Bacterial chitinases and chitin-binding proteins as virulence factors

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Bacterial chitinases (EC 3.2.1.14) and chitin-binding proteins (CBPs) play a fundamental role in the degradation of the ubiquitous biopolymer chitin, and the degradation products serve as an important nutrient source for marine- and soil-dwelling bacteria. However, it has recently become clear that representatives of both Gram-positive and Gram-negative bacterial pathogens encode chitinases and CBPs that support infection of non-chitinous mammalian hosts. This review addresses this biological role of bacterial chitinases and CBPs in terms of substrate specificities, regulation, secretion and involvement in cellular and animal infection.

Introduction

Glycoside hydrolases (GHs) are enzymes that cleave the glycosidic bonds of glycans. They are increasingly being recognized as virulence factors of bacterial pathogens, and examples include enzymes belonging to GH2 galactosidases, GH13 pullulanases, GH18 endo-β-N-acetylglucosaminidases, GH20 β-hexosaminidases and β-N-acetylglucosaminidases, GH30 glycosylceramidases, GH33 sialidases, GH73 muramidases/lysozymes, GH84 hyaluronidases and GH101 N-acetylglucosaminidases (Canard et al., 1994; Garbe & Collin, 2012; Kim et al., 2009; Lenz et al., 2003; van Bueren et al., 2007; Renzi et al., 2011; Sjögren et al., 2011).

Chitinases belong to GH families 18 and 19, and are ubiquitous enzymes produced by bacteria and other life forms (Gooday, 1990). They hydrolyse the second most abundant polysaccharide in nature, chitin, which comprises linear β 1,4-N-acetylglucosamine (GlcNAc) residues. This polymer is a structural component of crustacean shells, the fungal cell wall, the exoskeleton of arthropods and the cysts of various protozoans. Chitinases often work synergistically with chitin-binding proteins (CBPs). Bacterial CBPs contain carbohydrate-binding modules of family 33 (CBM33) and sometimes family 5/12 (CBM5/12). CBM33s are thought to facilitate chitinase accessibility to crystalline chitin matrices by introducing breaks in the chitin chains. Recently, it has become apparent that some CBM33 domains of bacterial CBPs are able to enzymically cleave chitin. Therefore, a more descriptive term, lytic polysaccharide monoxygenases, has been proposed for these proteins (Aachmann et al., 2012) and they are now reclassified as auxiliary activity family 10 (AA10) (CAZY, 2013).

The biological roles of bacterial chitinases and CBPs are easily understood in an environmental context, typically exemplified by chitin cycling in the marine environment by numerous taxa, including Vibrio spp. (Keyhani & Roseman, 1999). There is an increasing amount of direct or indirect evidence suggesting that some chitinases and CBPs additionally serve as virulence factors for bacterial pathogens during infection. Examples include chitinases from Legionella pneumophila (DebRoy et al., 2006), Listeria monocytogenes (Chaudhuri et al., 2010; Larsen et al., 2010) and Salmonella enterica serovar Typhimurium (S. Typhimurium) (Larsen et al., 2011) as well as a CBP from Vibrio cholerae (Kirn et al., 2005). A summary of cell culture/in vivo indications of this role is presented in Tables 1 and 2. Potential targets of the chitinases and CBPs during infection include glycoproteins and glycolipids of host organisms that contain GlcNAc (Fig. 1), the monomer that also composes chitin.

The assessment of bacterial chitinases as virulence factors is complicated by the fact that the majority of bacterial pathogens that encode GH family 18 and 19 enzymes harbour up to several different variants of these proteins. Furthermore, enzymic activity targeting chitin per se may in some cases be perceived as being involved in virulence. This concerns bacterial pathogens infecting invertebrate hosts that contain chitin as a constituent either of their exoskeleton (arthropods) or their intestinal peritrophic membrane (annelids and some arthropods). This virulence aspect will not be discussed further here, as it falls outside the scope of this review, but it has been dealt with elsewhere (Gooday, 1999). Instead, we will focus on what is known regarding bacterial chitinases and CBPs as virulence factors through their interaction with target substrates other than chitin.
### Table 1. Examples of bacterial GH family 18 chitinases as putative virulence factors

