RESEARCH ARTICLE

Identification and characterization of genes underlying chitinolysis in *Collimonas fungivorans* Ter331

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Abstract

Through a combinatorial approach of plasposon mutagenesis, genome mining, and heterologous expression, we identified genes contributing to the chitinolytic phenotype of bacterium *Collimonas fungivorans* Ter331. One of five mutants with abolished ability to hydrolyze colloidal chitin carried its plasposon in the *chiI* gene coding for an extracellular endochitinase. Two mutants were affected in the promoter of *chiP-II* coding for an outer-membrane transporter of chitooligosaccharides. The remaining two mutations were linked to chitobiose/*N*-acetylglucosamine uptake. Thus, our model for the *Collimonas* chitinolytic system assumes a positive feedback regulation of chitinase activity by chitin degradation products. A second chitinase gene, *chiII*, coded for an exochitinase that preferentially released chitobiose from chitin analogs. Genes *hexI* and *hexII* showed coding resemblance to *N*-acetylglucosaminidases, and the activity of purified HexI protein towards chitin analogs suggested its role in converting chitobiose to *N*-acetylglucosamine. The *hexI* gene clustered with *chiI*, *chiII*, and *chiP-II* in one locus, while chitobiose/*N*-acetylglucosamine uptake genes colocalized in another. Both loci contained genes for conversion of *N*-acetylglucosamine to fructose-6-phosphate, confirming that *C. fungivorans* Ter331 features a complete chitin pathway. No link could be established between chitinolysis and antifungal activity of *C. fungivorans* Ter331, suggesting that the bacterium's reported antagonism towards fungi relies on other mechanisms.

Introduction

The genus *Collimonas* belongs to the family *Oxalobacteraceae*, order *Burkholderiales*, class *Betaproteobacteria* (de Boer *et al.*, 2004). It consists of three recently recognized species, i.e. *fungivorans*, *arenae* and *pratensis* (Höppener-Ogawa *et al.*, 2008). First discovered (de Boer *et al.*, 1998) between the roots of Marram grass (*Ammophila arenaria*) at coastal dune sites of the island of Terschelling, The Netherlands, *Collimonas* strains have since been detected in various locations and environments around the world, by both culture-dependent and -independent methods (Wilson *et al.*, 2003; Opelt & Berg, 2004; Aspray *et al.*, 2005; Mahmood *et al.*, 2005; Hamamura *et al.*, 2006; Ikeda *et al.*, 2006; Mannisto & Haggblom, 2006; Schmidt *et al.*, 2006; Adesina *et al.*, 2007; Höppener-Ogawa *et al.*, 2007; Offre *et al.*, 2007; Uroz *et al.*, 2007).

A defining property of the original *Collimonas* bacteria from Terschelling is the ability to grow at the expense of living fungal hyphae (de Boer *et al.*, 2001). This phenomenon has been named bacterial mycophagy (de Boer *et al.*, 2005; Fritsche *et al.*, 2006) and represents a novel type of bacterial–fungal interaction (Leveau & Preston, 2007). A related characteristic of many collimonads is their antagonistic activity towards fungi. All 22 strains first described suppressed the growth of fungi in confrontation on agar plates (de Boer *et al.*, 1998). Similar *in vitro* antifungal activity has been reported for collimonads isolated from mosses along the Baltic sea coast of Germany (Opelt & Berg, 2004). Recently, it was demonstrated that *Collimonas fungivorans* strain Ter331 is antagonistic *in vivo* against the plant-pathogenic fungus *Fusarium oxysporum* f.sp. *radicis-lycopersici*, the causal agent of tomato foot and root rot (Kamilova *et al.*, 2007).

Another shared characteristic of most *Collimonas* bacteria is the ability to hydrolyze chitin, the β(1 → 4) linear homopolymer of *N*-acetylglucosamine (GlcNAc). The original collimonads from Terschelling were all isolated as CFUs capable of halo formation on water–agar plates containing colloidal chitin (de Boer *et al.*, 1998). Similarly, chitinolytic bacteria recovered from different soils in the Netherlands were identified as belonging to the *Collimonas* genus (Höppener-Ogawa *et al.*, 2007). The ability to hydrolyze chitin is not a delimiting property of collimonads, as nonchitinolytic strains have also been reported. For example, out of 14 Finnish Lapland soil isolates that were first identified as *Collimonas* strains and subsequently tested for chitinolytic activity, three scored negative (Mannisto & Haggblom, 2006). It is worth noting, however, that chitinolysis by *Collimonas* species varies with medium composition: for example, it was shown for some strains that the presence of glucose or tryptic soy broth inhibited halo formation (de Boer *et al.*, 1998; Höppener-Ogawa *et al.*, 2007).

It was suggested (de Boer *et al.*, 1998) that the chitinolytic activity of *Collimonas* is linked to its antifungal properties. Chitin is a major structural component of most fungi, and in several other cases, bacterial chitinase activity has been linked to antifungal properties (Chernin *et al.*, 1995; Kobayashi *et al.*, 2002; Chang *et al.*, 2003; Dahiya *et al.*, 2005). Chitinolysis has also been implemented in *Collimonas* mycophagy based on the observation that the chitinase inhibitor allosamidin reduced the proliferation of *Collimonas* on fungal substrates (de Boer *et al.*, 2001).

Our long-term goal is to understand in greater detail the biology and ecology of bacteria belonging to the genus *Collimonas*, specifically with regard to their interactions with fungi. A proper understanding of the role of chitinolysis in these interactions requires an insight into the genes that contribute to this phenotype. In this study, we present a characterization of the chitinolytic system of *C. fungivorans* Ter331 through a combinatorial approach involving loss-of-function mutants, genome mining, and heterologous expression and activity of candidate genes.

Materials and methods

Bacterial strains and culture conditions

The phylogeny (de Boer *et al.*, 2004), antifungal and chitinolytic properties (de Boer *et al.*, 1998), and mycophagous character (de Boer *et al.*, 2001) of *C. fungivorans* strain Ter331 have been described previously, as has the construction and screening of a plasposon mutant library (Leveau *et al.*, 2006). Three nonchitinolytic mutants 17H10, 18D9, and 23B7 and their plasposon insertion sites have been reported elsewhere (Leveau *et al.*, 2006). In this study, we identified two more mutants with reduced ability to form haloes on 2% agar plates supplemented with 2 g colloidal chitin, 0.1 g yeast agar, 1 g KH_2PO_4, and 5 g NaCl L^{-1}. All five mutants grew as well on these plates as the wild-type strain, i.e. chitinolysis was not required for growth. DNA sequences flanking the plasposon insertion sites in the two newly identified mutants were determined as described previously (Leveau *et al.*, 2006). *Collimonas fungivorans* Ter331 and its nonchitinolytic mutants were maintained on King's B (KB). Where appropriate, kanamycin was added to a final concentration of 50 μg mL^{-1}. The antifungal properties of nonchitinolytic mutants of *C. fungivorans* Ter331 were compared with those of the wild-type strain in plate confrontation assays as described previously (de Boer *et al.*, 1998) using *Fusarium culmorum*, *Cladosporium* sp. and *Trichosporon guehoae* as target fungi.

Insertional inactivation of the *chiI* gene

A 1095-bp DNA fragment of the *chiI* gene was amplified by PCR using the Expand High Fidelity PCR System (Roche, Mannheim, Germany) and the primers 700f_seq (5′-CACGGTCAGCTACAACGGCGTC-3′) and QAer_rev (5′-GAGATGAAGGCGTTGGTGAAG-3′). The 50-μL PCR reaction contained 1 × reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer, and 0.75 μL DNA polymerase. The temperature profile was as follows: 95 °C for 3 min and 25 cycles 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. During the last 15 cycles, the elongation time at 72 °C was increased by 5 s per cycle. Cycling was completed by a final elongation step at 72 °C for 10 min. The PCR product was ligated into vector pCR4-TOPO (Invitrogen) and transformed into *Escherichia coli* Top10. The resulting plasmid pCR4chiI was isolated and introduced into *C. fungivorans* Ter331 by electroporation as described before (Kamilova *et al.*, 2007) using a Gene Pulser Xcell Microbial System (Biorad, Veenendaal, the Netherlands). The ability of kanamycin-resistant transformants to form haloes was tested on chitin yeast agar containing 50 μg kanamycin mL^{-1}. The single-crossover integration of pCR4chiI into the chromosomal DNA was verified using colony PCR.

