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**Issue Date:** 2015-10-28
Chapter 3

Chitin degrading abilities and antifungal activity of bacterial strains differing in complexity of their chitinolytic systems and growth form

Yani Bai, Johannes A. van Veen, Wietse de Boer
**Bacteria strains differ in chitinolytic activity**

**Abstract**

Chitinolytic bacteria have different abilities with respect to chitin degradation and antifungal properties. This might be partially explained by differences in numbers of chitinases and/or possession of additional enzymes. In order to get a deeper understanding of the factors determining the use of chitinases for chitin degradation and antifungal activity, we selected 13 bacteria from different phyla and tested their chitin degrading ability in liquid cultures and the antifungal activity on agar plates. Next we compared the degradation rates and antifungal activity with the composition of the chitinolytic system of the strains as based on their annotated genomes. Degradation rates of chitin resources differed strongly between bacteria. The results supported the recently suggested importance of chitin-binding proteins in degradation of crystalline chitin as numbers of chitin-binding proteins on genomes rather than numbers of chitinases correlated positively with degradation of chitin particles. Although hyphal growth form has been indicated as an important trait for degradation of crystalline chitin, we found strong variation in the degradation abilities of both filamentous and non-filamentous bacteria. There were no clear indications for a relationship between the composition of the chitinolytic system and antifungal activities of the strains.
Introduction

Chitin is one of the most abundant biopolymers in nature (Chater et al 2010, Gooday 1990). Chitin degradation, which is mainly a microbial process, is important in global carbon and nitrogen cycling. Chitinases are the major chitin decomposing enzymes (Bhattacharya et al 2007). Bacterial chitinases are typically divided into two families according to their amino acid sequence homology: family 18 and 19 of glycoside hydrolases (Henrissat & Davies 2000). The majority of the known bacterial chitinases belong to family 18. Chitinases of both families have also been reported to exhibit antifungal activity (Kawase et al 2006, Prasanna et al 2013).

The ability to hydrolyze chitin is taxonomically widespread in bacteria and is found for both Gram-negative and Gram-positive genera (Gooday 1990). Yet, bacteria differ strongly in their ability to degrade chitin. This has been ascribed to differences in chitinolytic systems, i.e. different numbers of chitinases and different types of chitinases (Bai et al 2015). In addition, Vaaje-Kolstad et al (2005a) showed that chitin binding proteins, also called CBM33 proteins, contribute to the efficiency of chitinases. These proteins were reported to catalyze oxidative cleavage of glycosidic bonds in crystalline chitin, thereby making the chitin fibers more accessible for chitinases (Vaaje-Kolstad et al 2005b). Hence, these proteins can contribute to the chitinolytic performance of bacteria.

Furthermore, next to physiological properties also morphological properties can influence chitin degrading capabilities. Hyphal forming bacteria, such as Actinomycetes, are reported to be better capable of degrading chitin particles than most non-hyphal bacteria (De Boer et al 1999). Filamentous bacteria were often found to be the dominant chitin-degrading bacteria when chitinous material was added to soil (Krsek & Wellington 2001, Sato et al 2010). One of the possible reasons for the good chitin degrading abilities of Actinomycetes might be that the hyphae can penetrate the chitin particles.
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thereby extending the area where chitinases are in close contact with the chitin polymers.

The overall hypothesis of this study is that the chitin degrading performance and antifungal activity of chitinolytic bacteria is determined by the number of chitinases (including family 18 and 19 chitinase), the number of chitin binding proteins and the bacterial growth form. In the current study this was tested using a selection of 13 bacterial chitinolytic bacterial strains for which genome information is available. These bacteria were exposed to different forms of chitin in liquid media to test their chitin degrading abilities. In addition, their antifungal activity capabilities were tested in an in-vitro confrontation assay with different fungi. The aim of the study was to indicate the possible importance of the chitinolytic system complexity and morphological characteristics for bacterial chitin degrading capacities and antifungal activities.