ND, Not determined; CF, cystic fibrosis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein name/ID</th>
<th>Accessory domain*</th>
<th>Endogenous upregulation†</th>
<th>Exogenous upregulation†</th>
<th>Cell culture/ in vivo phenotype</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
<td>eChi18A/EF0361</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>Endogenous upregulation†</td>
<td>Chitin</td>
<td>Leisner et al., 2009; Vebo et al., 2009; Vebo et al., 2010</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>ChiA/b3338</td>
<td>CBM5/12</td>
<td>ND</td>
<td>Mutant with reduced adhesion to epithelial cells‡</td>
<td>In vitro human urine and horse blood</td>
<td>Chitin</td>
<td>Francetic et al., 2000a; Tran et al., 2011</td>
</tr>
<tr>
<td><strong>F. tularensis</strong></td>
<td>ChiA/FTT_0715</td>
<td>CBM5/12, FnIII</td>
<td>ND</td>
<td>Mutant not attenuated in mice</td>
<td>Mutant not attenuated in mice</td>
<td>Chitin</td>
<td>Kadzhaev et al., 2009; Mortensen et al., 2010; Twine et al., 2006</td>
</tr>
<tr>
<td><strong>Legionella pneumophila</strong></td>
<td>ChiA/lpg1116</td>
<td>–</td>
<td>Virulence regulator</td>
<td>No effect in cell cultures; reduced recovery of mutant from mice lungs</td>
<td>In vivo mice spleen§</td>
<td>Chitin</td>
<td>Debray et al., 2006; Galka et al., 2008</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td>ChiA/lmo1883</td>
<td>–</td>
<td>Virulence regulators¶</td>
<td>No effect in cell cultures; reduced mutant recovery from mice spleen and liver</td>
<td>No effect in cell cultures; reduced mutant recovery from mice spleen and liver</td>
<td>Chitin, LacdiNAc</td>
<td>Chatterjee et al., 2006; Chaudhuri et al., 2010; Kazmiereczak et al., 2003; Larsen et al., 2010; Maheil et al., 2011; B. G. Storgaard, M. M. Palcic, J. J. Leisner and others, unpublished results; Toledo-Arana et al., 2009; van der Veen et al., 2007</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>ChiC/PA2300</td>
<td>CBM5/12, FnIII</td>
<td>Virulence regulators¶</td>
<td>Stationary phase, in vivo mice intestine, heat-shock, GlcNAcII, chitinII</td>
<td>No effect in cell cultures; reduced mutant recovery from mice spleen and liver</td>
<td>Chitin, LacdiNAc</td>
<td>Chugani &amp; Greenberg, 2007; Folders et al., 2001; Lesic et al., 2007; Salunkhe et al., 2005; Fung et al., 2010</td>
</tr>
</tbody>
</table>
An additional issue that complicates the picture is that most bacterial chitinases are classified in the GH family 18 (CAZY, 2013; Cantarel et al., 2009), which also harbours some bacterial endoglycosidases (Fig. 2) (endo-β-N-acetylglucosaminidases; EC 3.2.1.96), promoting endohydrolysis of the chitobiose core of various N-linked glycoproteins. The endoglycosidases resemble chitinases in amino acid sequences but no, or only little, chitinolytic activity has been demonstrated for these enzymes. Examples of such endoglycosidases include enzymes produced by Enterococcus faecalis (Collin & Fischetti, 2004; Bøhle et al., 2011) and Streptococcus pyogenes (Collin & Ölsén, 2001). We will not describe the role of these enzymes as virulence factors in any detail, since this has been the topic of another recent review (Garbe & Collin, 2012).

Reports in the literature also describe some of the enzymes in the GH families 20 and 46 as chitinases. They are not included in this review as these families in our opinion should more appropriately be regarded as β-N-acetylglucosaminidases/hexosaminidases and chitosanases, respectively, as outlined by Henrissat (1999).

Classification of hydrolytic domains of chitinases

As mentioned above, based on their primary structures the hydrolytic domains of chitinases can be allocated to the GH families 18 and 19. These families attain the triosephosphate isomerase (TIM) barrel and bilobal secondary structures, respectively, and differ in their reaction mechanisms, with family 18 enzymes operating with overall retention of the anomic configuration at the cleavage point, and family 19 operating by inversion of the anomic configuration (Henrissat, 1999). The two families most likely evolved from different ancestors (Bussink et al., 2007). Family 18 chitinases occur in viruses, bacteria, archaea and eukaryotes (Fig. 2), while family 19 chitinases have been associated mainly with plants, although it is now clear that many bacteria encode these enzymes as well.

Based on sequence analyses the GH family 18 enzymes have been separated into different groups (Karlsson & Stenlid, 2009; Svitil & Kirchman, 1998; Watanabe et al., 1993). This classification illuminates the phylogenetic relationships between catalytic domains, but has so far not offered insights regarding distinct roles of biological significance. The TIM barrel of GH family 18 enzymes contains in the active site a catalytic motif of DXDXE residues (Fig. 2) that includes a glutamic acid which protonates the oxygen in scissile glycosidic bonds (Vaaje-Kolstad et al., 2004). Based on conservation of the active site structure and essential catalytic residues, the GH 18 family enzymes are suggested to be part of an enzyme superfamily also including GH 20 β-hexosaminidases and N-acetylglucosaminidases, GH 25 lysozymes, GH 56 hyaluronidases, GH 84 O-GlcNAcases and GH 85 endo-β-N-glucosaminidases (Martinez-Fleites et al., 2009). GHs can be classified as either exo or endo depending on whether the enzyme cleaves the substrate...
Table 2. Examples of bacterial CBPs/lytic polysaccharide monooxygenases as putative virulence factors