Protein expression in *E. coli* and purification

Candidate genes for chitinase or *N*-acetylglucosaminidase were overexpressed in pASK-IBA5 or -3 (IBA, Goettingen, Germany) as Strep-tag II fusion proteins without (if applicable) their predicted signal peptide sequences in *E. coli* TOP10, following the instructions of the supplier. For this, specific tailed primers were designed with the PRIMERD'-SIGNER software (IBA) and used in a PCR reaction with *C. fungivorans* Ter331 genomic DNA as a template. The following primer pairs were used (tail in italics): ChiI-f

(5′-*ATGGTAGGTCTCAAATG*AGCCCCCTGATAGCAGCGAG C-3′), ChiI-r (5′-*ATGGTAGGTCTCAGCGCT*CAGTCCCAGT TTCGAGATGAAGG-3′), ChiII-f (5′-*ATGGTAGGTCTCAGCG CC*GCGCCCTATAAAGTAGTTGCTTAC-3′), ChiII-r (5′-*ATG GTAGGTCTCATATCAC*CCTGCCCTTGCGATTCTACTA-3′), ChiIII-f (5′-*ATGGTAGGTCTCAAATG*CCTGCCATCTCCCAA AACCAGC-3′), ChiIII-r (5′-*ATGGTAGGTCTCAGCGCT*TTT GAGCGCAAGGATCTGCATCG-3′), HexI-f (5′-*ATGGTAGG TCTCAAATG*AGTAGCACCGATAGCAAATCCGA-3′), HexI-r (5′-*ATGGTAGGTCTCAGCGCT*TGTCAGTTGCCGCTGTGGG CG-3′), HexII-f (5′-*ATGGTAGGTCTCAAATG*AGCAGTGAGA CTCACACGAAAAAA-3′), and HexII-r (5′-*ATGGTAGGTCT CAGCGCT*CAGCAGCCTTGCACCAGCG-3′). PCR amplification was carried out using the Expand High Fidelity PCR System (Roche) in 50 μL containing 1 × reaction buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 40 pmol of each primer, and 0.8 μL DNA polymerase. For the amplification of *hexI*, dimethyl sulfoxide (DMSO) was added to the reaction mix at a final concentration of 3%. Thermal cycling included an initial denaturation step of 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 66 °C (*chiI*, *chiII*), 64 °C (*chiIII*, *hexII*), or 63 °C (*hexI*) for 30 s, and elongation at 72 °C for 60–90 s. The elongation time of the last 20 cycles was increased by 5 s per cycle. Cycling was completed by a final step at 72 °C for 10 min. PCR products were digested with BsaI, ligated into pASK-IBA5 (*chiII* product) or pASK-IBA3 (*chiI*, *chiIII*, *hexI*, *hexII* products), and introduced into *E. coli* Top10. After chemical lysis of the *E. coli* cells, the proteins were purified with Strep-Tactin spin columns (IBA) following the recommended protocols. Extracts that were still viscous after an optional RNAse digestion step were diluted with 1 vol. buffer B before application onto the spin column. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on cell extracts of *E. coli* or on purified proteins using a MiniProtean 3 electrophoresis cell and ready gel precast Tris-HCl gels (Biorad). A broad-range protein marker (2–212 kDa, New England Biolabs, Beverly, MA) was used as a size standard. Proteins were stained with Biosafe Coomassie (Biorad).

Enzyme and protein assay

The glycohydrolase activity of cell extracts or purified proteins was measured using the chitin analogs 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (MU-NAG), 4-methylumbelliferyl-*N*,*N*′-diacetyl-β-D-chitobioside (MU-diNAG) and 4-methylumbelliferyl-β-D-*N*,*N*′,*N*″-triacetylchitotrioside (MU-triNAG) (Sigma-Aldrich, Zwijndrecht, the Netherlands). In brief, 10 μL of a protein extract and 5 μL of each chitin analog (200 μM, final concentration 10 μM) were added to 85-μL potassium phosphate buffer (100 mM, pH 6.5) and incubated at 30 °C for 45–75 min. After addition of 100-μL ice-cold ethanol and 22.5 μL Tris, fluorescence was detected using a 360/40-nm excitation filter and a 460/40-nm emission filter on a Synergy HT1 plate reader (Biotek, Winooski, VT). A standard curve was prepared with 0–10 μM 4-methylumbelliferone (MU, Sigma-Aldrich). Protein content was determined using the Roti-Quant protein assay (Carl Roth, Karlsruhe, Germany). Glycohydrolase activity was expressed as pmol MU released per minute per microgram of protein.

Sequence analysis

The complete genome sequence of *C. fungivorans* Ter331 has been determined recently and its full analysis will be reported elsewhere (Leveau *et al.*, unpublished data). Mining of the genome sequence was facilitated by the GenDB (Meyer *et al.*, 2003) and RAST/SEED (Overbeek *et al.*, 2005) genome annotation systems. Signal peptide sequences, transmembrane regions, and functional domains were predicted or confirmed using InterProScan (http://www.ebi.ac.uk/InterProScan). Sequence similarity searches were performed using the basic local alignment search tool (BLAST) at the National Center for Biotechnology. Promoter searches were carried out using Softberry's BPROM (www.softberry.com).

Results

Screening of a plasposon mutant library of *C. fungivorans* Ter331 (Leveau *et al.*, 2006) revealed five unique clones (9G3, 17H10, 18D9, 21E8, and 23B7) with abolished ability to form haloes on water–yeast agar plates containing colloidal chitin. Mapping the DNA sequences that flanked the plasposon insertion sites onto the genome sequence of *C. fungivorans* Ter331 revealed that each of the five mutants was affected in one of two distinct loci on the genome, i.e. *chi* locus A (Fig. 1a) or B (Fig. 1b). The complete nucleotide sequence of both loci has been submitted to GenBank (accession numbers EU599185 and EU599184, respectively).

On the genome of *C. fungivorans* Ter331, we identified five candidate genes coding for chitinolytic activity (Table 1). Two of the three putative chitinase genes were located in close proximity to each other within a cluster of ORFs in *chi* locus A (Fig. 1a). Their predicted products ChiI and ChiII resembled chitinases belonging to the glycosyl hydrolase family 18 (Table 1). Both products featured a conserved catalytic activity domain (SXGG; GXDXDXE) (Watanabe *et al.*, 1993) as well as a type-II N-terminal secretion signal. ChiI also contained a type-3 chitin-binding domain (ChtBD3) and a 20-aa proline/threonine-rich linker between ChtBD3 and the catalytic domain. Attempts to overexpress the *chiI* gene in *E. coli* in order to assess its product's activity towards chitin analogs (Table 2) were unsuccessful. In chitinolytic mutant 23B7, we identified a plasposon insertion in the predicted promoter region of the *chiI* gene (Fig. 2). By insertional inactivation of the *chiI* ORF by

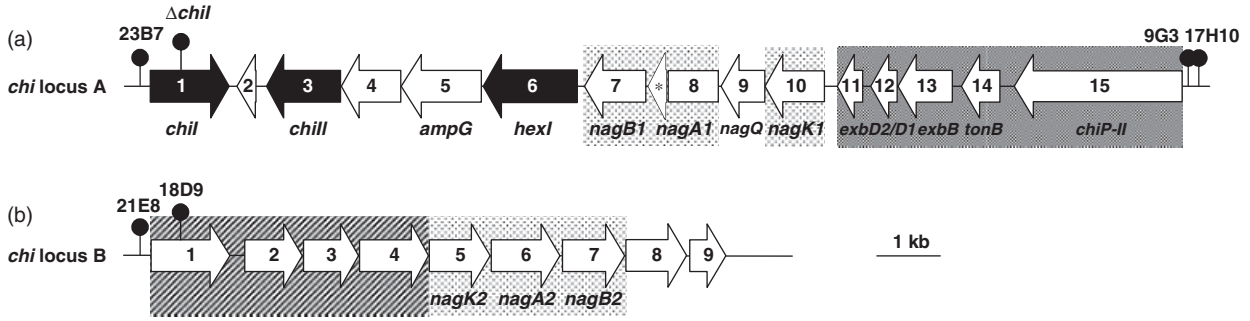

Fig. 1. Graphical representation of *chi* loci A (top) and B (bottom) of *Collimonas fungivorans* Ter331. ORFs are numbered and represented as arrows, with the proposed gene name, if applicable. In black (ORFs A1, A3, and A6) are those proposed to encode for chitinase or *N*-acetylglucosaminidase activity (Table 1). ORFs presumed to be contributing to the same function are given in the same background shading: transport of chitooligosaccharides across the outer membrane (densely stippled), transport of $(GlcNAc)_2$ across the inner membrane (diagonal stripes), and metabolism of GlcNAc (lightly stippled). The plasposon insertion sites in nonchitinolytic mutants 9G3, 17H10, 18D9, 21E8, and 23B7, as well as the site of inactivation in mutant *ΔchiI* (see Materials and methods) are indicated by black circles. The asterisk indicates the position of an in-frame stop codon in *nagA1*.