Method

Strains

We selected 13 chitinolytic bacterial strains that had been isolated from soil and for which genome information is publicly available (National Center for Biotechnology Information database: http://www.ncbi.nlm.nih.gov). These bacteria are from different phyla, have different number of chitinases and have different morphological characteristics (hyphal versus unicellular). The selected strains are listed in Table 1.
### Table 1: Characteristics of the selected bacterial strains

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Gram</th>
<th>Order</th>
<th>Chitinase number</th>
<th>Filamentous</th>
<th>Chitin binding proteins number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces coelicolor</em> A3 (2)</td>
<td>+</td>
<td>Actinomycetales</td>
<td>8</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> TK24</td>
<td>+</td>
<td>Actinomycetales</td>
<td>9</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptomyces clavuligerus</em> ATCC 27064</td>
<td>+</td>
<td>Actinomycetales</td>
<td>13</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptomyces viridochromogenes</em> DSM 40736</td>
<td>+</td>
<td>Actinomycetales</td>
<td>4</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td><em>Kribbella flavida</em> DSM 17836</td>
<td>+</td>
<td>Actinomycetales</td>
<td>7</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>Conexibacter woesei</em> DSM 14684</td>
<td>+</td>
<td>Solirubrobacterales</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>Micromonospora aurantiaca</em> ATCC 27029</td>
<td>+</td>
<td>Actinomycetales</td>
<td>4</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td><em>Catenulispora acidipila</em> DSM 44928</td>
<td>+</td>
<td>Actinomycetales</td>
<td>10</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td><em>Chitinophaga pinensis</em> DSM 2588</td>
<td>-</td>
<td>Sphingobacterales</td>
<td>6</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td><em>Flavobacterium johnsoniae</em> UW101</td>
<td>-</td>
<td>Flavobacteria</td>
<td>4</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>Ktedonobacter racemifer</em> DSM 44963</td>
<td>+</td>
<td>Ktedonobacterales</td>
<td>5</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus mycoides</em> DSM 2048</td>
<td>+</td>
<td>Bacillales</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><em>Collimonas fungivorans</em> Ter331</td>
<td>-</td>
<td>Burkholderiales</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>
**Chitin resources and bacterial growth**

We used four different chitin sources: *N*-acetylglucosamine (chitin monomer), colloidal chitin, small particles of crystal chitin (<0.5 mm) and big particles of crystal chitin (> 2 mm). Purified crystal chitin (poly-N-acetyl-1, 4-β-D-glucosamine, Sigma-Aldrich) was manufactured from shrimp shells. For use in this experiment it was sieved through 2 (big size) or 0.5 (small size) mm mesh filters. Colloidal chitin was prepared from crystal chitin according to Hsu & Lockwood (1975). Their procedure includes the dissolution of bleached chitin in concentrated HCl, suspending the dissolved chitin by adding water, and removing the HCl in several washing steps until the pH of the suspension was between 2.5 to 3.0.

The chitin containing liquid growth medium was prepared by mixing either 0.1 g colloid chitin, 0.5 g *N*-acetylglucosamine, 1 g big (>2.0 mm) or small (< 0.5 mm) size chitin powder in 1 L medium containing 40 mg MgSO$_4$·7 H$_2$O, 20 mg CaCl$_2$·2 H$_2$O, 1 g KH$_2$PO$_4$. The chitin sources were the only carbon- and energy substrates for bacterial growth. The final pH of media was adjusted to 6.5. The medium was sterilized by heating at 121 °C for 15 minutes.

Bacterial inocula were prepared by transferring bacterial biomass from agar plates to 1 ml sterilized phosphate buffer to make suspensions with an OD of 0.01 (approximately 10$^7$ cells/ml), 0.2 ml of these bacterial suspensions was transferred to 100 ml of liquid media with different chitin resources. After inoculation, flasks were put on a shaker (100 rpm) and incubated at 20 °C. Every two days 1.5 ml samples were taken from the flask and centrifuged, after which the supernatant was stored at – 20 °C. We took samples for a total period of 24 days.

Chitin degradation was quantified by measuring the amount of NH$_4^+$-N released from chitin in liquid media (De Boer et al 1996). NH$_4^+$-N was determined
using an auto analyzer QUATRO (SEAL). A control treatment, without N-acetylglucosamine or chitin in the growth medium, was included.

**Antifungal activity**

Antifungal activity of the selected bacteria was tested by an *in vitro* confrontation assay as shown in Figure 1. Bacteria were grown on water-yeast agar for one week in area B of the plate. Next, a PDA agar block of 1 cm ø from the growing margin of a fungal colony was placed in area A. As a control fungal strains were transferred to agar without the presence of bacteria in area B. After sealing the plates were incubated at 20°C for 2 weeks.

![Figure 1 Design of antifungal activity test of bacteria. See text for explanation of the letters](image)

Antifungal activity of bacteria was determined by comparing the fungal mycelium development in area C of control plates with that of bacteria-containing plates. The fungal strains used in the test were: *Mucor hiemalis* (zygomycete) isolated from Dutch coastal dunes, *Apergillus niger* (ascomycete) obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and *Rhizoctonia solani* (basidiomycete) obtained from the Instituut voor Rationele Suikerproductie (IRS), Bergen op Zoom, The Netherlands.