ND, Not determined.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein name/ID</th>
<th>Domain*</th>
<th>Secretion mechanism</th>
<th>Endogenous regulation†</th>
<th>Exogenous upregulation†</th>
<th>Cell culture/in vivo phenotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td>GbpA/VCA0811</td>
<td>CBM33, CBM5/12</td>
<td>Type II</td>
<td>ND</td>
<td>Quorum sensing‡</td>
<td>Reduced mutant adherence to epithelial cells; reduced colonization of mice intestine</td>
<td>Kirn et al., 2005; Bhowmick et al., 2008; Jude et al., 2009; Wong et al., 2012</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Lmo2467</td>
<td>CBM33, FnIII, CBM5/12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Macrophage cytosol, stationary phase Reduced recovery of mutant from mice spleen and liver</td>
<td>Chaudhuri et al., 2010; Chatterjee et al., 2006; Toledo-Arana et al., 2009</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>CbpD/PAO852</td>
<td>CBM33</td>
<td>Type II</td>
<td>Quorum sensing‡</td>
<td>Biofilm formation</td>
<td>Upregulated in CF isolates but not in wound isolates‡</td>
<td>Folders et al., 2000; Sriramulu et al., 2005; Kay et al., 2006</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>CBP21</td>
<td>CBM33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Chitin, GlcNAc uptake Reduced mutant adherence to epithelial cells</td>
<td>Kawada et al., 2008</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>efCBM33A/EF0362</td>
<td>CBM33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>In vitro human urine and horse blood</td>
<td>Vebø et al., 2009; Vebø et al., 2010</td>
</tr>
</tbody>
</table>

*CBM33 is now classified as AA10.
†Data from microarray studies if not noted otherwise.
‡Protein analysis.
from a terminus or randomly at internal sites along the polymer, respectively. The endochitinases tend to be non-processive and have shallow substrate-binding clefts, while exochitinases are often processive with deep substrate-binding clefts. The closed architecture of the exochitinases seems to be correlated with the presence of an insertion domain in the TIM barrel of these enzymes (Horn et al., 2006; Suzuki et al., 1999).

Based on tertiary structure, the catalytic domain of family 19 chitinases has been allocated to the lysozyme superfamily which also includes GH 22, GH 23 and GH 24 lysozymes, and the GH 46 chitosanases. Enzymes of this superfamily are believed to have diverged from a common ancestor (Holm & Sander, 1994; Wohlkönig et al., 2010). The catalytic domains of these enzyme families share no overall sequence similarity, but all attain a common fold constituted by an all α-helix domain containing a catalytic glutamate residue and a β-hairpin. Considering the chemical similarity between chitin and peptidoglycan, it is not so surprising that lysozymes can often hydrolyse chitin, and that chitinases sometimes cleave peptidoglycan, though less efficiently than their natural substrates (Wohlkönig et al., 2010). In agreement with that, the phylogenetic tree of the bacterial family 19 chitinases (Fig. 3) illustrates that some enzymes belonging to this family may more appropriately be annotated as lysozymes or as bifunctional chitinases/lysozymes.

**Classification of chitin-binding modules of chitinases and CBPs**

Protein domains responsible for the binding of carbohydrates are, based on primary sequence similarity, classified
**Fig. 2.** Phylogenetic tree of catalytic domains of GH 18 family endoglycosidases. In blue, slime moulds; in green, viruses; in purple, trypanosomatids. Proteins lacking predicted signal peptides are indicated by an asterisk. Amino acid positions of catalytic domains are also indicated after the accession numbers.

