Enzymatic cocktails produced by *Fusarium graminearum* under submerged fermentation using different lignocellulosic biomasses

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Abstract

*Fusarium graminearum* was grown on four lignocellulosic substrates (corn cobs, wheat bran, hop cell walls, and birchwood) and glucose as the sole carbon source. Proteomic studies performed on the resulting enzymatic cocktails highlighted a great diversity in the number and type of proteins secreted. The cell wall-degrading enzymes (CWDE) proportion varied greatly from 20% to 69%. Only one of the 57 CWDEs detected in this study was common to the five proteomes. In contrast, 35 CWDEs were specific to one proteome only. The polysaccharide-degradation activities were different depending on the cocktail and the polysaccharide used. *F. graminearum* strongly modifies the enzymatic cocktail it secretes as a function of the biomass used for growth.

Introduction

Various lignocellulosic materials have been tested as potential substrates for biofuel production, including wood, crop byproducts, herbaceous plants, beet pulp, municipal wastes, and paper industry wastes (Carroll & Somerville, 2009). The processes of enzymatic biomass degradation may depend on the specificities of cell wall-degrading enzymes (CWDEs).

The ‘minimal enzyme cocktail’ concept (Sørensen et al., 2007) is defined as the lowest number of selected enzymes sufficient for the complete digestion of a biomass. Another method is to mimic natural processes using the more diverse enzymatic cocktails that are produced by microorganisms, especially fungi. The widely used fungus *Trichoderma reesei* is known to produce large amounts of cellulases (essentially cellobiohydrolases), but is relatively poor in the other enzymes required for biomass bioconversion (Martinez et al., 2008). Thus, other fungi have been investigated for their ability to produce more diverse and efficient cocktails or to complement *T. reesei* enzymes (Gottschalk et al., 2010; Ma et al., 2011).

*Fusarium graminearum* is a well-known pathogen of cereals (Munkvold, 2003) and dicots (Urban et al., 2002), and several *Fusarium* isolates were recovered from hops gardens (Hatsch et al., 2002; Phalip et al., 2004). The metabolism of one of these isolates, F9, was investigated in the presence of plant cell wall components. Its entire genome expression was studied after growth on cellulose, xylan, and hop cell wall (Carapito et al., 2008). Depending on the carbon source, the number of CWDE-encoding genes that were over-expressed (as compared with their expression on glucose) was variable, the largest proportion being observed when F9 grew on plant cell wall (19% of over-expressed genes). As CWDE-encoding genes correspond to only c. 0.5% of the genome, F9 cell metabolism was dramatically directed toward cell wall degradation. Furthermore, the genes encoding cellulases, hemicellulases, and pectinases were equally over-expressed.
when the fungus was grown on plant cell wall, whereas cellulases, respectively hemicellulases, were over-expressed when the fungus was grown on cellulose, respectively on xylan. These results suggest that each polysaccharide represents specific signals to the fungus and consequently induces a specific response.

A proteomic study was performed previously with the *F. graminearum* F9 strain grown either on glucose or on hop cell wall (Phalip *et al.*, 2005). On hops, the fungus secreted higher amounts of more diverse proteins, and 45% of these proteins were putative CWDEs, corresponding to 11 putative cellulases, 25 putative hemicellulases, and 19 putative pectinases. The fungus clearly responds to plant cell walls by secreting enzymes capable of digesting each primary cell wall component. Since then, other authors used a similar culture media to study *F. graminearum* secretomes on biomasses (Paper *et al.*, 2007; Ravalason *et al.*, 2012).

In this study, we characterized cocktails produced from the fermentation of four lignocellulosic substrates (two poorly lignified substrates, corn cobs and wheat bran, and two more lignified, birchwood and hop cell wall) and glucose by *F. graminearum*. The fungus produced variable protein cocktails depending on the biomass that was used as the substrate. These cocktails varied in a considerable magnitude (notably much more in comparison with previous works) in terms of protein quantity, the proportion of CWDE determined by proteomics, and the enzymatic activities.

