gianinazzi S (2000) Hydrolytic enzyme activity of *Paenibacillus* sp. strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. *Appl Soil Ecol* **15**: 191–199.

Chagas GM, Oliveira BM, Campello AP & Kluppel ML (1992) Mechanism of citrinin-induced dysfunction of mitochondria. II. Effect on respiration, enzyme activities, and membrane potential of liver mitochondria. *Cell Biochem Funct* **10**: 209–216.

Cheong H, Park SY, Ryu CM, Kim JHF, Park SH & Park CS (2005) Diversity of root-associated *Paenibacillus* spp. in winter crops from the southern part of Korea. *J Microbiol Biotechnol* **15**: 1286–1298.

Chernin L, Ismailov Z, Haran S & Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* **61**: 1720–1726.

Choi OH, Kim JW, Ryu CM & Park CS (2004) Colonization and population changes of a biocontrol agent, *Paenibacillus polymyxa* E681, in seeds and roots. *Plant Pathol J* **20**: 97–102.

Choi SK, Park SY, Kim R, Lee CH, Kim JF & Park SH (2008) Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681. *Biochem Biophys Res Commun* **365**: 89–95.

Da Lozzo EJ, Oliveira MB & Carnieri EG (1998) Citrinin-induced mitochondrial permeability transition. *J Biochem Mol Toxicol* **12**: 291–297.

Da Lozzo EJ, Mangrich AS, Rocha ME, de Oliveira MB & Carnieri EG (2002) Effects of citrinin on iron-redox cycle. *Cell Biochem Funct* **20**: 19–29.

Devi P, Naik CG & Rodrigues C (2006) Biotransformation of citrinin to decarboxycitrinin using an organic solvent-tolerant marine bacterium, *Moraxella* sp. MB1. *Mar Biotechnol* **8**: 129–138.

de Weert S, Kuiper I, Lagendijk EL, Lamers GE & Lugtenberg BJ (2004) Role of chemotaxis toward fusaric acid in colonization of hyphae of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by *Pseudomonas fluorescens* WCS365. *Mol Plant Microbe Interact* **17**: 1185–1191.

Dijksterhuis J, Sanders M, Gorris LG & Smid EJ (1999) Antibiosis plays a role in the context of direct interaction during antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. *J Appl Microbiol* **86**: 13–21.

Duffy B, Schouten A & Raaijmakers JM (2003) Pathogen self-defense: mechanisms to counteract microbial antagonism. *Annu Rev Phytopathol* **41**: 501–538.

Dunne C, Moenne-Loccoz Y, de Bruijn FJ & O'Gara F (2000) Overproduction of an inducible extracellular serine protease improves biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* strain W81. *Microbiology* **146**: 2069–2078.

Grau FH & Wilson PW (1962) Physiology of nitrogen-fixation by *Bacillus polymyxa*. *J Bacteriol* **83**: 490–496.

Heimbrook ME, Wang WL & Campbell G (1989) Staining bacterial flagella easily. *J Clin Microbiol* **27**: 2612–2615.

Hoehler D, Marquardt RR, McIntosh AR & Xiao H (1996) Free radical generation as induced by ochratoxin A and its analogs in bacteria (*Bacillus brevis*). *J Biol Chem* **271**: 27388–27394.

Hogan D & Kolter R (2002) *Pseudomonas–Candida* interactions: an ecological role for virulence factors. *Science* **296**: 2229–2232.

Jeong HY, Kim JF, Park YK, Kim SB, Kim CH & Park SH (2006) Genome snapshot of *Paenibacillus polymyxa* ATCC 842^{T}. *J Microbiol Biotechnol* **16**: 1650–1655.

Jijakli HM & Lepoivre P (1998) Characterization of an exo-β-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrylis cinerea* on apples. *Phytopathology* **88**: 335–343.

Lebuhn M, Heulin T & Hartmann A (1997) Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. *FEMS Microbiol Ecol* **22**: 325–334.

Lee JS, Kim DH, Liu KH, Oh TK & Lee CH (2005) Identification of flavonoids using liquid chromatography with electrospray ionization and ion trap tandem mass spectrometry with an MS/MS library. *Rapid Commun Mass Spectrom* **19**: 3539–3548.