**GH 18 active site consensus sequences**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
<th>Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>TIGR4</td>
<td>DGE</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Aba49</td>
<td>DGL</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>V853</td>
<td>DGL</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>ATCC4579</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>M66-2, ChiA</td>
<td>DGL</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>EGD-e, ChiA</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PAO1, ChiC*</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>, chitinase C*</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Yersinia pseudotuberculosis</em></td>
<td>YPII, glycosidehydrolyase family 18</td>
<td>DGF</td>
</tr>
<tr>
<td><em>Franciella tularensis</em></td>
<td>subsp. tularensis FSC198, chitinase*</td>
<td>NGi</td>
</tr>
<tr>
<td><em>Franciella tularensis</em></td>
<td>subsp. tularensis FSC198, FT00806</td>
<td>NSE</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>subsp. enterica serovar Typhimurium str. LT2, ChiA*</td>
<td>SVE</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>enterica serovar Typh (EPI)</td>
<td>SVE</td>
</tr>
<tr>
<td><em>Cronobacter sakazakii</em></td>
<td>E899, CSE899_07525*</td>
<td>SVE</td>
</tr>
<tr>
<td><em>Sodalis glossinidius</em></td>
<td><em>morsitans</em>, exochitinase*</td>
<td>GIE</td>
</tr>
<tr>
<td><em>Dictyostelium purpureum</em></td>
<td>, DICPUDRAFT_96643*</td>
<td>TIE</td>
</tr>
<tr>
<td><em>Polysphondylium pallidum</em></td>
<td>PNS000, glycosylhydrolase*</td>
<td>TIE</td>
</tr>
<tr>
<td><em>Rickettsia felis</em></td>
<td>URRWXCa2, chinase*</td>
<td>KIQ</td>
</tr>
<tr>
<td><em>Xenorhabdus nematophila</em></td>
<td>ATCC 19061, putative chitinase*</td>
<td>SHL</td>
</tr>
<tr>
<td><em>Xenorhabdus nematophila</em></td>
<td>ATCC 19061, Chi*</td>
<td>SAT</td>
</tr>
<tr>
<td><em>Paramecium bursaria</em></td>
<td>Chlorella virus 1</td>
<td>NIS</td>
</tr>
<tr>
<td><em>Rhodnius prolixus</em></td>
<td>M66, chitinase 18 protein</td>
<td>DGV</td>
</tr>
<tr>
<td><em>Mammalian</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bombyl mori</em></td>
<td><em>nucleopolyhedrovirus, chitinase</em></td>
<td>DGV</td>
</tr>
<tr>
<td><em>Bombyl mori</em></td>
<td><em>nucleopolyhedrovirus, chitinase</em></td>
<td>DGV</td>
</tr>
<tr>
<td><em>Autographa californica</em></td>
<td><em>nucleopolyhedrovirus, ChiA</em></td>
<td>DGV</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>M66-2, chitinase</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Streptomyces plicatus</em>, chitinase 63</td>
<td>DGI</td>
<td>D</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>ATCC 14579, chitinase C</td>
<td>DGV</td>
</tr>
<tr>
<td><em>Serratia marcescens</em>, chitinase A</td>
<td>DGV</td>
<td>V</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>EGD-e, ChiB</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Clostridium difficil</em>, piroxerodin/chitinase*</td>
<td>DGI</td>
<td>D</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila</em></td>
<td>subsp. hydrophila ATCC 7966, chitodextrinase</td>
<td>DGV</td>
</tr>
<tr>
<td><em>Serratia marcescens</em>, chitinase A</td>
<td>DGI</td>
<td>D</td>
</tr>
<tr>
<td><em>Citrobacter koseri</em></td>
<td>ATCC 895, COK_02217</td>
<td>DGV</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>ATCC 10987, glycosylhydrolyase*</td>
<td>DGV</td>
</tr>
<tr>
<td><em>Leishmania mexicana</em>, chitinase</td>
<td>DGI</td>
<td>D</td>
</tr>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td>1710b, glycosylhydrolyase</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>BW2952, periplasmic endochitinase</td>
<td>KVL</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em>, Dugway SJ108-111, chitinase*</td>
<td>DGI</td>
<td>D</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>subp. pneumophila, str. Philadelphia 1</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>V583, EF2863</td>
<td>DGI</td>
</tr>
<tr>
<td><em>Streptomyces plicatus</em>, EndoH</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>subp. pneumophila, str. Philadelphia 1, ChiA</td>
<td>DGI</td>
</tr>
</tbody>
</table>
Serratia marcescens

Members of the CBM33 family are mostly found in bacterial genomes, mainly chitin-binding modules belonging to families 1, 2, 3, 5, 12, 14, 18, 19, 33, 37 and 50 (CAZY, 2013). CBM5 is related to CBM12, and will hence be referred to as CBM5/12. In bacterial chitinases, as well as the CBM33 of CBPs. CBM2 domains are frequently found in both family 18 and 19 chitinases, as well as the CBM33 of CBPs. CBM2 domains are found in bacterial family 18 chitinases, while CBM5/12s are frequently found in both family 18 and 19 chitinases, as well as in some CBPs (CAZY, 2013; Punta et al., 2012; Karlsson & Stenlid, 2009). Functional studies on chitin-binding properties of CBM2 and CBM5/12 have mainly focused on the role of the latter, but both domains have been shown to enhance substrate affinity and increase catalytic efficiency of chitinases (Watanabe et al., 1994; Hashimoto et al., 2000; Svitil & Kirchman, 1998; Nakamura et al., 2008), in a manner likely dependent on certain conserved surface-exposed aromatic residues (Morimoto et al., 1997).

Besides the chitin-binding domains, both chitinases and CBPs can contain additional domains, as discussed below.

**Expression and secretion of chitinases and CBPs during infection**

From DNA microarray studies as well as deletion mutant studies, it is clear that a number of endogenous factors may promote expression of chitinase-encoding genes during infection in Listeria monocytogenes, Legionella pneumophila, Pseudomonas aeruginosa and Chromobacterium violaceum (Table 1) (Stauff & Bassler, 2011; Winson et al., 1995; Kay et al., 2006). External parameters are also important in this regard. Transcription of the virulence-related chitinase ChiB of Listeria monocytogenes and a chitinase from Enterococcus faecalis (eCh18A) has been shown to be upregulated in vitro in body fluids, such as blood and urine (Toledo-Arana et al., 2009; Vebø et al., 2009; Vebø et al., 2010). Similarly, chitinase upregulation has also been seen during growth of P. aeruginosa in artificial cystic fibrosis sputum (Fung et al., 2010), which is in agreement with the fact that the expression of a P. aeruginosa chitinase is enhanced in clinical isolates as compared with that in a laboratory strain (Salunkhe et al., 2005).