**Methods**

**Biomasses used**

The lignocellulosic substrates that were used for the fermentations included hop cell wall (H, prepared as described in Sposato *et al.*, 1995), corn cobs (Cc, grits, 100–200 mesh) obtained from Eurocobs (France), wheat bran (Wb, from flour, Pomacle, France), and birchwood sawdust (Bi) from INRA (Nancy, France).

**Strain, growth conditions, and enzyme production**

*Fusarium graminearum* (Gibberella zeae) strain F9 was isolated in our laboratory from diseased hops and identified by CABI Bioscience (UK). Cultivations were conducted at 25 °C on M3 medium (Mitchell *et al.*, 1997) supplemented with either glucose, hop cell wall, birch, wheat bran, or corn cobs, each at 10 g L⁻¹ (Phalip *et al.*, 2005). Culture flasks (Falcon 175 cm²) were filled with 125 mL of medium and inoculated with 1000 ± 50 macroconidia. The cultivations continued for 6–9 days for the medium to be sufficiently colonized (Phalip *et al.*, 2005), and the culture media were then separated from the fungus by centrifugation (10 min, 10 000 g). The proteins were precipitated with 95% ammonium sulfate (Sigma) at 4 °C, re-suspended in 1 mL of 100 mM sodium phosphate buffer at pH 6, and dialyzed overnight using the same buffer. Then, they were concentrated to < 100 μL using ultrafiltration devices (Vivaspin, 10 kDa; Sartorius, Germany). For each concentrate, a sample volume equivalent to an 830 μL starting supernatant volume was loaded onto SDS-PAGE. The gels were stained with PageBlue (Fermentas, Lithuania).

**Proteomics**

**In-gel digestion and mass spectrometry analysis**

The gels were cut into slices (2 mm), and in-gel digests were performed using an automated protein digestion system, MassPREP Station (Waters, UK). The gel slices were washed three times in a mixture containing 25 mM NH₄HCO₃: CH₃CN (1 : 1, v/v), and the cysteine residues were reduced using 50 μL of 10 mM dithiothreitol at 57 °C and alkylated using 50 μL of 55 mM iodoacetamide. After dehydration with CH₃CN, the proteins were cleaved in the gel using 40 μL of 12.5 ng μL⁻¹ modified porcine trypsin (Promega) in 25 mM NH₄HCO₃ at 37 °C for 4 h. Tryptic peptides were then extracted using 60% CH₃CN in 0.5% formic acid followed by a second extraction with 100% CH₃CN before nanoLC-MS/MS analysis.

Mass spectrometry analyses were performed on a nanoACQUITY Ultra-Performance-LC system (UPLC) coupled to a Q-TOF mass spectrometer (micrOTOFQ, Bruker Daltonics, Germany) and an Agilent 1100 series nanoLC-Chip/MS system (Agilent Technologies) coupled to an ion trap (amaZon, Bruker Daltonics, Germany) as described in Supporting Information, Data S3.

**Protein identification**

The collected mass data were searched using a local Mascot server (version 2.2.0, MatrixScience, UK) against an in-house-generated database composed of protein sequences downloaded from the MIPS *F. graminearum* Genome Database (http://mips.helmholtz-muenchen.de). Searches were performed without any molecular weight or isoelectric point restriction, trypsin was selected as the enzyme, and carbamidomethylation of cysteine (+ 57 Da) and oxidation of methionine (+ 16 Da) were set as variable modifications. The mass tolerances of the precursor and fragment ions of 10 ppm and 0.04 Da were used, respectively, for micrOTOFQ analysis, and 250 ppm and...
0.5 Da used for the corresponding values, respectively, for amaZon analysis. Mascot (.dat) results were loaded into the SCAFFOLD software (version 2.2.0, Proteome Software Inc.) and filtered to evaluate the false discovery rate (Elias et al., 2007). Protein identification was confirmed when at least two peptides with high-quality MS/MS spectra (above MASCOT’s threshold score of identity at 95% confidence level) were identified. A more stringent filter was applied for single-peptide identification, and the score of a unique peptide had to be more than 30 points above the MASCOT’s threshold score of identity at the 95% confidence level. These thresholds led to protein identification with a false discovery rate of < 1%.

