

SHORT REPORT

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Bacterial and fungal chitinase *chiJ* orthologs evolve under different selective constraints following horizontal gene transfer

Wimal Ubhayasekera¹ and Magnus Karlsson^{2*}

Abstract

Background: Certain bacteria from the genus *Streptomyces* are currently used as biological control agents against plant pathogenic fungi. Hydrolytic enzymes that degrade fungal cell wall components, such as chitinases, are suggested as one possible mechanism in biocontrol interactions. Adaptive evolution of chitinases are previously reported for plant chitinases involved in defence against fungal pathogens, and in fungal chitinases involved in fungal-fungal interactions. In this study we investigated the molecular evolution of chitinase *chiJ* in the bacterial genus *Streptomyces*. In addition, as *chiJ* orthologs are previously reported in certain fungal species as a result from horizontal gene transfer, we conducted a comparative study of differences in evolutionary patterns between bacterial and fungal taxa.

Findings: *ChiJ* contained three sites evolving under strong positive selection and four groups of co-evolving sites. Regions of high amino acid diversity were predicted to be surface-exposed and associated with coil regions that connect certain α -helices and β -strands in the family 18 chitinase TIM barrel structure, but not associated with the catalytic cleft. The comparative study with fungal *ChiJ* orthologs identified three regions that display signs of type 1 functional divergence, where unique adaptations in the bacterial and fungal taxa are driven by positive selection.

Conclusions: The identified surface-exposed regions of chitinase *ChiJ* where sequence diversification is driven by positive selection may putatively be related to functional divergence between bacterial and fungal orthologs. These results show that *ChiJ* orthologs have evolved under different selective constraints following the horizontal gene transfer event.

Keywords: Chitinase, Functional divergence, Homology modelling, Horizontal gene transfer, *Hypocrea*, Protein evolution, *Streptomyces*, *Trichoderma*

Findings

Background

Chitin is an insoluble linear β -1,4-linked polymer of *N*-acetylglucosamine which is an essential structural component of fungal cell walls. Chitinases (EC 3.2.1.14) are hydrolytic enzymes that catalyse the degradation of chitin and are classified into glycoside hydrolase families 18 and 19, based on amino acid sequence similarity [1,2]. These two families possess different three-dimensional structural folds as well as modes of action.

Family 18 chitinases is based on a triosephosphate isomerase (TIM) barrel structure and use a substrate-assisted double-displacement with net retention of the configuration of the anomeric carbon during the catalysis [3].

Biological control of pathogenic fungi causing diseases on important crop plants is an attractive alternative to the extensive use of chemical fungicides in modern agriculture, which may have negative environmental impacts and result in evolution of fungicide resistance in pathogen populations. Certain bacteria from the genus *Streptomyces* and fungi from the genera *Hypocrea* (anamorph *Trichoderma*) and *Bionectria* (anamorph *Clonostachys*) are currently used as biological control agents. The biocontrol ability of these microbes are suggested to be the result of several mechanisms that act

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synergistically, including direct antagonism by toxins and cell wall degrading enzymes (including chitinases), induced resistance in plants, or competition for nutrients and space [4].

Streptomyces bacteria are well known for their production of secreted hydrolytic enzymes and secondary metabolites [5], illustrating their ecological niche as ubiquitous soil bacteria. The genetically best studied species is *S. coelicolor* which is reported to possess 12 different chitinase genes, ten family 18 members (*chiA, B, C, D, E, H, I, J, K, L*) and two family 19 members (*chiF, G*) [6]. The ten family 18 chitinases can be phylogenetically divided into subfamilies A, B and C, and subgroups II, IVa and VI [6,7]. Seven genes, *chiA, B, C, D, F, I* and *J*, are reported to be induced to varying degrees in the presence of colloidal chitin [6,8], while studies of enzymatic activity are reported for *S. coelicolor* ChiA, C, D, F and G [6,9]. Recently, *chiJ* is shown to have an ortholog in *Hypocrea* species (*chi18-15/chit36*) and in *Bionectria ochroleuca* (*ech37*), acquired through horizontal gene transfer (HGT) [7,10,11].

Certain *Hypocrea* and *Bionectria* species are mycoparasitic, i.e. they have the ability to parasitize other fungi and use them as a source of nutrients. Studies of gene family evolution show that the mycoparasitic lifestyle has resulted in selection for increased number of cell wall degrading chitinases in *Hypocrea* [10]. In addition, certain *Hypocrea* chitinases evolve rapidly in a manner consistent with co-evolutionary interactions between parasite and host [10], similar to certain plant chitinases involved in defence against fungal pathogens [12,13].