Host intracellular environments can also promote chitinase transcription, as demonstrated for Listeria monocytogenes during infection of macrophage cell cultures (Mraheil et al., 2011), and for epithelial and macrophage cell cultures infected by S. Typhimurium (Eriksson et al., 2003; Haufert et al., 2008). Other microarray data show, however, that the distinct Salmonella chitinase-encoding gene, chiA, is not always expressed during intracellular infection, as no upregulation was observed for Salmonella enterica serovar Typhi infecting macrophage cells (Faucher et al., 2006).
A Northern blot study demonstrated that the central Listeria monocytogenes virulence regulators PrfA and σB significantly increase transcription of the two chitinase genes chiA and chiB in this organism (Larsen et al., 2010). However, the overall evaluation of the role of Listeria monocytogenes chitinases as virulence factors is complicated by the fact that the expression of both enzymes is also induced by chitin, while only expression of chiA and not chiB is induced by GlcNAC (Larsen et al., 2010).

The above-described in vitro data are supported by in vivo findings for several pathogens, such as chitinase gene upregulation in Listeria monocytogenes infecting mice intestine (Toledo-Arana et al., 2009) and in S. Typhimurium infecting chicken intestines (Harvey et al., 2011). Similarly, the chitinase from Francisella tularensis, the agent causing tularemia, has been shown by proteomics to be highly expressed during in vivo infection of mice spleens (Twine et al., 2006).

Secretion to the outside of the bacterial cell is needed during the infection process for the chitinases to interact with host cell glycans. Information on secretion mechanisms for bacterial chitinases so far comes mostly from bioinformatical prediction of the presence of signal sequences. Such analyses have predicted that the eCh18A chitinase (Table 1) from the Gram-positive Enterococcus faecalis is secreted by the Sec pathway. The Gram-negative Escherichia coli, Legionella pneumophila and V. cholerae chitinases also carry signal sequences and are secreted by means of the type II secretion system (Table 1) (DebRoy et al., 2006; Francetic et al., 2000b; Sikora et al., 2011), whereas this does not seem to be the case for P. aeruginosa (Foldes et al., 2001). A number of chitinases (Fig. 2) lack the typical signal peptide sequence but most of these are predicted by bioinformatics tools (SecretomeP 2.0 Server) to be secretory proteins, which suggests that their substrates are outside the bacterial cell. It remains to be determined whether these enzymes are indeed secreted or instead are located on the outer bacterial surface (Larsen et al., 2011; B. G. Storgaard, M. M. Palcic, J. J. Leisner and others, unpublished results).

Five CBPs with suspected roles in virulence (Table 2) all carry signal peptides associated with Sec- or Tat-dependent export mechanisms. Lmo2467 from the Gram-positive Listeria monocytogenes is probably secreted by the Sec pathway, as secretion by the Tat pathway is a rare event in this species (Desvaux & Hébraud, 2006). The CbpD and GbpA proteins from the Gram-negatives P. aeruginosa and V. cholerae, respectively, are secreted by the type II secretion system (Foldes et al., 2000; Kirn et al., 2005), while the secretion mechanism for CPB21 from Serratia marcescens and eCBM33A from Enterococcus faecalis is unknown. The endogenous factors that regulate expression of these CBPs are not well described. GbpA is, as most virulence factors in V. cholerae are, detected at low cell density only, and is cleaved by quorum sensing-regulated proteases at high cell density (Jude et al., 2009). CbpD is likewise expressed in concert with quorum-sensing regulated virulence factors, however, at high cell density (Kay et al., 2006). Expression of the CBP eCBM33A from Enterococcus faecalis is upregulated in the presence of blood and urine (Vebo et al., 2009; Vebo et al., 2010) and has been proposed as a potential virulence factor (Paulsen et al., 2003).

Taken together, these results indicate that for several bacterial pathogens, the expression/secretion of chitinases and CBPs is related to the infective ability of the organisms.

What do we know from cellular and animal studies?

A straightforward approach to assess the effect of chitinases or CBPs on bacterial virulence consists of comparing wild-type strains with deletion mutants. Data from such studies are presented in Tables 1 and 2. In some instances chitinase-encoding genes are important for virulence in in vivo animal models but not in in vitro cellular assays. Examples include the intracellular pathogens Legionella pneumophila and Listeria monocytogenes in which chitinase deficiency causes defective growth in mice lungs and spleens, respectively (Chaudhuri et al., 2010; DebRoy et al., 2006). However, the ability of the Legionella pneumophila mutants to survive in macrophage cell cultures is similar to that of the wild-type. This is also the case for Listeria monocytogenes mutants infecting epithelial and macrophage cell cultures (Chaudhuri et al., 2010; DebRoy et al., 2006; Larsen et al., 2010), despite the notion of enhanced intracellular chitinase transcription in macrophages (Mraheil et al., 2011). The in vivo virulence effects of chitinases might not be limited to bacteria, as the trypanosomatid protozoan pathogen, Leishmania mexicana, produces a chitinase that contributes to formation of lesions in a mouse model as well as promoting survival in host macrophage cells (Joshi et al., 2005).