Table 1. Predicted chitinase and *N*-acetylglucosaminidase genes of *Collimonas fungivorans* Ter331

Gene name	ORF name (see Fig. 1a)	Size (aa/kDa) of predicted protein	Predicted function; EC number (if available)	Most similar protein; organism; % identity/% similarity (length of alignment in amino acids); accession no.	Best experimental hit; organism; % identity/% similarity (length of alignment in amino acid); accession no.
chiI	A1	414/42.7	Chitinase; 3.2.1.14	Chitinase pCA8 ORF; *Aeromonas* sp. 10S24; 62/73 (392); BAA36459	Chitinase pCA8 ORF; *Aeromonas* sp. 10S24; 62/73 (392); BAA36459
chiII	A3	386/42.6	Chitinase; 3.2.1.14	Catalytic domain chitinaseA1; *Bacillus circulans* Wl-12; 49/60 (422); P20533	Catalytic domain chitinase A1; *Bacillus circulans* Wl-12; 49/60 (422); P20533
chiIII	n/a	216/24.0	Chitinase/lytic protein	Predicted family 19 chitinase; *Magnetospirillum gryphiswaldense* MSR-1; 53/67 (182); CAM74777	Lytic enzyme; *Pseudomonas aeruginosa*; 49/62 (191); BAA83168
hexI	A6	521/55.5	β-*N*-Acetyl-glucosaminidase; 3.2.1.52	Probable glycosyl hydrolase family 3; *Chromobacterium violaceum*; 54/68 (266); AAQ57938	β-*N*-Acetylglucosaminidase; *Clostridium paraputrificum*; 36/56 (292); BAC56177
*hexII**	n/a	349/37.1	β-*N*-Acetyl-glucosaminidase; 3.2.1.52	β-*N*-Acetylglucosaminidase; *Minibacterium massiliensis*; 74/84 (329); YP_001353065	β-*N*-Acetylglucosaminidase; *Vibrio furnissii*; 43/57 (340); P96157

*GenBank accession number EU616822.

marker exchange, producing a Ter331Δ*chiI* mutant, we confirmed that this gene is essential for halo formation on colloidal chitin (not shown). Based on these observations and on the fact that *chiI* seems to constitute a single-gene transcriptional unit, we hypothesize that ChiI is a major if not the sole contributor to chitinolysis by *C. fungivorans* Ter331.

The *chiII* gene product showed a high degree of identity (49%) to the catalytic domain of chitinase A1 from *Bacillus circulans* Wl-12 (Watanabe *et al*., 1990b, 1993). This chitinase has been shown to release predominantly *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$ from colloidal chitin (Watanabe *et al*., 1990a). We made a similar observation with ChiII protein that was overexpressed in *E. coli* (Fig. 3) and subsequently StrepTag-purified: MU-$(GlcNAc)_2$, and to a lesser extent MU-$(GlcNAc)_3$, were readily accepted as a substrate, whereas MU-GlcNAc was not (Table 2).

We identified a third putative chitinase gene (*chiIII*) elsewhere on the genome of *C. fungivorans* Ter331 (GenBank accession number EU616821). The amino acid sequence of its predicted product resembled glycosyl hydrolases from family 19, which includes chitinases (Table 1). It also showed significant homology (49% identity) to an enzyme in *Pseudomonas aeruginosa* that when overexpressed in *E. coli* or *P. aeruginosa* caused cell lysis following treatment with chloroform to allow access of the cytosolic enzyme to peptidoglycan in the periplasmic space (Nakayama *et al*., 2000). We overexpressed and purified the ChiIII protein (Fig. 3), but did not detect activity towards $(MU\text{-}GlcNAc)_{1–3}$ substrates (Table 2). However, we did

Table 2. Heterologously expressed Chi and Hex proteins and their activities towards chitin analogs

Gene name	Type of StrepTag fusion overexpressed in *E. coli*	Purified	Activity* of purified protein towards		
			MU-GlcNAc	MU-$(GlcNAc)_2$	MU-$(GlcNAc)_3$
chiI	C-terminal	No†	–	–	–
chiII	N-terminal	Yes	< 0.7	25	1.8
chiIII	C-terminal	Yes	< 0.1	< 0.1	< 0.1
hexI	C-terminal	Yes	2.9	4.5	4.2
hexII	C-terminal	No†	–	–	–

*Expressed as the release of 4-methylumbelliferone (MU) in pmoles per minute per microgram of protein.

†No ChiI or HexII could be purified; corresponding *Escherichia coli* crude extracts showed no activity towards MU-$(GlcNAc)_2$.

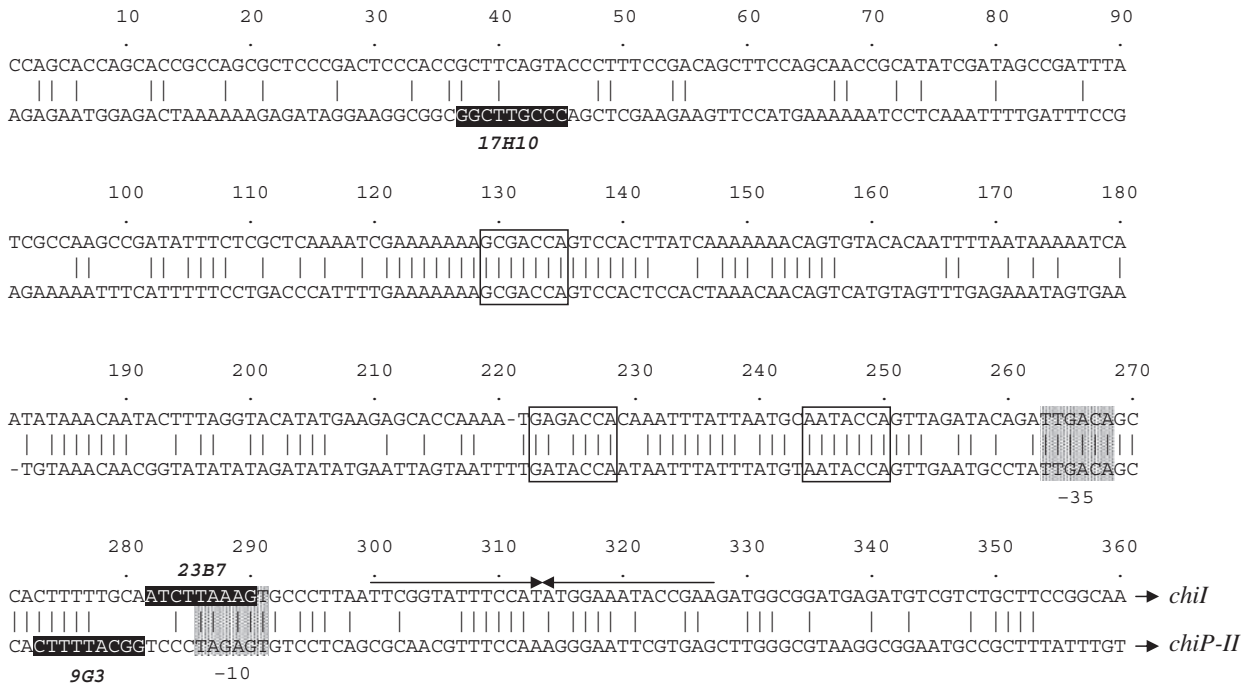

Fig. 2. Pairwise comparison of the *chiI* (top) and *chiP-II* (bottom) promoter regions (in the 5′–3′ direction). Predicted −35 and −10 regions are indicated by background shading. Plasposon insertion sites in mutants 9G3, 17H10, and 23B7 are highlighted in white letters with a black background. The 17H10 insertion has been described before (Leveau *et al.*, 2006), but without its genomic context. Fully or partially conserved sequences that resemble the proposed NagQ consensus binding site (5′-TGGTATT-3′) are boxed (Yang *et al.*, 2006). Also indicated is a perfect 14-bp inverted repeat with unknown function in the transcriptional start region of *chiI*. This repeat contains two more possible NagQ-binding sites (5′-cGGTATT-3′).

observe spontaneous lysis after storage at −20 °C of *E. coli* cells expressing the *chiIII* gene. Given the resemblance of ChiIII to the lytic protein of *P. aeruginosa*, we hypothesize that the *chiIII* gene of *C. fungivorans* Ter331 codes for hydrolytic activity towards peptidoglycan, a heteropolymer of GlcNAc and *N*-acetyl-D-muramic acid. A possible link of ChiIII activity to *Collimonas* chitinolysis will be addressed in the Discussion.

In addition to the three chitinase genes, we identified two putative genes for *N*-acetylglucosaminidases on the *C. fungivorans* Ter331 genome. Both predicted gene products (HexI and HexII) contained a glycosyl hydrolase family 3 N-terminal domain and a KHFPGHG-x4-DSH motif, which is believed to play a role in binding of the *N*-acetyl group (Mayer *et al.*, 2006). For neither protein was a clear N-terminal signal peptide predicted, suggesting they are cytoplasmic proteins. Overexpressed and purified HexI showed activity towards all three tested chitin analogs (Table 2), which is consistent with previous reports on *N*-acetylglucosaminidases (Chernin *et al.*, 1995). We were not able to StrepTag-purify HexII from overexpressing *E. coli* cells (Table 2).