**Data analysis**

The results were statistically examined using Microsoft Excel 2010.
Bacteria strains differ in chitinolytic activity

Correlation between chitin degradation rates or antifungal activity and chitinase numbers, chitin binding protein numbers of the bacteria was calculated in PAST (Hammer et al 2001). Antifungal activity was classified in 5 different levels, namely 1 to 5 (see Table 5). These classification levels were used to calculate correlations with other parameters. Hyphal-forming morphology bacteria was categorized by 1 (presence of hyphae) and 0 (no hyphal formation). Effects of hyphal morphology were tested for significance using the non-parametric Kruskall-Wallis test in PAST.

Results

Bacterial chitin degradation in liquid media

The release of \( \text{NH}_4^+ \) from the different chitin resources was determined at 12 time points, every other day of the total incubation period. In Table 2 \( \text{NH}_4^+ \)-N concentration are given at day 1, 12 and 24. For colloidal chitin and crystal chitin we calculated the \( \text{NH}_4^+ \)-N production for two periods namely the accumulation of \( \text{NH}_4^+ \)-N from day 1 to day 12, and from day 12 to day 24. For both periods, we calculated the release rate of \( \text{NH}_4^+ \)-N on a daily basis. Several bacteria showed an increase of \( \text{NH}_4^+ \)-N production during the first 24 h followed by a slow accumulation during the next 11 or 23 days. We attributed this initial quick release to decomposition of easily accessible chitin on the outside of the particles or of soluble chitin oligomers present in the sieved chitin fractions. Therefore, we used the \( \text{NH}_4^+ \)-N release rate during the subsequent period (day 1 – day 12 and day 12 – day 24) as proxy for chitin degrading rate of these bacteria. Since rapid decomposition is to be expected for \( N \)-acetylglucosamine the \( \text{NH}_4^+ \)-N production during the first 24 h was included in the calculations. The highest rates of substrate degradation are shown in Table 3.
Table 2 NH$_4^+$-N concentrations at day 1, 12 and 24 after start of the incubations

<table>
<thead>
<tr>
<th></th>
<th>N-acetylglucosamine</th>
<th>Colloidal chitin</th>
<th>Small size crystal chitin</th>
<th>Big size crystal chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.163</td>
<td>-</td>
<td>-</td>
<td>0.045</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em> A3 (2)</td>
<td>0.148</td>
<td>0.641</td>
<td>1.394</td>
<td>0.026</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> TK24</td>
<td>0.178</td>
<td>0.282</td>
<td>0.57</td>
<td>0.047</td>
</tr>
<tr>
<td><em>Streptomyces clavuligerus</em> ATCC 27064</td>
<td>0.885</td>
<td>1.122</td>
<td>1.419</td>
<td>0.043</td>
</tr>
<tr>
<td><em>Streptomyces viridochromogenes</em> DSM 40736</td>
<td>0.941</td>
<td>1.7</td>
<td>2.853</td>
<td>0.058</td>
</tr>
<tr>
<td><em>Kribbella flavida</em> DSM 17836</td>
<td>0.272</td>
<td>0.742</td>
<td>0.985</td>
<td>0.036</td>
</tr>
<tr>
<td><em>Catenulispora acidiphila</em> DSM 44928</td>
<td>1.262</td>
<td>1.326</td>
<td>1.36</td>
<td>0.031</td>
</tr>
<tr>
<td><em>Micromonospora aurantiaca</em> ATCC 27029</td>
<td>0.839</td>
<td>0.909</td>
<td>0.909</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Collimonas fungivorans</em> Ter331</td>
<td>1.815</td>
<td>2.842</td>
<td>2.876</td>
<td>0.047</td>
</tr>
</tbody>
</table>
**Bacteria strains differ in chitinolytic activity**