However, bacterial chitinases may also affect the infection of cell cultures. The transcription of the S. Typhimurium LT2 chiA gene is enhanced upon infection of epithelial and macrophage cells (Eriksson et al., 2003; Hautefort et al., 2008). We have very recently found that deletion of chiA in this strain moderately reduces the ability of Salmonella to invade epithelial cells and macrophages (R. F. Frederiksen and others, unpublished results). The role of this phenomenon in an in vivo model has not been investigated.

Animal studies show that absence of two CBPs, the GbpA and Lmo2467 from V. cholerae and Listeria monocytogenes, respectively, attenuates bacterial virulence in mice (Chaudhuri et al., 2010; Kirn et al., 2005). The possible underlying mechanisms will be discussed below.

Substrate specificities of catalytic domains relevant to virulence

In several studies chitinases have been suggested as virulence factors although the target molecules in the host have not been identified. Indeed, the absence of endogenous chitin in
vertebrate hosts immediately suggests that other GlcNAc-containing glycans are the target(s). Bacterial pathogens encounter host GlcNAc-containing glycans in the form of glycolipids, glycoproteins and/or as part of extracellular matrices during infection. Extracellular pathogens interact with such substrates located in tissues or lumens of, for example, the intestine or blood vessels, while intracellular pathogens encounter substrates also within the host cells, e.g. in phagosomes (Fig. 1). The GlcNAc residues are found in the two major types of protein-linked glycans, the asparagine-linked glycans (N-glycans) and the serine/threonine-linked glycans (O-glycans) as well as in glycolipids and extracellular matrix proteins, such as glucosaminoglycans (Fig. 1).

N-glycans contain a common pentasaccharide structure composed of an asparagine-linked chitobiose core \([\text{GlcNAc}_2]\) and three terminal mannose residues \([\text{Man}_3]\), which act as the starting point for carbohydrate branches (Fig. 1). N-glycans carrying branches of only mannose residues are termed high-mannose N-glycans, while complex-type N-glycans include other residues, e.g. GlcNAc, galactose (Gal) and sialic acid. Many N-glycans have features shared by both high-mannose and complex-type and are classified as hybrid-type. O-glycans are based on a serine/threonine-linked N-acetylgalactosamine (GalNAc) residue extended with, for example, galactose and GlcNAc residues resulting in eight common core structures. Both the chitobiose core, \(\text{N},\text{N}'\)-diacyctylacetosamine (LacdiNAc) (GalNAc\(\beta\)-1-4GlcNAc) and \(\text{N}\)-acetyllactosamine (LacNAc) (Gal\(\beta\)-1-4GlcNAc) structures of O-glycans and N-glycans are putative targets for bacterial GH family 18 enzymes.

The chitobiose core of N-glycans can be cleaved by GH18 family endoglycosidases, which, as mentioned previously, are phylogenetically related to chitinases (Fig. 2) (Collin & Olsen, 2001; Garbe & Collin, 2012). It would be of interest to examine whether ‘true’ chitinases exhibit endoglycosidase-like activity towards chitobiose cores.

It has recently been demonstrated that a diverse range of bacterial chitinases hydrolyse GlcNAc-containing model substrates of LacdiNAc and to a lesser extent also LacNAc, though at low rates (Larsen et al., 2011; Murata et al., 2005; Shoda et al., 2006; B. G. Storgaard, M. M. Palcic, J. J. Leisner and others, unpublished results). LacNAc structures have been identified in O-glycans and N-glycans of glycoproteins as well as in glycolipids of eukaryotic cells (Varki et al., 2009), and are abundant in proteins of the human immune system (Marth & Grewal, 2008). The possibility that bacteria might be using LacNAc structures in O-glycans as substrates has been proposed for a commensal of the human gut, \textit{Bacteroides thetaiotaomicron} (Martens et al., 2008). This species encodes GH family 18 enzymes predicted to cleave LacNAc repeats of O-glycan chains. LacNAc also constitutes part of the carbohydrate backbone of mucins, which form a protective physical barrier of the gastrointestinal tract, and chitinase B from \textit{Serratia marcescens} has been described to target mucins (Sanders et al., 2007). The mechanism of hydrolysis has not been elucidated.

LacdiNAc is present in the N-glycans of some human host proteins, e.g. lactoferrin, but is in general more commonly found in the insect glycome (Van den Eijnden et al., 1995). So far, activity towards LacdiNAc and/or LacNAc structures in specific glycoproteins or glycolipids has not been demonstrated.