The entire experiment from fungus growth to protein identification by mass spectrometry was performed twice in an independent manner (see Data S1 and S2 for crude results of both experiments). The results being highly similar, the two protein datasets were merged for interpretation (Table S1).

**Protein assay and enzyme activity measurements**

The protein concentrations were determined using the Bradford assay with bovine serum albumin as a standard (Bradford, 1976). AZCL (Azurine-Crosslinked)-xylan from birch, AZCL-arabinoxylan from wheat, AZCL-HE-cellulose, and AZCL-tamarind xyl glucan (tamarind) were obtained from Megazyme (Ireland). The substrates were prepared at 0.25% in 100 mM sodium phosphate buffer, pH 6. Then, 500 ng of protein from each concentrated enzyme cocktail, prepared as described previously, was added to 500 μL of substrate-buffer solution. Immediately after mixing, 100 μL was collected and frozen in liquid nitrogen, and other 100-μL aliquots were recovered after different incubation times at 37 °C. After a brief centrifugation (1 min, 2000 g), the OD_{595 nm} was measured to quantify the enzymatic activities.

**Composition of the starting biomasses**

Biomasses (300 mg) were soaked in 12 M H₂SO₄ for 2 h at room temperature and subsequently diluted with water to 1.5 M H₂SO₄ for total hydrolysis at 100 °C for 2 h (Seeman et al., 1954). The glucan and xylan contents were estimated via the determination of glucose and xylose concentrations using the chromogenic 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulfonate) (Tabka et al., 2006) and an enzymatic kit (Megazyme, Ireland), respectively. Klason lignin was quantified and was found in the acid-insoluble material that remained after sulfuric acid hydrolysis of lignocellulosic substrates (Monties, 1984).

**Results and discussion**

**Composition of the four substrates used for fermentation**

Table 1 shows the composition of the substrates that were used in this study. The data confirmed that the four biomasses varied greatly in composition. Hops contained the most Klason lignin, found in the acid-insoluble material (30.9%), whereas the least lignified biomass was corn cobs (6.5%). The carbohydrate centesimal compositions of corn cob, wheat bran, and birch were similar to those previously described (Lequart et al., 1999; Blondeau & Jeanmart, 2012; Menon & Rao, 2012), except, that in our experiments, the xylose contents tended to be lower. Compared with the others, the hop cell wall contained a small amount of xylose (2.7%).

**Protein production and enzyme activities in the different enzyme cocktails**

Fermentation of *F. graminearum* was performed using hop, wheat bran, corn cobs, birch, and glucose as substrates. The protein concentrations in the supernatants were the following: 13 μg mL⁻¹ for hop, 27 μg mL⁻¹ for wheat bran, 35 μg mL⁻¹ for birch, 30 μg mL⁻¹ for corn cobs, and < 1 μg mL⁻¹ for glucose. The small amount of proteins secreted by *F. graminearum* when grown on glucose, an easily diffusible and metabolizable molecule, has been observed previously (Phalip et al., 2005). In contrast, when the fungus was in contact with complex and polymeric substrates, it secreted hydrolytic enzymes (CWDE) to degrade these substrates to support its growth. Accordingly, in our experiments, the protein concentration was much higher than in the enzyme cocktail obtained on glucose (see above). The differences observed in the catalytic potentialities of these enzyme cocktails were even more interesting. In addition to the small quantities of proteins, weak activities toward cellulose and hemicellulose analogs were found for enzymes produced during growth on glucose (Table 2). Corn cobs and birch appeared to be the best biomasses for growth.
regarding to the secretion of CWDE, particularly when cellulose degradation was concerned (at least 10-times more activity compared with other cocktails). Wheat bran was also efficient for the production of xylanases and arabinoxylanases, whereas the hop enzymatic cocktail appeared significantly less efficient as was the glucose one. Higher activities were measured when using AZCL (Azurine-Crosslinked)-arabinoxylan as a substrate with the wheat bran, birchwood, and corn-cob cocktails, and the same cocktails also hydrolyzed AZCL-xylan but with a lower efficiency.