Martin W, Lorkowski G, Creppy EE, Dirheimer G & Roschenthaler R (1986) Action of citrinin on bacterial chromosomal and plasmid DNA *in vivo* and *in vitro*. *Appl Environ Microbiol* **52**: 1273–1279.

Mavingui P & Heulin T (1994) *In vitro* chitinase antifungal activity of soil, rhizosphere and rhizoplane populations of *Bacillus polymyxa*. *Soil Biol Biochem* **26**: 801–803.

Merino S, Shaw JG & Tomas JM (2006) Bacterial lateral flagella: an inducible flagella system. *FEMS Microbiol Lett* **263**: 127–135.

Nielsen P & Sorensen J (1997) Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. *FEMS Microbiol Ecol* **22**: 183–192.

Notz R, Maurhofer M, Dubach H, Haas D & Défago G (2002) Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 *in vitro* and in the rhizosphere of wheat. *Appl Environ Microbiol* **68**: 2229–2235.

Pandya S, Iyer P, Gaitonde V, Parekh T & Desai A (1999) Chemotaxis of *Rhizobium* SP.S2 towards *Cajanus cajan* root exudate and its major components. *Curr Microbiol* **38**: 205–209.

Pichard JP, Larue D & Thouvenot JL (1995) Gavaserin and Saltavalin, new peptide antibiotics produced by *Bacillus polymyxa*. *FEMS Microbiol Lett* **133**: 215–218.

Pitt JI (2002) Biology and ecology of toxigenic *Penicillium* species. *Mycotoxins and Food Safety* (De Vries JW, Trucksess MW & Jackson LS, eds), pp. 29–41. Kluwer Academic, Plenum Publishers, New York.

Raistrick H & Smith G (1941) Antibacterial substances from moulds. I. Citrinin, a metabolic product of *Penicillium citrinum*. *Thom Chem Ind* **60**: 828–830.

Ribeiro SM, Chagas GM, Campello AP & Kluppel ML (1997) Mechanism of citrinin-induced dysfunction of mitochondria. V. Effect on the homeostasis of the reactive oxygen species. *Cell Biochem Funct* **15**: 203–209.

Ribeiro SM, Campello AP, Chagas GM & Kluppel ML (1998) Mechanism of citrinin-induced dysfunction of mitochondria. VI. Effect on iron-induced lipid peroxidation of rat liver mitochondria and microsomes. *Cell Biochem Funct* **16**: 15–20.

Ryu CM, Kim JW, Choi OH, Park SY, Park SH & Park CS (2005) Nature of a root-associated *Paenibacillus polymyxa* from field-grown winter barley in Korea. *J Microbiol Biotechnol* **15**: 984–991.

Ryu CM, Kim JW, Choi OH, Kim SH & Park CS (2006) Improvement of biological control capacity of *Paenibacillus polymyxa* E681 by seed pelleting on sesame. *Biol Control* **39**: 282–289.

Schnider-Keel U, Seematter A, Maurhofer M *et al.* (2000) Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182**: 1215–1225.

Schouten A, van den Berg G, Edel-Hermann V, Steinberg C, Gautheron N, Alabouvette C, de Vos CH, Lemanceau P & Raaijmakers JM (2004) Defense responses of *Fusarium oxysporum* to 2,4-diacetylphloroglucinol, a broad-spectrum antibiotic produced by *Pseudomonas fluorescens*. *Mol Plant Microbe Interact* **17**: 1201–1211.

Storm KS, Rosenthal V & Swanson A (1977) Polymixin and related peptide antibiotics. *Annu Rev Biochem* **46**: 723–763.

Stormer FC & Hoiby EA (1996) Citrinin, ochratoxin A and iron. Possible implications for their biological function and induction of nephropathy. *Mycopathologia* **134**: 103–107.

Timmusk S, Nicander B, Granhall U & Tillberg E (1999) Cytokinin production by *Paenibacillus polymyxa*. *Soil Biol Biochem* **31**: 1847–1852.

Ueno Y & Kubota K (1976) DNA-attacking ability of carcinogenic mycotoxins in recombination-deficient mutant cells of *Bacillus subtilis*. *Cancer Res* **36**: 445–451.

Wang Y, Hong FK, Hwang FT & Fan CS (1947) Citrinin as an antibiotic. *Science* **106**: 291–292.

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. *J Exp Bot* **52**: 487–511.