Interestingly, the *hexI* gene was part of the same *chi* locus A on the *C. fungivorans* Ter331 genome as *chiI* and

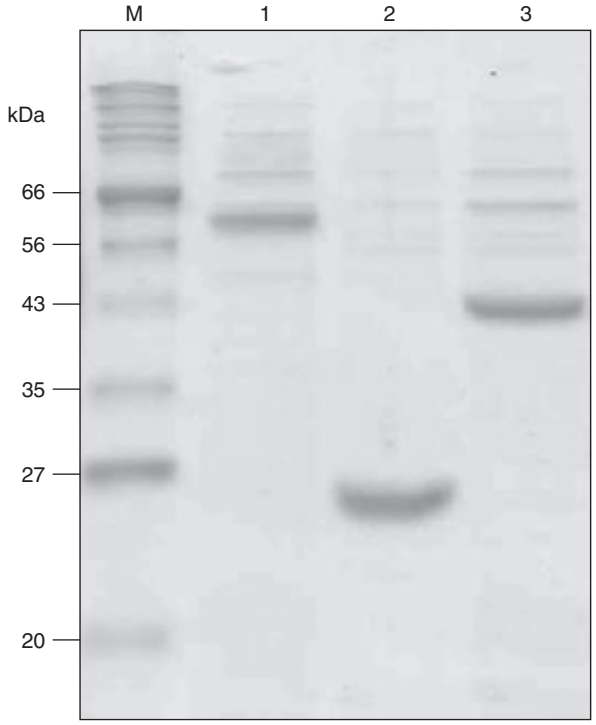

Fig. 3. SDS-polyacrylamide gel electrophoresis (PAGE) of heterologously expressed and purified HexI (lane 1), ChiII (lane 2), and ChiI (lane 3). Predicted sizes of the proteins without their signal peptide but including the StrepTag were 56.7, 25.2, and 42.0 kDa, respectively (Table 1). Lane M contained a standard (sizes, in kDa, are given to the left). To each lane, 2 μg of protein was applied.

chiII (Fig. 1a). In fact, *hexI* and *chiII* belonged to a cluster of genes with the same orientation within this locus (Fig. 1a). Table 3 lists a coding prediction for each of the ORFs in this cluster. Based on amino sequence homology of their products, ORFs A10, A8, and A7 probably code for the three-step conversion of GlcNAc to fructose-6-phosphate by a kinase NagK1 (EC 2.7.1.59), a deacetylase NagA1 (EC 3.5.1.25) and a deaminase NagB1 (EC 3.5.99.6), respectively. The *nagA1* gene actually contained an in-frame stop codon TGA, but it is unclear whether this means that *nagA1* codes for a truncated and thus nonfunctional protein, because *C. fungivorans* Ter331 possesses the genetic determinants to produce the alternative amino acid selenocysteine and incorporate it into growing peptide chains using the TGA codon (not shown). ORF A9 codes for a predicted GntR-type repressor protein, provisionally named NagQ (Yang *et al.*, 2006), with high coding similarity to DasR (Table 3). In several *Streptomyces* species, the *dasR* gene product controls the uptake and metabolism of GlcNAc (Rigali *et al.*, 2006) and/or $GlcNAc_2$ (Seo *et al.*, 2002; Saito *et al.*, 2007), as well as chitin utilization (Rigali *et al.*, 2006).

Products of ORFs A11 through A14 resembled components of TonB–ExbB–ExbD protein complexes (Braun, 1995). The predicted product of ORF A15 showed significant homology to ChiP-II proteins of *Shewanella* species and other *Alteromonadales* and *Xanthomonadales*, inferred to encode TonB-dependent outer-membrane receptors for chitooligosaccharides (Yang *et al.*, 2006). *Collimonas* mutants 17H10 and 9G3 carried a plasposon insertion upstream of ORF A15 (*chiP-II*, Fig. 2). The 9G3 insertion was located in between the predicted -35 and -10 region of the predicted promoter of *chiP-II* (Fig. 2). During the course of this analysis, we recognized that the promoter regions of *chiP-II* and *chiI* shared substantial similarity, including conserved regions with partial homology to proposed regulatory sites (Fig. 2). This promoter conservation may suggest a common mode of regulation between the two genes.

Within the context of chitinolysis, the roles of ORFs A2, A4, and A5 within *chi* cluster A remain unclear (Table 3). ORF A2 showed coding similarity to conserved hypothetical proteins with unknown function in several bacteria from the family *Burkholderiaceae*. ORF A4 codes for a peptide with a glycosyl hydrolase family 16 signature, with low homology to enzymes with demonstrated function (Table 3). ORF A5 codes for an AmpG-like transporter, possibly involved in peptidoglycan recycling by taking up peptidoglycan degradation products into the bacterial cytosol, where *N*-acetylglucosamine is released by the activity of *N*-acetyl-glucosaminidases (Lindquist *et al.*, 1993; Cheng & Park, 2002). This scenario fits well with the observed clustering of ORF A4 with the *hexI* gene and the *nag* genes for GlcNAc metabolism (Fig. 1a).

As did *chi* locus A, locus B (Fig. 1b) contained genes involved in GlcNAc metabolism (Table 4). The products of ORFs B5, B6, and B7 exhibited a high level of amino acid sequence identity (i.e. 52%, 72% and 82%, respectively) with those coded for by ORFs A10, A8, and A7, respectively, and most likely represent a second set of enzymes involved in the conversion of *N*-acetylglucosamine to fructose-6-phosphate. Interestingly, unlike ORF A8 (*nagA1*), ORF B6 (*nagA2*) did not feature an in-frame nonsense codon. ORFs B1, B2, B3 and B4 showed substantial coding homology to components of several ATP-binding cassette (ABC)-type transporters of mono-, di- and oligosaccharides (Table 4). Two of these are the Ngc system from *Streptomyces olivaceoviridis* and the Das system from *Streptomyces coelicolor* A3(2), both of which mediate the uptake of $(GlcNAc)_2$ and GlcNAc (Xiao *et al.*, 2002; Saito & Schrempf, 2004; Saito *et al.*, 2007). Given the nonchitinolytic nature of mutants 21E8 and 18D9 and their plasposon insertion sites upstream of and within ORF B1, respectively (Fig. 1), these observations would suggest that uptake and/or metabolism of chitin degradation products is essential for chitinolysis by *C. fungivorans* Ter331. The role of the other two ORFs in the locus B gene cluster is unclear. The ORF B8 product resembled known oxidoreductase domain proteins and

Table 3. Genes in *chi* locus A and homology of their products*

ORF	Gene name	Size (aa/kDa) of predicted protein	Most similar protein; organism; % identity/% similarity (length of alignment in amino acids); accession no.	Best experimental hit; organism; % identity/% similarity (length of alignment in amino acids); accession no.
A2	–	111/11.6	Hypothetical protein Reut_A2597; *Ralstonia eutropha* JMP134; 56/72 (90); YP_296802	–
A4	–	306/33.9	Hydrolase (secreted protein); *Shewanella woodyi* ATCC51908; 41/55 (302); ZP_01541624	β-Glucanase precursor; *Paenibacillus macerans*; 23/39 (236); P23904
A5	*ampG*	433/46.6	Putative signal transducer; *Bacteroides fragilis* YCH46; 48/66 (406); YO_098614	AmpG1-signal transducer; *E. coli*; 23/40 (405); CAA57651
A7	*nagB1*	336/35.5	Probable aminotransferase; *Chromobacterium violaceum*; 65/76 (335); AAQ58233	Glutamine-fructose-6-phosphate transaminase; *Thermus aquaticus*; 31/51 (331); S69793
A8a	*nagA1a*	103/11.0	*N*-Acetylglucosamine-6-phosphate deacetylase; *Burkholderia ambifaria* MC40-6; 75/82 (98); EAV66210	*N*-Acetylglucosamine-6-phosphate deacetylase; *Lysinibacillus sphaericus* 44/60 (89); Q84F86
A8b	*nagA1b*	275/28.9	Putative *N*-acetylglucosamine-6-phosphate deacetylase; *Pseudomonas aeruginosa* UCBPP-PA14; 65/77 (254); ABJ12992	*N*-Acetylglucosamine-6-phosphate deacetylase; *Lysinibacillus sphaericus*; 39/59 (228); Q84F86,
A9	*nagQ*	244/27.2	Probable transcriptional regulator, GntR family; *Chromobacterium violaceum*; 61/76 (239); AAQ58231	HTH-type transcriptional repressor DasR; *Streptomyces griseus*; 33/47 (242); Q8VV01
A10	*nagK*	324/33.4	Probable *N*-acetylglucosamine kinase; *Chromobacterium violaceum*; 48/61 (304); AAQ60564	*N*-Acetylglucosamine kinase; proteobacterium AI21; 33/48 (297); ABE96868
A11	*exbD2*	144/15.4	Biopolymer transport protein ExbD/TolR; *Burkholderia xenovorans* LB400; 65/77 (144); ABE29982	ExbD2; *Xanthomonas campestris* pv. *campestris*; 34/57 (136); AAM89269
A12	*exbD1*	142/15.7	Biopolymer transport protein ExbD/TolR; *Ralstonia pickettii* 12D; 64/85 (141); EDN40948	ExbD2; *Xanthomonas campestris* pv. *campestris*; 38/53 (140); AAM89269
A13	*exbB*	306/31.8	Putative biopolymer transport protein ExbB; *Azoarcus* sp. BH72; 68/81 (235); CAL94947	ExbB; *Xanthomonas campestris*; 40/57 (213); CAB08609
A14	*tonB*	216/23.0	Periplasmic protein TonB; *Magnetospirillum magneticum* AMB-1; 38/56 (211); BAE52353	TonB; *Plesiomonas shigelloides*; 28/42 (200); AAG23396
A15	*chiP-II*	890/95.6	TonB-dependent receptor; *Stenotrophomonas maltophilia*; 47/63 (889); EAX22079	Citrate-dependent iron transporter FecA; *Stenotrophomonas maltophilia*; 25/41 (774); AMB53764