In summary, the key information obtained from these data was that the activities were different when considering the cocktail (Table 2, vertical view) and the substrate that was used (Table 2, horizontal view). It could be concluded that *F. graminearum* adapts itself to the biomass it has encountered by the secretion of a specific cocktail.

### Proteomic analysis of cocktails

*Fusarium graminearum* grown on several carbon sources produced and secreted variable amounts of proteins, and this finding was confirmed by SDS-PAGE (Fig. 1). When the same volume equivalent of culture supernatant (enzymatic cocktail) was loaded on a gel, the lanes corresponding to wheat bran, birchwood, and corn-cob cocktails, and the same cocktails also hydrolyzed AZCL-xylan but with a lower efficiency.

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#### Table 2. Enzymatic activities measured on enzyme cocktails after growth of *Fusarium graminearum* on different plant materials and glucose

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hop</th>
<th>Wheat bran</th>
<th>Birch</th>
<th>Corn cobs</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZCL-HE-Cellulose</td>
<td>&lt; 0.10</td>
<td>&lt; 0.10</td>
<td>0.79</td>
<td>0.30</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>AZCL-Xylan</td>
<td>0.17</td>
<td>8.82</td>
<td>12.36</td>
<td>22.66</td>
<td>0.21</td>
</tr>
<tr>
<td>AZCL-Xyloglucan</td>
<td>&lt; 0.10</td>
<td>0.04</td>
<td>0.44</td>
<td>&lt; 0.10</td>
<td>&lt; 0.10</td>
</tr>
<tr>
<td>AZCL-Arabinoxylan</td>
<td>0.13</td>
<td>41.20</td>
<td>42.72</td>
<td>54.32</td>
<td>&lt; 0.10</td>
</tr>
</tbody>
</table>

AZCL (Azurine-Crosslinked)-xylan (birch), AZCL-arabinoxylan (wheat), AZCL-HE-cellulose, and AZCL-xyloglucan (tamarind) were prepared at 0.25% in 0.1 M phosphate buffer, pH 6. Five-hundred nanogram of protein were added, and reaction mixes were incubated at 37 °C with agitation. Enzymatic activities are expressed as ΔOD₅₉₅ nm/h. Data are means of two determinations.

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Table 3. CWDE characterized by the proteome analysis

<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
<th>Predicted EC</th>
<th>Predicted CAZy</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGSG_00184*</td>
<td>Endo-1,3(4)-β-glucanase</td>
<td>3.2.1.6</td>
<td>GH16</td>
<td>Cellulose</td>
</tr>
<tr>
<td>FGSG_02022</td>
<td>Probable 1,3-β-glucanoyltransferase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGSG_02354*</td>
<td>Related to chitinase</td>
<td>3.2.1.14</td>
<td>GH18</td>
<td>Chitin</td>
</tr>
<tr>
<td>FGSG_02866*</td>
<td>Conserved hypothetical protein</td>
<td></td>
<td>GH88</td>
<td></td>
</tr>
<tr>
<td>FGSG_03017*</td>
<td>Probable glucanase</td>
<td></td>
<td>GH16</td>
<td>Cellulose</td>
</tr>
<tr>
<td>FGSG_03591</td>
<td>Probable endochitinase</td>
<td>3.2.1.14</td>
<td>GH18</td>
<td>Chitin</td>
</tr>
<tr>
<td>FGSG_03906*</td>
<td>Probable pectate lyase</td>
<td>4.2.2.2</td>
<td></td>
<td>Pectin</td>
</tr>
<tr>
<td>FGSG_04842</td>
<td>Probable exo-α-sialidase</td>
<td>3.2.1.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGSG_07551*</td>
<td>Probable exopolygalacturonase</td>
<td>3.2.1.15</td>
<td>GH28</td>
<td>Pectin</td>
</tr>
<tr>
<td>FGSG_07794*</td>
<td>Probable pectate lyase</td>
<td>4.2.2.2</td>
<td></td>
<td>Pectin</td>
</tr>
<tr>
<td>FGSG_09289</td>
<td>Related to family of putative glycosidases</td>
<td></td>
<td>GH16</td>
<td></td>
</tr>
<tr>
<td>FGSG_11163*</td>
<td>Probable pectate lyase</td>
<td>4.2.2.2</td>
<td></td>
<td>Pectin</td>
</tr>
<tr>
<td>FGSG_11280*</td>
<td>Probable acetyesterase (pectin esterase)</td>
<td>3.1.1.11</td>
<td>CE12</td>
<td>Pectin</td>
</tr>
</tbody>
</table>