In this study we investigate the molecular evolution of *Streptomyces* chitinase ChiJ, under the hypothesis that its involvement in interactions with soil fungi have resulted in sequence diversification between taxa. In addition, the HGT event presents an opportunity for a comparative investigation of the molecular fate of ChiJ orthologs evolving in bacterial and fungal species, respectively. We hypothesize that bacterial and fungal ChiJ orthologs evolve differently due to the fundamentally different organismal environments.

Materials and methods

Identification of genes and phylogenetic analysis

Streptomyces genome sequences available at the National Center for Biotechnological Information (NCBI) were screened for the presence of chitinase *chiJ* [GenBank: NP_626743], DNA recombinase A *recA* [GenBank: NP_629894] and DNA polymerase III subunit beta *dnaN* [GenBank:NP_628065] using BLAST [14]. GenBank [15] sequences were screened with ChiJ using BLAST. Sequences were aligned with Clustal X [16]. Phylogenetic analysis was performed using maximum likelihood methods implemented in PhyML-aLRT ver. 2.4.5 [17,18].

The JTT amino acid substitution model [19] was used, the proportion of invariable sites was set to 0, one category of substitution rate was used and gaps were treated as unknown characters. The starting tree to be refined by the maximum likelihood algorithm was a distance-based BIONJ tree estimated by the program [17]. Statistical support for phylogenetic grouping was assessed by approximate likelihood-ratio tests based on a Shimodaira-Hasegawa-like procedure [18] and by bootstrap analysis (1000 resamplings).

Homology modelling

Similar sequences were located by BLAST in the protein entries of GenBank and aligned using Clustal W [20]. Similar chitinase catalytic module structures (Additional file 1) were obtained from the Protein Data Bank (PDB [21]), then superimposed and compared with the program O [22]. Multiple sequence alignments were used to identify the best pair-wise alignment of the *Streptomyces* sp. Mg1 [GenBank:ZP_04999219] enzyme with that of *Lactococcus lactis* subsp. *lactis*. This pair-wise alignment was the basis of creating a homology model, with PDB entry [PDB:3IAN] (*L. lactis* subsp. *lactis*) as the template in the program SOD [23]. The model was adjusted in O, using rotamers that would improve packing in the interior of the protein. The model is available upon request from the authors. The figure was prepared using O, MOLSCRIPT [24] and Molray [25].

Reverse conservation analysis

Amino acid diversity in sequence alignments were analysed using Reverse Conservation Analysis (RCA). Amino acid sequences were aligned by Clustal X [16] and amino acid conservation at each position was analysed by RCA analysis as described by Lee (2008) [26]. In short, Rate4Site (Version 2.01) was used to calculate the degree of conservation (S score) for each amino acid position using the empirical Bayesian method [27,28]. A sliding window-average ($n = 7$) S score was plotted (W mean score) and significant peaks were defined by intensity (I) values of 0.5, as recommended by Lee (2008) [26].

Codon-based analyses

The rate of non-synonymous (dN) and synonymous (dS) substitutions at each codon, and identification of sites evolving under positive or negative selection, was determined using random effects maximum likelihood models (REL) [29] implemented in the HyPhy software package [30] through the Datamonkey webserver [31]. The optimal nucleotide substitution model was estimated for each gene separately [32] and included the following modifications to the general reversible nucleotide

model [29] for *chiJ*: C↔G: R₁, C↔T: R_{AC}, G↔T: R_{AT}; for *recA*: A↔C: R₁, A↔T: R₁, C↔G: R_{CG}, C↔T: R_{CT}, G↔T: R_{GT}, and for *dnaN*: A↔C: R₁, A↔T: R₁, C↔G: R_{CG}, G↔T: R₁. A Bayes factor value ≥ 50 (default) was used as an indication of strong positive selection at a site, while values between 10 and 49 were considered to indicate weak support of positive selection [29].

Identification of co-evolving sites was done using the Spidermonkey/BGM program [33] implemented in the HyPhy software package [30] through the Datamonkey webserver [31]. The same nucleotide substitution models as in REL analysis was used, global dN/dS values were estimated by the program, ambiguous characters were averaged, a two-parent, directed network was used and