The non-sulfated glycosaminoglycan hyaluronan, a chief component of many extracellular matrices, could also be a target. However, the \textit{S. Typhimurium} ChiA and \textit{Listeria monocytogenes} ChiA and B enzymes do not show hydrolytic activity towards this substrate (B. G. Storgaard, M. M. Palcic, J. J. Leisner and others, unpublished results). Other GlcNAc-containing glycosaminoglycans, such as heparin and keratan sulfate, have not yet been tested as substrates for GH family 18 and 19 enzymes.

Unexpected targets may also exist. Interestingly, 28S rRNA N-glucosidase activity, characteristic for ribosome-inactivating protein, has been shown for some plant chitinases (Shih et al., 1997; Xu et al., 2008). These enzymes were, nevertheless, not fully characterized.

Chitinases from bacterial pathogens may also target the peptidoglycan-containing cell wall of other members of the gastrointestinal microbiome or their own cell wall to accommodate cell growth and division. Only a few studies on \textit{a Bacillus pumilus} GH family 18 chitinase and two \textit{P. aeruginosa} chitinases of unspecified GH family have, however, shown such hydrolytic activity (Ghasemi et al., 2011; Wang & Chang, 1997), whereas \textit{Enterococcus faecalis}, S. Typhimurium and \textit{Listeria monocytogenes} GH family 18 chitinases were inactive in this regard (Leisner et al., 2009; Larsen et al., 2011). It is of interest that the bacterial GH family 19 chitinases include a group annotated as having similarities with the lysozymes belonging to the lysozyme superfamily (Fig. 3). Unfortunately, data on lysozymic activity of GH family 19 chitinases are currently only available for chitinases of plant origin (Beintema & Terwisscha van Scheltinga, 1996).

Interestingly, for most of the virulence-related chitinases found in Table 1 (efChi18A, B3338, FTT-0715, Lpg1116, Lmo1883 and PA2300) homology modelling (Phyre2 server; data not shown) predicted the absence of an insertion domain in their hydrolytic domain, which indicates that these enzymes are endochitinases. It is conceivable that the open architecture of the catalytic domains of these predicted endochitinases might be prone to accommodate recognition of non-chitinous host glycans, but experimental evidence is warranted.

**Substrate specificities of chitin-binding domains relevant to virulence**

Although CBM33 (AA10) domains of CBPs are mainly associated with binding and cleavage of chitin, alternative
substrates such as cellulose have been observed (Forsberg et al., 2011). Host-related alternative binding substrates have also been described, with the virulence-related GbpA protein (Table 2) from V. cholerae as an example. GbpA consists of two domains allocated to attachment to the bacterial cell surface, and two domains capable of binding to chitin, of which the CBM33 domain also binds GlcNAc residues of mucin. This tripartite binding capacity indicates that the protein may be involved in adhesion to chitinous exoskeletons as well as matrix proteins of host cells (Wong et al., 2012; Bhowmick et al., 2008). A CBP consisting of a single CBM33 module from Lactobacillus plantarum is also able to bind both chitinous and cell-surface-type substrates, such as mucin (Sánchez et al., 2011). Also, bacterial CBPs have been proposed to mediate adhesion to host cells through interactions with mammalian chitinases or chitolecitons (Kawada et al., 2008). It remains to be studied whether the observed monooxygenase activity towards chitin (Vaaje-Kolstad et al., 2010; Vaaje-Kolstad et al., 2012) might be of relevance regarding related substrates in the host.

CBMs as accessory domains of bacterial enzymes functioning as virulence factors and toxins have been reported. As might be expected, these domains contribute to virulence through sugar-binding properties that promote adhesion to extracellular or intracellular targets (Guillén et al., 2010). Chitin-binding domains, other than CBM33, that accompany chitinases and CBPs might contribute to virulence in this manner. As described earlier, these domains belong to CBM2 and CBM5/12 (CAzY, 2013), but so far only CBM 5/12 is found to be associated with bacterial chitinases and CBPs related to virulence (Tables 1 and 2). It is conceivable that these domains as part of virulent chitinases could play a role in adhesion similar to that proposed for the CBM33 chitin-binding domains of CBPs such as GbpA and Lmo2467 (Bhowmick et al., 2008; Chaudhuri et al., 2010). However, it should be noted that the CBM5/12 domain (domain 4) of GbpA was found to be dispensable for virulence, and thus such a role remains to be demonstrated (Wong et al., 2012).

**Functional roles of other accessory modules**

The majority of bacterial chitinases exhibit a complex, multi-domain architecture, including sometimes catalytic domains belonging to other GH families, but, more frequently, additional domains with no obvious catalytic role. Besides the chitin-binding domains, these include other domains of essentially unknown function, such as fibronectin type III (FnIII) or cadherin-like domains and polycystic kidney disease (PKD) domains. The functionality of such domains is mostly deduced by their annotation and further descriptive studies are clearly warranted.