Proteins present in hop proteome only (13)

Proteins present in birch proteome only (14)

Proteins present in wheat bran proteome only (7)

Protein present in corn cobs proteome only (1)

Proteins present in both wheat bran and birch proteomes (5)

Proteins present in both wheat bran and hop proteomes (7)

Proteins present in both hop and birch proteomes (2)
polysaccharide-mimicking substrates (Table 2). This result means that when the same volume of supernatant was taken, lower quantities of protein (Fig. 1) and less-active CWDEs (Table 2) were recovered from hop although a high diversity of proteins was observed (101 unique proteins).

Among a total of 180 unique proteins (Table S1), 57 different CWDEs were found (Table 3). The repartition of these enzymes regarding the carbon source used for growth is diverse (Fig. 2): only two were found in the glucose-induced proteome, nine were identified in the corn cob-induced proteome, and 25–28 CWDEs were identified when the three other substrates were used. Most interesting is the comparison of these data to the number of unique proteins. The percentages of CWDEs among the total proteins secreted when the complex biomasses were used for growth were variable from 20% for corn cobs to as much as 69% for birch (Fig. 2). These data are in accordance with the enzymatic activities (Table 2) because the cocktail generated after growth on birch was clearly the most diverse, presenting the highest activity on two of the substrates and the second highest activity on two other substrates. In quite similar conditions, Paper et al. (2007) did not evidence such differences. By adding no carbon source, sucrose, corn, carrot or xylan, 91, 98, 96, 98, or 97 different proteins were identified, respectively. Furthermore, the proportion of enzymes potentially acting on cell wall polysaccharides was markedly less variable than in our study. As enzymatic activities were absent, the full comparison of the two studies cannot be achieved. In contrast, the closely related fungus F. verticillioides behaved remarkably similarly than F. graminearum when grown on corn material. Indeed, it secreted 25% of enzymes able to act on cell wall polysaccharides (Ravalason et al., 2012) close to the 20% in our study. As the in silico predicted secretome contain about 19% of these enzymes (Brown et al., 2012), higher proportions of CWDE in a secretome indicate an active response of the fungus. This metabolism reorientation was particularly obvious in the case of birchwood (Fig. 2).

The occurrence of a given protein in only one exoproteome or in several exoproteomes is of interest. As many as 35 of the 57 CWDEs were present in only one proteome (Table 3), 15 were recovered from two proteomes, and only one was common to the five proteomes. In contrast, some CWDEs were more widespread (Table 3). A cellobiohydrolase (FGSG_00571), a probable endoglucanase (FGSD_11037), an endoxylanase (FGSD_06445), and an arabinoarabinofuranosidase (FGSD_03813) were found in the four cocktails from the complex biomasses, hop, wheat bran, corn cobs, and birch. These four proteins were also found on corn- and carrot-based media (Paper et al., 2007). The complexity and the heterogeneity of CWDE repartition within the different cocktails are in good agreement with their previously mentioned differences in enzymatic activities (Table 2). However, even if the enzymes composing the cocktails were different, these four cocktails displayed putative proteins that were, more or less, able to attack the three major components of the