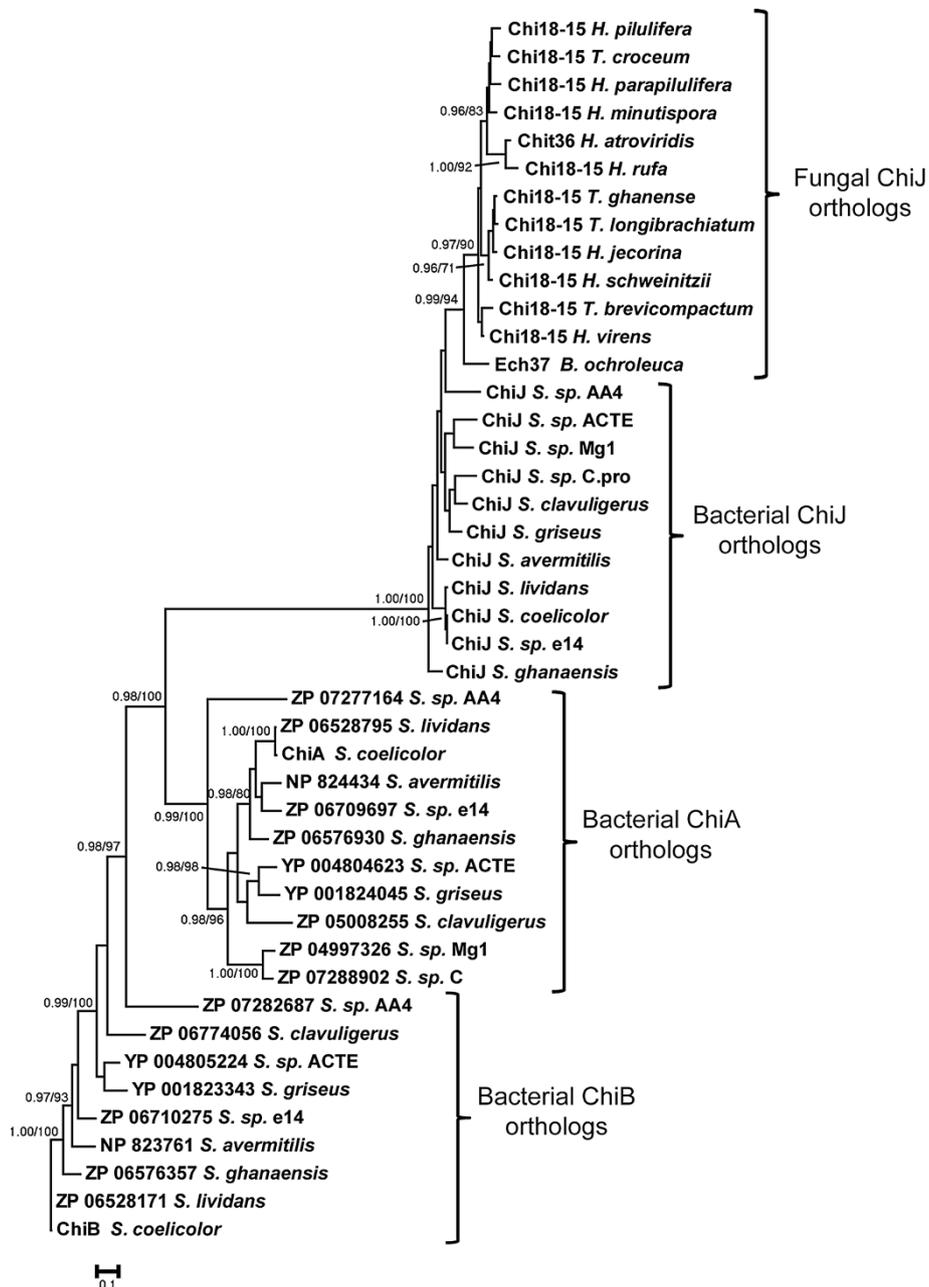


Figure 1 Phylogenetic relationships of *Streptomyces* ChiJ and related proteins. Phylogenetic analyses were performed using maximum likelihood methods as implemented in PhyML-aLRT, based on an alignment of chitinase amino acid sequences. Branch support values (approximate likelihood-ratio test probabilities (> 0.95) / bootstrap proportions (> 70%)) are associated with nodes. The bar marker indicate the number of amino acid substitutions per 100 amino acids. *Streptomyces* ChiA, ChiB, ChiJ and fungal ChiJ orthologs are indicated. Sequence identifiers include protein name or locus ID.

sites were selected based on non-synonymous branch counts (threshold ≥ 3). A posterior probability value ≥ 0.5 (default) was used as the definition of association between sites.