PKD domains have been shown to directly mediate binding to chitin (Orikoshi et al., 2005). PKD domains may also promote protein–protein interactions, but the importance of this ability with regard to bacterial chitinases has not been investigated.

FnIII-like domains are widely distributed among family 18 bacterial chitinases and other glycosyl hydrolases (Little et al., 1994), but have not been reported for any family 19 chitinases (CAzY, 2013; Punta et al., 2012). They appear to contribute to chitin hydrolysis (Watanabe et al., 1994), possibly by functioning as a flexible linker between the catalytic and chitin-binding domain (Lee et al., 2002). Such FnIII-like domains are probably acquired from animals (Bork & Doolittle, 1992; Lee et al., 2002), in which they are involved in cell adhesion and extracellular matrix assembly, mainly through protein–protein interactions between domains of fibronectin, integrin and heparin (Pankov & Yamada, 2002; Potts & Campbell, 1996). It remains to be investigated whether bacterial FnIII domains, despite some structural differences (Lee et al., 2002), have retained animal-like properties and thereby serve analogous roles during infection as hypothesized for the Campylobacter jejuni FlpA protein, which binds fibronectin and facilitates host epithelial cell adhesion (Konkel et al., 2010).

**Conclusions**

The work summarized in this review indicates that GH family 18 chitinases and CBPs from bacterial pathogens play roles as virulence factors, although not necessarily through a single unifying mechanism. We do not yet have data for GH family 19 chitinases to evaluate their role in virulence in this regard. However, the frequent occurrence of family 19 chitinases in plants and their often noticed anti-fungal activity suggest that these enzymes may primarily be involved in resistance towards fungal pathogens (Goody, 1999; Kawase et al., 2006). The GH family 18 chitinases of bacteria may exert easily understood roles during infection of chitin-containing hosts, whereas their biological function in hosts lacking chitin cannot be explained simply by chitinolytic activity. In the case of GH family 18 chitinases, the existence of non-chitinolytic activity is suggested by a structure/function relationship that may easily direct the development of new functions from the scaffold of the parent proteins (Funkhouser & Aronson, 2007). The substrate specificities of several bacterial chitinases towards LacNAc and particularly LacdiNAc model substrates serve as a possible example of such new functions. However, additional studies are needed to confirm that alternative substrates of bacterial chitinases do play a role during infection. Also, it should be mentioned that the molecular mechanisms may involve only attaching to the proper substrate, such as is most likely the case for some of the bacterial CBPs, e.g. the V. cholerae GbpA protein (Kirn et al., 2005).

It should also be considered that some bacterial CBPs and chitinases that serve as virulence factors may be attached to the bacterial cell surface as suggested for other bacterial glycosyl hydrolases with similar roles (Ficko-Blean &
Boraston 2012). In this scenario they may play a particular role in the host–bacteria intervention by mediating adhesion of the entire bacterial cell.

Some of the enzymes belonging to the GH family 18 are described as endoglycosidases (e.g. endo-H) rather than chitinases. Indeed, significant chitinolytic activity remains to be demonstrated by most, if not all, such enzymes. Future studies should clarify to what extent bacterial GH 18 enzymes defined as endoglycosidases or chitinases have similar or different substrate specificities with regard to their roles as virulence factors.

It is evident that any firm assessment of the biological role of bacterial chitinases and CBP s requires a careful assessment of their importance during infection, by use of both cellular and mammalian animal models. Here, also host-specific reactions need to be considered. One example involves a gene encoding a human CBP, a chitinase 3-like 1 protein, that may interact with bacterial components (e.g. CBPs) (Kawada et al., 2008; Tran et al., 2011) and appears to have a functional role during bacterial infections (Johansen et al., 2005).

Mammalian chitinases and chitolectins, initially thought of as primarily providing a defence towards chitinous pathogens, i.e. moulds (Lee et al., 2008), exert numerous other effects in the mammalian host, including pathological conditions such as autoimmune, neurological or lysosomal storage diseases in humans (Brinkman et al., 2005; Boot et al., 1999; Bussink et al., 2006; Di Rosa et al., 2006; Lautner et al., 2011; Lee et al., 2011, 2012; Mattsson et al., 2011; Sotgiu et al., 2008; Verbeek et al., 2010) and crystalline pneumonia in mice (Hoenerhoff et al., 2006; Liu et al., 2009). It will be of interest to examine such biologically relevant activities of mammalian chitinases and chitolectins in order to improve the understanding of the roles of similar proteins from bacterial pathogens.

A thorough examination of the bacterial chitinases, as discussed above, will hopefully lead to the ultimate goal: a precise definition of the extent to which they can be perceived as virulence factors. This will provide an escape from the situation outlined by Lewis Caroll, aptly used by Dubos (1945) in his pioneering treatise of bacterial virulence: ‘When I use a word’, Humpty Dumpty said, in a rather scornful voice, ‘It means just what I choose it to mean – neither more nor less’.

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