Table 3 Release rate of NH$_4^+$-N from chitinous substrates in liquid media. Data represent the amount of NH$_4^+$-N (mmol/l) released per day over the periods day 1 – day 12 or day 12 – day 24 (highest rate of the two periods is presented). For N (N-acetylglucosamine) substrate, data represent the highest rate for the periods of day 0- day 1, day 1- day 12 and day 12- day 24.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>C</th>
<th>S</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces coelicolor</em> A3 (2)</td>
<td>0.063</td>
<td>0.042</td>
<td>0.160</td>
<td>0.088</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> TK24</td>
<td>0.024</td>
<td>0.030</td>
<td>0.044</td>
<td>0.033</td>
</tr>
<tr>
<td><em>Streptomyces clavuligerus</em> ATCC 27064</td>
<td>0.722</td>
<td>0.012</td>
<td>0.099</td>
<td>0.052</td>
</tr>
<tr>
<td><em>Streptomyces viridochromogenes</em> DSM 40736</td>
<td>0.778</td>
<td>0.047</td>
<td>0.103</td>
<td>0.028</td>
</tr>
<tr>
<td><em>Kribbella flavida</em> DSM 17836</td>
<td>0.109</td>
<td>0.014</td>
<td>0.051</td>
<td>0.013</td>
</tr>
<tr>
<td><em>Conexibacter woesei</em> DSM 14684</td>
<td>1.099</td>
<td>0.015</td>
<td>0.026</td>
<td>0.005</td>
</tr>
<tr>
<td><em>Micromonospora aurantiaca</em> ATCC 27029</td>
<td>0.676</td>
<td>0.012</td>
<td>0.080</td>
<td>0.020</td>
</tr>
<tr>
<td><em>Catenulispora acidiphila</em> DSM 44928</td>
<td>0.024</td>
<td>0.015</td>
<td>0.006</td>
<td>0.005</td>
</tr>
<tr>
<td><em>Chitinophaga pinensis</em> DSM 2588</td>
<td>1.330</td>
<td>0.004</td>
<td>0.016</td>
<td>0.026</td>
</tr>
<tr>
<td><em>Flavobacterium johnsoniae</em> UW101</td>
<td>0.585</td>
<td>0.136</td>
<td>0.036</td>
<td>0.032</td>
</tr>
<tr>
<td><em>Ktedonobacter racemifer</em> DSM 44963</td>
<td>ND</td>
<td>0.006</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td><em>Bacillus mycoides</em> DSM 2048</td>
<td>0.163</td>
<td>0.004</td>
<td>0.008</td>
<td>0.022</td>
</tr>
<tr>
<td><em>Collimonas fungivorans</em> Ter331</td>
<td>1.652</td>
<td>0.058</td>
<td>0.010</td>
<td>0.042</td>
</tr>
</tbody>
</table>

N: N-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin

For most of the bacterial strains the degrading rates of N-acetylglucosamine were much higher than for the other substrates (Table 3). The highest rate of N-acetylglucosamine degradation was seen for *Collimonas fungivorans* Ter331 followed by *Chitinophaga pinensis* DSM 2588. For degradation of colloidal chitin, the highest degrading rates were observed for *Flavobacterium johnsoniae* UW101 followed by *Collimonas fungivorans* Ter331. Highest rates of
degradation of crystal chitin were observed for Streptomyces spp. Degradation rates of small and big crystal chitin were correlated (Table 4).

Table 4 Pearson correlation coefficients for degradation rates of different substrates. Significant correlations (P < 0.05) are indicated in bold

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>C</th>
<th>S</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degrating rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>-</td>
<td>0.18</td>
<td>-0.17</td>
<td>0.016</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.74</td>
</tr>
</tbody>
</table>

N: N-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin

**Antifungal activities**

The scores for antifungal activities of the chitinolytic bacterial strains are given in Table 5. The results indicated that bacterial strains showed different antifungal activities in the *in vitro* assay. Overall, most of the *Streptomyces* strains showed a broader and stronger antifungal activity than other bacteria. *Flavobacterium johnsoniae* UW 101, *Ktedonobacter racefmifer* DSM 44963, *Bacillus mycoides* DSM 2048 and *Collimonas fungivoras* Ter331 showed also strong antifungal activity against one or two fungi.

Two strains, *Conexibacteri woesei* and *Chitinophage pinesis* did not grow well on the media used here. This is most likely the reason why there was no antifungal activity observed for these strains.
Bacteria strains differ in chitinolytic activity

Table 5 Classification of *in vitro* antifungal activities of the selected chitinolytic bacteria