Results

Identification of *chiJ* orthologs

The translated *chiJ* gene from *S. coelicolor* was used as the template for screening *Streptomyces* genome

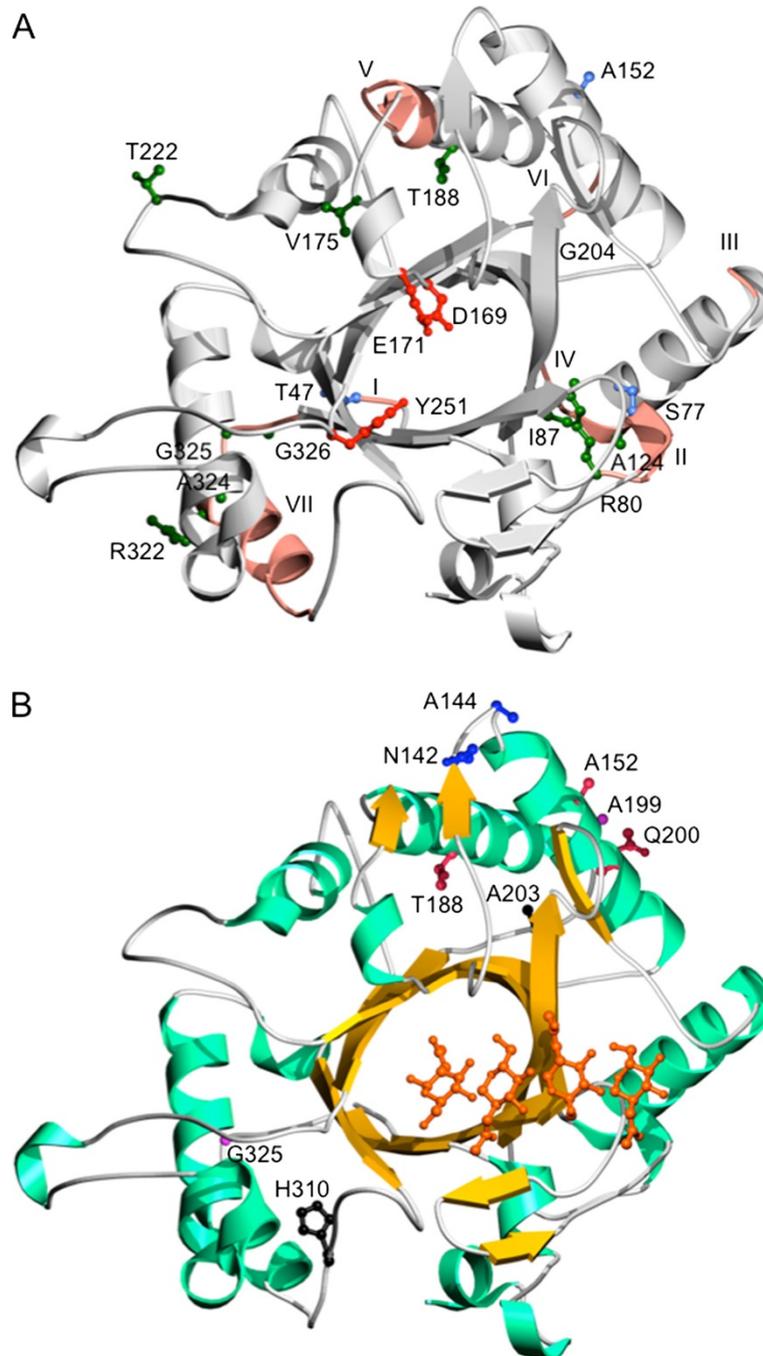


Figure 2 Homology model of *Streptomyces* sp. Mg1 ChiJ chitinase. A. Catalytically important residues are marked in red. Amino acids under strong and weak positive selection are shown in royal blue and dark green respectively. Variable regions from reverse conservation analysis are coloured in salmon and marked I-VII (I = 44-47, II = 75-80, III = 110-111, IV = 123-127, V = 178-185, VI = 200-203, VII = 312-327). **B.** Secondary structure elements of the enzyme are coloured differently. Side chains of co-evolving amino acids are shown in balls and sticks. Co-evolving groups of residues are illustrated in the same colour. Chito-tetraose is modelled to show the substrate-binding cleft.

sequences available at NCBI. *ChiJ* orthologs were present in ten species, *S. avermitilis*, *S. clavuligerus*, *S. ghanaensis*, *S. griseus*, *S. lividans*, *S. sp. AA4*, *S. sp. ACTE*, *S. sp. C*, *S. sp. e14* and *S. sp. Mg1*, in addition to *S. coelicolor*. With the exception of *S. sp. Mg1* and *S. sp. C* that only contained one additional similar gene (E-value $\leq 1e^{-5}$), the other nine species contained two additional similar genes (E-value $\leq 1e^{-5}$). A phylogenetic analysis of translated amino acid sequences confirmed that the identified proteins represented the three *Streptomyces* chitinase paralogs ChiA, ChiB and ChiJ in the IVa group [6,7], and confirmed the orthologous status of the identified *chiJ* sequences (Figure 1). The translated *S. coelicolor* ChiJ protein was 358 amino acids long and consisted of a signal peptide for secretion and a family 18 chitinase catalytic module, but no additional substrate-binding modules [34]. *Streptomyces recA* and *dnaN* were present in the selected bacterial genomes, and were retrieved for comparative purposes.