*For ORFs A1 (*chiI*), A3 (*chiII*), and A6 (*hexI*), see Table 1.

Table 4. Genes in *chi* locus B and homology of their products

ORF	Gene name	Size (aa/kDa) of predicted protein	Most similar protein; organism; % identity/% similarity (length of alignment in amino acids); accession no.	Best experimental hit; organism; % identity/% similarity (length of alignment in amino acids); accession no.
B1	–	419/46.4	Extracellular solute-binding protein, family 1; *Marinomonas* sp. MWYL1; 60/74 (405); ABR70577	Sugar-binding protein MsmE; *Streptococcus mutans* UA159; 26/46 (301); Q00749
B2	–	307/34.3	ABC-type sugar transport system, permease component; *Hahella chejuensis* KCTC2396; 66/83 (302); ABC33596	Sugar ABC transport system, permease component XynF; *Geobacillus stearothermophilus*; 31/56 (237); ABI49933
B3	–	295/32.4	ABC-type sugar transport system, permease component; *Hahella chejuensis* KCTC2396; 69/86 (269); ABC33595	Transmembrane protein NgcG; *Streptomyces olivaceoviridis*; 32/54 (284); CAD10685
B4	–	373/40.3	ABC-type sugar transport system, ATPase component; *Hahella chejuensis* KCTC2396; 63/78 (363); ABC33594	MtlK; *Pseudomonas fluorescens*; 50/70 (362); AAC04471
B5	*nagK2*	321/33.0	*N*-Acetyl glucosamine kinase; *Chromobacterium violaceum*; 48/64 (300); AAQ60564	GlcNAc kinase; proteobacterium AI21; 32/47 (290); ABE96868
B6	*nagA2*	373/39.6	*N*-Acetylglucosamine-6-phosphate deacetylase; *Chromobacterium violaceum*; 68/79 (373); AAQ58232	*N*-Acetylglucosamine-6-phosphate deacetylase; *Lysinibacillus sphaericus*; 38/56 (358); Q84F86
B7	*nagB2*	336/35.4	Sugar isomerase (glucosamine-fructose-6-P-aminotransferase; *Pseudomonas fluorescens* PfO-1; 63/75 (335); ABA72748	Glutamine-fructose-6-phosphate transaminase; *Thermus aquaticus*; 32/50 (330); S69793
B8	–	329/35.6	Oxidoreductase domain protein; *Roseiflexus castenholzi*; 49/67 (325); ABU59269	NADP: D-xylose dehydrogenase; *Hypocrea jecorina*; 32/48 (364); ABO33081
B9	–	189/20.5	Flavin reductase domain protein; *Ralstonia pickettii*; 72/82 (182); EAX41873	Reductase component of 6-nitrophenol monooxygenase; *Rhodococcus opacus* SAO101; 29/48 (167); BAD30041

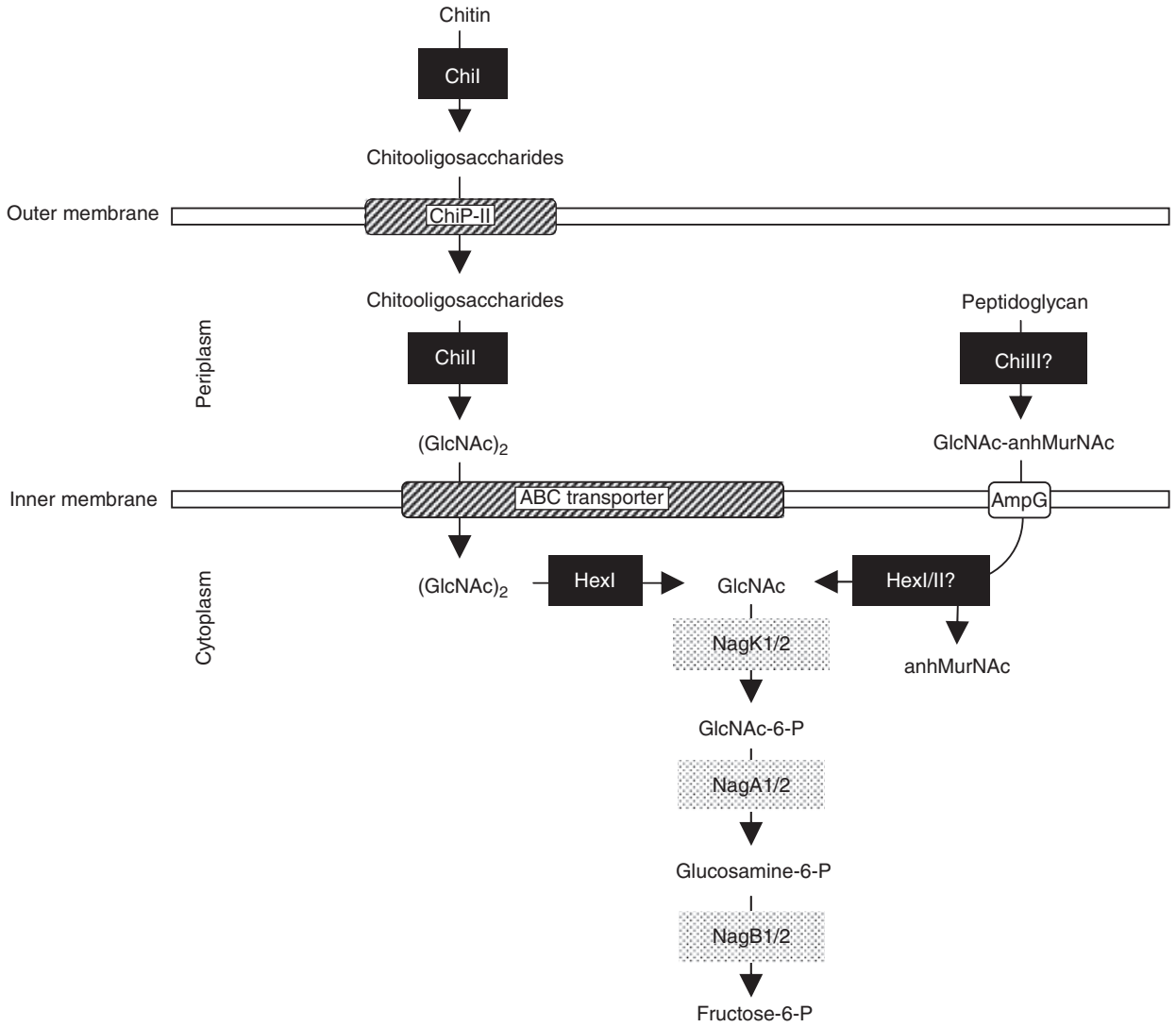

Fig. 4. Proposed null model for chitin metabolism in *Collimonas fungivorans* Ter331. Chitin is depolymerized extracellularly by ChiI to chitooligosaccharides that are taken up by TonB-dependent outer-membrane receptor ChiP-II into the periplasm. Here, they are degraded by ChiII predominantly to $(GlcNac)_2$, which is then transported across the inner membrane by an ABC-type sugar transporter. In the cytoplasm, $(GlcNac)_2$ is degraded to GlcNac by HexI. Subsequent conversion to fructose 6-P (Nag pathway) is catalyzed by NagK1/2, NagA1/2, and NagB1/2. GlcNac may also enter the Nag pathway via recycling of peptidoglycan by a hydrolase (possibly ChiIII) to GlcNAc-anhydro-*N*-acetyl muramic acid (anhMurNAc), uptake of the latter by AmpG (encoded by ORF A5) into the cytoplasm, and release of GlcNAc by HexI or HexII.

featured a putative signal peptide, while ORF B9 coded for a putative flavin reductase domain protein.