<table>
<thead>
<tr>
<th>Strains</th>
<th>M</th>
<th>A</th>
<th>R</th>
<th>Extra information</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces coelicolor</em> A3 (2)</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> TK24</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces clavuligerus</em> ATCC 27064</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces viridochromogenes</em> DSM 40736</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Kribbella flavida</em> DSM 17836</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Conexibacter woesei</em> DSM 14684</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Did not grow well</td>
</tr>
<tr>
<td><em>Micromonospora aurantiaca</em> ATCC 27029</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Catenulispora acidiphila</em> DSM 44928</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><em>Chitinophaga pinensis</em> DSM 2588</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Did not grow well</td>
</tr>
<tr>
<td><em>Flavobacterium johnsoniae</em> UW101</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>Ktedonobacter racemifer</em> DSM 44963</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus mycoides</em> DSM 2048</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><em>Collimonas fungivorans</em> Ter331</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

M: *Mucor hiemalis*; A: *Aspergillus niger*; R: *Rhizoctonia solani*. (Classification of antifungal activity: 1: no inhibition; 2: weak inhibition at only one time point; 3: weak inhibition at both time points; 4: weak inhibition at one time point and strong inhibition at another time point; 5: strong inhibition at both time points)

**Correlation analysis**

The degradation rates of crystal chitin particles, both big and small size were significantly positive related with the number of genes encoding chitin binding proteins but not with the number of chitinase genes. Except this, there was no significant correlation between other parameters and degrading rates of different substrates (Table 6).

The number of chitin-binding proteins in bacteria was also significantly positive correlated with antifungal activity against *Mucor hiemalis*. Numbers of GH18 chitinases were significantly positively correlated with the bacteria’s antifungal activity against *Aspergillus niger* (Table 6).
Table 6 Pearson correlation coefficients for the relationship between degradation rates and in vitro antifungal activities with number of chitinases and chitin-binding proteins. Significant correlations (P < 0.05) are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>Degrading rate</th>
<th>Antifungal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>GH 18 chitinases numbers</td>
<td>-0.46</td>
<td>-0.21</td>
</tr>
<tr>
<td>GH 19 chitinases numbers</td>
<td>-0.22</td>
<td>-0.17</td>
</tr>
<tr>
<td>Chitin binding protein numbers</td>
<td>-0.29</td>
<td>-0.11</td>
</tr>
</tbody>
</table>

N: N-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: Mucor Hiemali; A: Aspergillus niger; R: Rhizoctonia solani

Table 7 Non-parametric Kruskall Wallis test Effects of growth morphology (hyphal growth versus unicellular growth) on degradation rates and in vitro antifungal activity (Non-parametric Kruskall Wallis test). Given values are Bonferroni corrected P-values.

<table>
<thead>
<tr>
<th></th>
<th>Degrading rate</th>
<th>Antifungal activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>C</td>
</tr>
<tr>
<td>Hyphal versus non-hyphal</td>
<td><strong>0.04</strong></td>
<td>0.94</td>
</tr>
</tbody>
</table>

N: N-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: Mucor Hiemali; A: Aspergillus niger; R: Rhizoctonia solani

The impact of hyphal morphology on degradation of the chitin substrates was only significant for N-acetylglucosamine, for which filamentous bacteria had a lower degradation rate than non-filamentous ones (Table 7).

Table 8 Pearson correlation coefficients for the relationships between degradation rates and in vitro antifungal activities. Significant correlations (P < 0.05) are indicated in bold.

<table>
<thead>
<tr>
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<th>Degradation rate</th>
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<tr>
<td></td>
<td>N</td>
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<tr>
<td>Antifungal activity</td>
<td></td>
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<tr>
<td>M</td>
<td><strong>-0.33</strong></td>
</tr>
<tr>
<td>A</td>
<td><strong>-0.60</strong></td>
</tr>
<tr>
<td>R</td>
<td>0.19</td>
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</tbody>
</table>

N: N-acetylglucosamine; B: big size crystal chitin; S: small size crystal chitin; C: colloidal chitin; M: Mucor Hiemali; A: Aspergillus niger; R: Rhizoctonia solani
Bacteria strains differ in chitinolytic activity

The *in vitro* antifungal activity against fungal strain *Mucor hiemalis* was significantly positively correlated with degradation rates of big crystal chitin particles but not with degradation rates of small size crystal chitin particles or colloidal chitin. Antifungal activity of chitinolytic bacteria against *Aspergillus niger* was significantly negatively correlated with their degrading rates of N-acetylglucosamine (Table 8).