### Table 3. Continued

<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
<th>Predicted EC</th>
<th>Predicted CAZy</th>
<th>Substrate</th>
</tr>
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<tbody>
<tr>
<td>FGSG_09366</td>
<td>Related to glucan 1,3-β-glucosidase</td>
<td>3.2.1.58</td>
<td>GH17</td>
<td></td>
</tr>
<tr>
<td>FGSG_05663</td>
<td>Related to chitin deacetylase</td>
<td>3.5.1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGSG_05757</td>
<td>Probable cell wall glucanosyltransferase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGSG_06278</td>
<td>Probable glucan 1,4-α-glucosidase</td>
<td>3.2.1.3</td>
<td>GH16</td>
<td></td>
</tr>
<tr>
<td>FGSG_03813</td>
<td>Probable α-L-arabinofuranosidase/xylosidase</td>
<td>3.2.1.37</td>
<td>GH54</td>
<td>Pec./Hemi.</td>
</tr>
<tr>
<td>FGSG_06445</td>
<td>Probable endo-1,4-β-xylanase</td>
<td>3.2.1.8</td>
<td>GH10</td>
<td>Hemicellulose</td>
</tr>
<tr>
<td>FGSG_11037</td>
<td>Endoglucanase I</td>
<td>3.2.1.4</td>
<td>GH12</td>
<td>Cell./Hemi.</td>
</tr>
<tr>
<td>FGSG_00571</td>
<td>Probable cellulose 1,4-β-cellobioseidase</td>
<td>3.2.1.91</td>
<td>GH7</td>
<td>Cellulose</td>
</tr>
</tbody>
</table>

Two independent biological experiments were performed, and data correspond to the union of the two datasets. The proteins are classified as a function of their occurrence in the different proteomes. The number of proteins in each category is given in parentheses. The predictions were given by the Fusarium graminearum Genome Database (http://mips.helmholtz-muenchen.de/genre/proj/fusarium). When a protein is able to use two substrates, the following abbreviations were used: Pec., for pectin; Hemi., hemicellulose; and Cell., cellulose. As in the SDS-PAGE (Fig. 1), the equivalent of the same volume was analyzed for each proteome.
primary cell wall: cellulose, hemicellulose, and pectin (Tables 2 and 3). The glucose proteome did not display such enzyme diversity, which was again in accordance with the low activities that were measured (Table 2) and the simplicity of the substrate assimilation. These data clearly demonstrate that the nature of the secreted CWDEs closely depends on, and is directed by, the biomass used for growth.

Other fungi have been used for enzyme cocktail production. When growing on corn cell wall, T. reesei produced a hydrolytic cocktail (Nagendran et al., 2009) that was different from the cocktails produced in this study by Fusarium. Gottschalk et al. (2010) reported that enzymes from T. reesei grown on corn-steep liquor (better for cellulose hydrolysis) and Aspergillus awamori grown on wheat bran (better for xylan hydrolysis) displayed different hydrolytic activities. Both cocktails acting together improved glucose release from steam-treated sugarcane, which supports the argument that fungi exhibit specific responses to different biomasses.

The supernatant yielded by the growth on birch was the only one containing glycosyl hydrolases from the GH 61 family (Table 3). These enzymes were first described as ‘cellulase-enhancing factors’, and it has been demonstrated that they were oxidative enzymes involved in the production of oxidized cellobextrins (Hemsworth et al., 2013). This behavior represents a strong specificity of the exoproteome resulting from growth on birchwood.

The cocktails generated in this study are potentially richer in the specific enzymes (especially accessory enzymes) that are necessary for the digestion of a given biomass than the invariable and predefinite enzyme mixes (enzymes) that are necessary for the digestion of a given biomass. One hypothesis is that the cocktail produced in this study by Fusarium graminearum (Hemsworth et al., 2003) was different from the cocktails produced in this study by Fusarium graminearum (Hemsworth et al., 2003). Once the secretome of the plant pathogenic fungus Fusarium graminearum: a refined comparative analysis. PLoS One 7: e33731.


References


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** List of all the proteins recovered in all the five proteomes (two independent experiments were performed and mixed in this table).

**Data S1.** Crude proteomics data generated by NanoAC-QUITY Ultra-Performance-LC system coupled to a microTOFQ (see section ‘Protein identification’ for details).

**Data S2.** Crude proteomics data generated by nanoLC-Chip/MS system coupled to amaZon ion trap (see section ‘Protein identification’ for details).

**Data S3.** Proteomics: supporting methods.