Three of the five mutants we identified as being affected in halo formation on colloidal chitin (i.e. 17H10, 18D9, and 23B7) as well as Ter331*ΔchiI* were tested for antifungal activity. All performed as well as the wild-type strain in these assays. Supernatants of *C. fungivorans* Ter331 grown in the presence of chitin showed clearing activity on colloidal chitin plates, suggesting the expression of ChiI, but had no effect on fungal growth. Possible explanations for this apparent lack of a link between chitinolysis and antifungal activity will be discussed below.

Discussion

Based on our observations, we propose a null model for the chitinolytic system of *C. fungivorans* Ter331 as shown in Fig. 4. It features obvious similarities to and interesting differences from the well-studied chitin metabolic pathways in other bacteria. Our model assumes that ChiI is an extracellular endochitinase, hydrolyzing the chitin polymer at random sites, catalyzing its rapid solubilization, and thus causing halo formation. Disabling this enzyme, for example, in mutants 23B7 or Ter331*ΔchiI* completely abolished chitin-clearing activity, suggesting that ChiI is solely

responsible for this phenotype. Apparently, inactivation of *chiI* in these mutants could not be rescued by the *chiII* or the *chiIII* gene product, suggesting that the latter do not contribute considerably to chitin solubilization on agar plates. ChiII lacks a chitin-binding site, compared with other ChiII-like proteins, and such sites have been shown to contribute considerably to the activity towards chitin (Watanabe *et al.*, 1994). Based on MU-$(GlcNAc)_n$ activity measurements (Table 2), colloidal chitin is probably not a good substrate for ChiIII either.

Given that the other four nonchitinolytic mutants (9G3, 17H10, 18D9 and 21E8) were affected in genes predicted to code for the transport of degradation products of chitin across the outer and inner membrane, we suspect that one (or several) of these products plays a role in stimulating the expression of the *chiI* gene. A similar scenario of positive feedback regulation has been described for other chitinolytic bacteria. For example, in *Serratia marcescens* 2170, $(GlcNAc)_2$ has been suggested as an inducer of chitin degradative genes (Watanabe *et al.*, 1997), and a mini-Tn*5*Km1 mutant of *S. marcescens* 2170 affected in the cytosolic uptake of $(GlcNAc)_2$ was found to be nonchitinolytic (Uchiyama *et al.*, 2003). $(GlcNAc)_2$ has also been suggested as an inducer of chitinase production in other bacteria, e.g. *Saccharophagus degradans* 2-40 (Howard *et al.*, 2003), *Streptomyces lividans* (Miyashita *et al.*, 2000) and *Vibrio furnissii* (Bassler *et al.*, 1991). Analogously, the nonchitinolytic nature of our *Collimonas* mutants 21E8 and 18D9 might suggest that the transport of $(GlcNAc)_2$ from the periplasm and its presence in the cytoplasm is required for the expression of *chiI* gene activity.

As ChiII resembled gene products from *Bacillus* species, ChiP-II gave the best hits with proteins from *Gammaproteobacteria*, and ORFs 1–4 of locus B showed high coding similarity to genes from *Alphaproteobacteria*, one can postulate that at least part of the chitinolytic system of the betaproteobacterium *C. fungivorans* Ter331 is a result of lateral gene transfer. We found no other circumstantial evidence to support this: the %G+C contents of loci A and B were not markedly different from those of their flanking DNA sequences, and there was no association with mobility signature genes such as the ones encoding transposases or tRNAs. This could mean that acquisition of these chitinolytic genes by *Collimonas* was not a recent event. Preliminary results from comparative genomic hybridization experiments (F. Mela *et al.*, unpublished data) revealed that four other chitinolytic strains of *Collimonas*, i.e. *C. fungivorans* Ter6 and Ter14, *Collimonas arenae* Ter10 and *Collimonas pratensis* Ter91 (Höppener-Ogawa *et al.*, 2008), contain a complete set of the genes listed in Tables 1, 3 and 4, which includes *chiII*, *chiP-II* and locus B ORFs 1–4. Such conservation would suggest that acquisition of these genes predates *Collimonas* speciation. The closest relatives of collimonads are bacteria from the genus *Herbaspirillum*, which are known as plant-associated nitrogen fixers but not chitin degraders. We found no homologs of *Collimonas* chitinase genes on the two published genome sequences of the *Oxalobacteracaea*, i.e. *Minibacterium massiliensis* (Audic *et al.*, 2007) and *Herminiimonas arsenicoxydans* (Muller *et al.*, 2007). These observations support the hypothesis of acquisition of chitin degradation genes by early collimonads and invite further speculation as to whether and to what degree such an event actually shaped the fate of the *Collimonas* genus and allowed niche-differentiation from related species.

The involvement of a TonB-dependent recognition and transport system for chitooligosaccharides has been proposed only recently based on positional clustering of *chiP-II* with *hex* and *nag* genes (Yang *et al.*, 2006). Here, with the description of mutants 9G3 and 17H10, we present, to the best of our knowledge, the first experimental evidence for the functionality of such a system in a bacterium. Interestingly, the gene for the outer-membrane receptor *chiP-II* is linked to those for a cognate TonB–ExbB–ExbD complex, which seems unique for *C. fungivorans* Ter331 compared with other *Proteobacteria* (Yang *et al.*, 2006). For several TonB-dependent receptors, it has been demonstrated that they have signaling capacity in addition to their transport function. For example, *E. coli* takes up ferric citrate through the outer membrane by the FecA receptor protein and the TonB–ExbB–ExbD complex (Enz *et al.*, 2000). Binding of ferric citrate to FecA also transmits a signal via FecR across the cytoplasmic membrane to FecI, a sigma factor, which then induces the expression of FecA, as well as FecBCDE, an ABC transporter for the uptake of ferric citrate or iron from the periplasm. Given the loose resemblance of this scenario to our proposed model for chitin metabolism (Fig. 4), future research efforts will be focused on exploring the possibility that recognition of chitooligosaccharides by ChiP-II relays a signal to activate the expression of the inner-membrane ABC transporter for $(GlcNAc)_2$.

The possession of an uptake system for chitooligosaccharides may be an important ecological advantage for *C. fungivorans* Ter331. Degradation of high-molecular-weight compounds by extracellular enzymes generally attracts 'cheaters' that do not invest in the production of extracellular enzymes but do take advantage of the degradation products (Allison, 2005). One strategy to undermine the effect of chitin cheaters is to use cell-associated chitinases, which would minimize diffusion of di- and monomers away from the chitinase-producing cell (Keyhani & Roseman, 1999). Another tactic is to degrade chitin only partially into oligomers that supposedly less bacteria can utilize and thus draw less cheaters, and to use an uptake system, for example, the ChiP-II system in *C. fungivorans* Ter331, that is specific for these degradation products.

Analogously, *Vibrio furnissii* uses an outer-membrane chitoporin for the uptake of chitooligosaccharides (Keyhani *et al.*, 2000). An additional advantage of such a strategy is that degradation of chitin would not release mono- (GlcNAc) and dimers $(GlcNAc)_2$ that are used by many bacteria as infochemicals for the presence of chitin. In ecological terms, there might actually be a trade-off between the ability to rapidly degrade chitin extracellularly to palatable compounds and the ability to compete with other degraders by sharing as little information as possible on the availability of chitin.