Discussion

Bacteria play an important role in the degradation of chitinous resources via hydrolysis of chitin polymers by chitinases (Hoell et al 2010). The chitinolytic system (number of chitinases, modular composition, additional proteins) differs considerably among bacteria and these differences may point at differences in ecological functioning (Bai et al 2015). Next to degradation of chitin, chitinases can be part of an antagonistic system involved in competition with fungi or in defense against bacteriolytic fungi. This antagonistic activity is based on the fact that chitinases can contribute to destabilization of the fungal exterior (cell wall and cell membrane) as chitin is an important structural component of the fungal cell wall (Bowman & Free 2006). In this study we tested, via correlation analysis, whether there is a relationship between the assembly of the chitinolytic system and chitin-degrading abilities or antifungal activities for a set of bacteria.

The results showed that the abilities to degrade the different chitin resources and the chitin monomer N-acetylglucosamine differed strongly among the bacterial strains. The positive correlation between degradation of small and big crystal chitin indicates that bacteria use similar mechanisms to degrade these substrates. In particular, strains from the genus *Streptomyces*, showed high decomposition activity with respect to both big and small chitin particles. It has been suggested that the formation of hyphae by these bacteria is important for the degradation of crystal chitin as it allows for penetration of chitin layers (Gooday 1994). Yet, several other hyphal *Actinomycetes* (such as *Kribbella flavida* DSM 17836 and *Catenulispora acidiphila* DSM 44928), showed slow degradation
of chitin particles, in particular of big chitin particles. Moreover, no statistically significant differences were observed in the degradation of chitin particles between hyphal and non-hyphal bacteria. This indicates that the ability to degrade crystal chitin is not necessarily strong for or limited to filamentous bacteria. The significant lower degradation of N-acetylglucosamine by hyphal bacteria is not surprising as the unicellular growth form is superior for compounds dissolved in aqueous media (De Boer et al 2005).

The production of multiple chitinases is thought to enable synergistic actions during chitin degradation (Horn et al 2006, Techkarnjanaruk & Goodman 1999). Although most of tested strains with higher numbers of chitinases, such as *Streptomyces* showed strong chitin degradation ability, strain *Micromonospora aurantia* DSM 43813 which produce 12 chitinases was a poor degrader of big size crystal chitin. Chitin binding proteins are also known as lytic polysaccharide monooxygenases (LPMOs). They belong to CAZy as Auxiliary Activity family 10 (Horn et al 2012, Levasseur et al 2013). These proteins were reported to introduce molecular oxygen, and then break the polysaccharide chains without the need of first “extracting” these chains from their crystalline matrix, which promotes further degradation by chitinases (Horn et al 2012, Vaaje-Kolstad et al 2010). In addition, chitin binding proteins of the chitinolytic system of *Streptomyces* have been reported to have direct contributions to chitin degradation (Hoell et al 2010, Schrempf 2001). Our study supports the importance of the role of chitin-binding proteins in crystal chitin degradation and antifungal activity.

Neither the number of chitin-binding proteins nor the number of chitinases was correlated with degradation of colloidal chitin. This shows that the structural property of this chitin substrate that is obtained after dissolution in strong acid and re-precipitation in water is different from that of crystal chitin. Indeed, the fungus *Aphanocladium album* was shown to excrete different chitinases when grown on crystal or colloidal chitin (Studer et al 1992). Interestingly, two non-filamentous bacteria with gliding motilities (*Flavobacterium*...
Bacteria strains differ in chitinolytic activity

johnsoniae UW101 and Collimonas fungivorans Ter331) were the fastest degraders of colloidal chitin. This indicates that motility may be an important property for unicellular bacteria to degrade the (very small) particles in colloidal chitin suspensions.

The in vitro inhibition test revealed different responses by the fungi to their confrontation with chitinolytic bacteria. Yet some patterns could be recognized. Streptomycete isolates showed a strong inhibition against all fungi. Streptomycetes are known as degraders of cellulose and other polymers in soil organic matter (Mccarthy & Williams 1992). They share this niche with fungi, and therefore the production of antifungal compounds may be part of their arsenal to compete with fungi (De Boer et al 2005). Based on the current results it is hard to assess the role of chitinases in this interference competition.

In summary, our study gave additional support for the importance of both the chitinolytic system and other abilities (gliding, hyphal growth) for the ability of bacteria to degrade different chitin resources. With respect to the chitinolytic system, our study lends support to the suggested prominent role for chitin-binding proteins in degradation of crystal chitin. No clear relationships were observed between chitin-degrading abilities and inhibition of fungi, underlining the ideas that a combination of factors is in involved in antifungal activity. Despite the fact that we realize that the incubation conditions used in this study (liquid, 20 °C) may not be optimal for all the strains, we feel that it is justified to draw the aforementioned general conclusions.