Future work will be addressing in more detail what *cis*- and *trans*-acting regulatory elements are involved in the regulation of chitinolysis by *C. fungivorans* Ter331. This will include an analysis of the striking similarity between the promoters of *chiI* and *chiP-II* (Fig. 2) to verify whether this underlies a similar mode of transcriptional regulation. Coregulated expression of genes for chitinases, *N*-acetylglucosaminidases, and putative chitooligosaccharide receptors is considered common in *Proteobacteria* and has been explained in terms of regulatory proteins that recognize specific DNA motifs that occur in all the promoter regions of the genes they regulate (Yang *et al.*, 2006). A most obvious candidate for such a regulator in *C. fungivorans* Ter331 is the *nagQ* product (Fig. 1a; Table 3). Its analog in *S. coelicolor* A3(2), DasR, controls many GlcNAc-related genes, including those encoding chitinolysis (Rigali *et al.*, 2006) by acting as a DNA-binding repressor. DNA binding of DasR is abolished by glucosamine-6-phosphate, a metabolite of the GlcNAc degradation pathway. It remains to be determined whether this intermediate also represents the actual effector of chitin utilization in *C. fungivorans* Ter331. Alternative effector candidates are $(GlcNAc)_2$ (Watanabe *et al.*, 1997) or GlcNAc-6-phosphate. The latter is the inducing signal for NagC, the transcriptional repressor of the *nag* operon in *E. coli* (Alvarez-Anorve *et al.*, 2005). Quite interestingly, GlcNAc-6-phosphate has also been shown to play an additional modulating role in *E. coli* (Alvarez-Anorve *et al.*, 2005) and *Shewanella oneidensis* MR-1 (Yang *et al.*, 2006), i.e. by allosteric activation of the NagB protein. The essence of this activation is that GlcNAc-6-phosphate lowers the apparent K_m value of NagB for its actual substrate glucosamine-6-P (Yang *et al.*, 2006). This invites a closer look at the nonsense mutation in the *nagA1* gene in *C. fungivorans* Ter331 (Fig. 1a) to determine how this mutation affects intracellular concentrations of the NagA-substrate GlcNAc-6-phosphate and thus NagB activity.

The positional clustering of chitin degradation genes with an *ampG*-like gene in *chi* locus A seems to be unique to *C. fungivorans* Ter331. The significance of this clustering is not clear. Peptidoglycan turnover and chitinolysis both release GlcNAc and perhaps the two processes just share the same genes for the channeling of GlcNAc into central carbon metabolism. For *S. coelicolor* A3(2), it has been suggested that peptidoglycan degradation is under the same control as chitin degradation (Rigali *et al.*, 2006). An intriguing possibility is that modification of the peptidoglycan layer is required during chitin utilization in order to increase the mobility of chitinases and/or chitooligosaccharides within the periplasmic space. The peptidoglycan layer of Gram-negative cells is considered to be a barrier for the diffusion of macromolecular structures (Demchick & Koch, 1996), and active hydrolysis of peptidoglycan for the purpose of transporting macromolecules across the periplasm has been implemented in flagellar assembly (Nambu *et al.*, 1999) and in the transport of phage genomes across the bacterial cell wall (Moak & Molineux, 2004). In this context, we might hypothesize that ChiIII, predicted to have lytic activity towards peptidoglycan, plays an accessory role in chitin utilization by *C. fungivorans* Ter331. A striking observation is the nonredundancy of ChiI function in *C. fungivorans* Ter331. Many chitinolytic bacteria have multiple chitinases (Howard *et al.*, 2003; Meibom *et al.*, 2004; Orikoshi *et al.*, 2005), and the functional synergy between these enzymes is assumed to be necessary for efficient chitin degradation (Suzuki *et al.*, 2002; Orikoshi *et al.*, 2005). By this rationale, the presence of only a single chitin-active gene in *Collimonas* may explain the relatively poor chitin-degrading ability of this strain compared with much more efficient bacterial degraders of chitin (de Boer *et al.*, 2001). Several chitinolytic bacteria have been described with only a single chitinase gene. One of these is biocontrol agent *Stenotrophomonas maltophilia* strain 34S1 (Kobayashi *et al.*, 2002). A *chiA* mutant of this strain was devoid of chitinase activity and not affected in its ability to inhibit growth of the fungus *Magnaporthe poae* in an *in vitro* antagonism test. This mirrors our own observations with *Collimonas* mutants 23B7 and *ΔchiI* in antifungal assays. However, the *chiA* mutant of strain 34S1 showed reduced ability to control summer patch disease on Kentucky bluegrass, caused by *M. poae*. In similar experiments (F. Kamilova *et al.*, unpublished data), mutant 23B7 did not differ significantly from wild-type *Collimonas* in the ability to control tomato foot and root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Kamilova *et al.*, 2007). Based on this, we conclude that *chiI* is probably not a major contributing factor to the antifungal activity of *C. fungivorans* Ter331 *in vivo*. A similar noninvolvement of chitinases has been reported for other chitinolytic bacteria with antifungal properties (Kamensky *et al.*, 2003; Meziane *et al.*, 2006). Preliminary experiments with *C. fungivorans* Ter331 showed that *in vitro* antifungal activity was not affected upon separation of the bacteria and their fungal target by a dialysis membrane with an 8-kDa cut-off (K. Fritsche *et al.*, unpublished data). This would moreover exclude an involvement of the chitinases we describe in this study (Table 1). To explain

the antifungal activity of *C. fungivorans* Ter331, our focus has thus shifted to other possible mechanisms, including low-molecular-weight compounds such as small proteins and secondary metabolites.

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References

Adesina MF, Lembke A, Costa R, Speksnijder A & Smalla K (2007) Screening of bacterial isolates from various European soils for *in vitro* antagonistic activity towards *Rhizoctonia solani* and *Fusarium oxysporum*: site-dependent composition and diversity revealed. *Soil Biol Biochem* **39**: 2818–2828.

Allison SD (2005) Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. *Ecol Lett* **8**: 626–635.

Alvarez-Anorve LI, Calcagno ML & Plumbridge J (2005) Why does *Escherichia coli* grow more slowly on glucosamine than on *N*-acetylglucosamine? Effects of enzyme levels and allosteric activation of GlcN6P deaminase (NagB) on growth rates. *J Bacteriol* **187**: 2974–2982.

Aspray TJ, Hansen SK & Burns RG (2005) A soil-based microbial biofilm exposed to 2,4-D: bacterial community development and establishment of conjugative plasmid pJP4. *FEMS Microbiol Ecol* **54**: 317–327.

Audic S, Robert C, Campagna B, Parinello H, Claverie JM, Raoult D & Drancourt M (2007) Genome analysis of *Minibacterium massiliensis* highlights the convergent evolution of water-living bacteria. *PloS Genet* **3**: 1454–1463.

Bassler BL, Yu C, Lee YC & Roseman S (1991) Chitin utilization by marine bacteria: degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. *J Biol Chem* **268**: 24276–24286.

Braun V (1995) Energy-coupled transport and signal-transduction through the Gram-negative outer-membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol Rev* **16**: 295–307.

Chang WT, Chen CS & Wang SL (2003) An antifungal chitinase produced by *Bacillus cereus* with shrimp and crab shell powder as a carbon source. *Curr Microbiol* **47**: 102–108.

Cheng QM & Park JT (2002) Substrate specificity of the AmpG permease required for recycling of cell wall anhydro-muropeptides. *J Bacteriol* **184**: 6434–6436.

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

Dahiya N, Tewari R, Tiwari R & Hoondal G (2005) Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World J Microbiol Biotechnol* **21**: 1611–1616.

De Boer W, Klein Gunnewiek PJA, Lafeber P, Janse JD, Spit BE & Woldendorp JW (1998) Anti-fungal properties of chitinolytic dune soil bacteria. *Soil Biol Biochem* **30**: 193–203.

De Boer W, Klein Gunnewiek PJA, Kowalchuk GA & Van Veen JA (2001) Growth of chitinolytic dune soil beta-subclass *Proteobacteria* in response to invading fungal hyphae. *Appl Environ Microbiol* **67**: 3358–3362.

De Boer W, Leveau JHJ, Kowalchuk GA *et al.* (2004) *Collimonas fungivorans* gen. nov., sp. nov., a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *Int J Syst Evol Microbiol* **54**: 857–864.

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

Demchick P & Koch AL (1996) The permeability of the wall fabric of *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* **178**: 768–773.

Enz S, Mahren S, Stroeher UH & Braun V (2000) Surface signaling in ferric citrate transport gene induction: interaction of the FecA, FecR, and FecI regulatory proteins. *J Bacteriol* **182**: 637–646.

Fritsche K, Leveau JHJ, Gerards S, Ogawa S, de Boer W & van Veen JA (2006) *Collimonas fungivorans* and bacterial mycophagy. *IOBC/WPRS Bull* **29**: 27–29.

Hamamura N, Olson SH, Ward DM & Inskeep WP (2006) Microbial population dynamics associated with crude-oil biodegradation in diverse soils. *Appl Environ Microbiol* **72**: 6316–6324.

Höppener-Ogawa S, Leveau JHJ, Smant W, van Veen JA & de Boer W (2007) Specific detection and real-time PCR quantification of potentially mycophagous bacteria belonging to the genus *Collimonas* in different soil ecosystems. *Appl Environ Microbiol* **73**: 4191–4197.

Höppener-Ogawa S, de Boer W, Leveau JHJ *et al.* (2008) *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov., isolated from (semi-) natural grassland soils. *Int J Syst Evol Microbiol* **58**: 414–419.

Howard MB, Ekborg NA, Taylor LE, Weiner RM & Hutcheson SW (2003) Genomic analysis and initial characterization of the chitinolytic system of *Microbulbifer degradans* strain 2-40. *J Bacteriol* **185**: 3352–3360.

Ikeda S, Omura T, Ytow N, Komaki H, Minamisawa K, Ezura H & Fujimura T (2006) Microbial community analysis in the rhizosphere of a transgenic tomato that overexpresses 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Microb Environ* **21**: 261–271.

Kamensky M, Ovadis M, Chet I & Chernin L (2003) Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biol Biochem* **35**: 323–331.

Kamilova F, Leveau JHJ & Lugtenberg B (2007) *Collimonas fungivorans*, an unpredicted *in vitro* but efficient *in vivo*

biocontrol agent for the suppression of tomato foot and root rot. *Environ Microbiol* **9**: 1597–1603.

Keyhani NO & Roseman S (1999) Physiological aspects of chitin catabolism in marine bacteria. *Biochim Biophys Acta* **1473**: 108–122.

Keyhani NO, Li XB & Roseman S (2000) Chitin catabolism in the marine bacterium *Vibrio furnissii*: identification and molecular cloning of a chitoporin. *J Biol Chem* **275**: 33068–33076.

Kobayashi DY, Reedy RM, Bick J & Oudemans PV (2002) Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. *Appl Environ Microbiol* **68**: 1047–1054.

Leveau JHJ & Preston GM (2007) Bacterial mycophagy: definition and diagnosis of a unique bacterial-fungal interaction. *New Phytol* **177**: 859–876.

Leveau JHJ, Gerards S, Fritsche K, Zondag G & van Veen JA (2006) Genomic flank-sequencing of plasposon insertion sites for rapid identification of functional genes. *J Microbiol Methods* **66**: 276–285.

Lindquist S, Westonhafer K, Schmidt H *et al.* (1993) AmpG, a signal transducer in chromosomal beta-lactamase induction. *Mol Microbiol* **9**: 703–715.

Mahmood S, Paton GI & Prosser JI (2005) Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environ Microbiol* **7**: 1349–1360.

Mannisto MK & Haggblom MM (2006) Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Syst Appl Microbiol* **29**: 229–243.

Mayer C, Vocadlo DJ, Mah M, Rupitz K, Stoll D, Warren RAJ & Withers SG (2006) Characterization of a beta-*N*-acetylhexosaminidase and a beta-*N*-acetylglucosaminidase/beta-glucosidase from *Cellulomonas fimi*. *FEBS J* **273**: 2929–2941.

Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S & Schoolnik GK (2004) The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci* **101**: 2524–2529.

Meyer F, Goesmann A, McHardy AC *et al.* (2003) GenDB – an open source genome annotation system for prokaryote genomes. *Nucleic Acids Res* **31**: 2187–2195.

Meziane H, Gavriel S, Ismailov Z, Chet I, Chernin L & Hofte M (2006) Control of green and blue mould on orange fruit by *Serratia plymuthica* strains IC14 and IC1270 and putative modes of action. *Postharvest Biol Technol* **39**: 125–133.

Miyashita K, Fujii T & Saito A (2000) Induction and repression of a *Streptomyces lividans* chitinase gene promoter in response to various carbon sources. *Biosci Biotechnol Biochem* **64**: 39–43.

Moak M & Molineux IJ (2004) Peptidoglycan hydrolytic activities associated with bacteriophage virions. *Mol Microbiol* **51**: 1169–1183.

Muller D, Médigue C, Koechler S, Barbe V, Barakat M *et al.* (2007) A tale of two oxidation states: bacterial colonization of arsenic-rich environments. *PLoS Genet* **3**: e53.

Nakayama K, Takashima K, Ishihara H *et al.* (2000) The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Mol Microbiol* **38**: 213–231.

Nambu T, Minamino T, Macnab RM & Kutsukake K (1999) Peptidoglycan-hydrolyzing activity of the FlgJ protein, essential for flagellar rod formation in *Salmonella typhimurium*. *J Bacteriol* **181**: 1555–1561.

Offre P, Pivato B, Siblot S, Gamalero E, Corberand T, Lemanceau P & Mougel C (2007) Identification of bacterial groups preferentially associated with mycorrhizal roots of *Medicago truncatula*. *Appl Environ Microbiol* **73**: 913–921.

Opelt K & Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea coast. *Appl Environ Microbiol* **70**: 6569–6579.

Orikoshi H, Nakayama S, Miyamoto K, Hanato C, Yasuda M, Inamori Y & Tsujibo H (2005) Roles of four chitinases (ChiA, ChiB, ChiC, and ChiD) in the chitin degradation system of marine bacterium *Alteromonas* sp. strain O-7. *Appl Environ Microbiol* **71**: 1811–1815.

Overbeek R, Begley T, Butler RM *et al.* (2005) The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res* **33**: 5691–5702.

Rigali S, Nothaft H, Noens EEE *et al.* (2006) The sugar phosphotransferase system of *Streptomyces coelicolor* is regulated by the GntR-family regulator DasR and links *N*-acetylglucosamine metabolism to the control of development. *Mol Microbiol* **61**: 1237–1251.

Saito A & Schrempf H (2004) Mutational analysis of the binding affinity and transport activity for *N*-acetylglucosamine of the novel ABC transporter Ngc in the chitin-degrader *Streptomyces olivaceoviridis*. *Mol Genet Genome* **271**: 545–553.

Saito A, Shinya T, Miyamoto K *et al.* (2007) The *dasABC* gene cluster, adjacent to *dasR*, encodes a novel ABC transporter for the uptake of *N*,*N*′-diacetylchitobiose in *Streptomyces coelicolor* A3(2). *Appl Environ Microbiol* **73**: 3000–3008.

Schmidt M, Prieme A & Stougaard P (2006) Bacterial diversity in permanently cold and alkaline ikaite columns from Greenland. *Extremophiles* **10**: 551–562.

Seo JW, Ohnishi Y, Hirata A & Horinouchi S (2002) ATP-binding cassette transport system involved in regulation of morphological differentiation in response to glucose in *Streptomyces griseus*. *J Bacteriol* **184**: 91–103.

Suzuki K, Sugawara N, Suzuki M, Uchiyama T, Katouno F, Nikaidou N & Watanabe T (2002) Chitinases A, B, and C1 of *Serratia marcescens* 2170 produced by recombinant *Escherichia coli*: enzymatic properties and synergism on chitin degradation. *Biosci Biotechnol Biochem* **66**: 1075–1083.

Uchiyama T, Kaneko R, Yamaguchi J *et al.* (2003) Uptake of *N*,*N*′-diacetylchitobiose [$(GlcNAc)_2$] via the phosphotransferase system is essential for chitinase production by *Serratia marcescens* 2170. *J Bacteriol* **185**: 1776–1782.

Uroz S, Calvaruso C, Turpaul MP, Pierrat JC, Mustin C & Frey-Klett P (2007) Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved

in mineral weathering in a forest soil. *Appl Environ Microbiol* **73**: 3019–3027.

Watanabe T, Oyanagi W, Suzuki K & Tanaka H (1990a) Chitinase system of *Bacillus circulans* Wl-12 and importance of chitinase A1 in chitin degradation. *J Bacteriol* **172**: 4017–4022.

Watanabe T, Suzuki K, Oyanagi W, Ohnishi K & Tanaka H (1990b) Gene cloning of chitinase A1 from *Bacillus circulans* Wl-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type-III homology units of fibronectin. *J Biol Chem* **265**: 15659–15665.

Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M & Tanaka H (1993) Identification of glutamic acid-204 and aspartic acid-200 in chitinase A1 of *Bacillus circulans* Wl-12 as essential residues for chitinase activity. *J Biol Chem* **268**: 18567–18572.

Watanabe T, Ito Y, Yamada T, Hashimoto M, Sekine S & Tanaka H (1994) The roles of the C-terminal domain and type-III domains of chitinase A1 from *Bacillus circulans* Wl-12 in chitin degradation. *J Bacteriol* **176**: 4465–4472.

Watanabe T, Kimura K, Sumiya T *et al.* (1997) Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J Bacteriol* **179**: 7111–7117.

Wilson MS, Herrick JB, Jeon CO, Hinman DE & Madsen EL (2003) Horizontal transfer of *phnAc* dioxygenase genes within one of two phenotypically and genotypically distinctive naphthalene-degrading guilds from adjacent soil environments. *Appl Environ Microbiol* **69**: 2172–2181.

Xiao X, Wang F, Saito A, Majka J, Schlosser A & Schrempf H (2002) The novel *Streptomyces olivaceoviridis* ABC transporter Ngc mediates uptake of *N*-acetylglucosamine and *N*,*N*′-diacetylchitobiose. *Mol Genet Genome* **267**: 429–439.

Yang C, Rodionov DA, Li XQ *et al.* (2006) Comparative genomics and experimental characterization of *N*-acetylglucosamine utilization pathway of *Shewanella oneidensis*. *J Biol Chem* **281**: 29872–29885.