Homology modelling of ChiJ

Homology modelling revealed that the *Streptomyces sp. Mg1* chitinase had a TIM barrel fold (Figure 2) with six insertions and three deletions compared to the chitinase structure from *L. lactis* subsp. *lactis*. In comparison to the hevine chitinase from rubber tree [35] the catalytic cleft of ChiJ was more open at the substrate entry side of the cleft. Catalytic proton donor (E₁₇₁) and the residues assisting in the catalysis (D₁₆₉ and Y₂₅₁) were conserved. Residues along the cleft that may play important roles in substrate binding were also conserved. The homology model of ChiJ displayed structural similarities to retaining endochitinases [36].

Distribution of amino acid diversity

Patterns of amino acid diversity can provide important information about the type of selective constraints that act on different parts of an enzyme. In distantly related orthologous sequences only functionally important regions are expected to be conserved between species, while other regions are expected to have a low degree of conservation due to accumulation of neutral mutations. In the case of closely related orthologs, such as ChiJ in the current work, a high degree of conservation is expected for most positions as the limited time since speciation has not allowed sufficient sequence diversification by genetic drift. However, as outlined by Lee (2008) [26], residues important for enzyme properties may be expected to display higher diversity than other positions in closely related orthologs due to selection for modified enzymatic properties between species.

Therefore, amino acid diversity in a ChiJ alignment (Additional file 2) was analysed by RCA. The first 36 amino acids, including the signal peptide, were excluded from the analysis as this part could not be reliably aligned. High amino acid diversity was distributed amongst seven regions, labelled I through VII, with W mean scores above the 0.5 standard deviation threshold from the RCA analysis (Figure 3). All predicted residues important for catalysis and substrate-binding (Additional file 3) were localised in conserved regions with low W mean scores (Figure 3). Structural positions of the identified regions were visualized on the ribbon cartoon of the homology model of *S. sp. Mg1* ChiJ (Figure 2A). *S. sp. Mg1* ChiJ was used as a reference sequence for residue positions throughout this paper. All seven regions with high amino acid diversity (W score ≥ 0.5) were predicted to be surface-exposed, but neither forms a part of the catalytic cleft (Figure 2A). A closer examination of the S scores for individual residue positions revealed that all seven regions of high amino acid diversity were associated with coil regions that connect certain α -helices and β -strands in the family 18 chitinase TIM barrel fold structure (Figure 3). More specifically, highly divergent residue positions (S scores ≥ 0.5) in region I were located in a coil region placed on the N-terminal side of a β -strand at residue positions 50–56. Regions II, III, V, VI and VII contained highly divergent residue positions located in coil regions adjacent to α -helices (pos. 77–79, 111–125, 183–198, 316–323) (Figure 2). Region IV was associated with a α -helix / β -strand junction (Figure 3).

Evolutionary patterns among sites

High sequence diversity in certain regions and sites between closely related orthologs may be the result of either low selective constraint, i.e. mutations do not alter enzyme function in a significant manner, or to positive selection where certain mutations provide a selective advantage that improve enzyme function. The ratio between non-synonymous and synonymous substitutions is expected to approach 1 in the case of low selective constraints and be > 1 in the case of positive selection.

Therefore, the rate of dN and dS at each codon and identification of sites evolving under positive or negative selection was determined using REL. The first 117 bp of *chiJ* was removed from the analysis as this part could not be reliably aligned. REL fits both dN and dS substitution rates into three discrete rate classes, yielding a total of nine different rate classes of dN/dS. For *chiJ*, one rate class was estimated to have a dN/dS value above 1 (dN (0.37) / dS (0.22) = 1.68). Three sites evolved under strong positive selection (Bayes factor ≥ 50) and an

Table 1 Positively selected sites in ChiJ

Amino acid position	Posterior probability	Bayes factor
T ₄₇	0.77	68
S ₇₇	0.73	53
R ₈₀	0.66	39
I ₈₇	0.34	10
A ₁₂₄	0.69	46
A ₁₅₂	0.91	197
V ₁₇₅	0.68	43
T ₁₈₈	0.65	38
G ₂₀₄	0.40	13
T ₂₂₂	0.63	34
R ₃₂₂	0.55	25
A ₃₂₄	0.45	16
G ₃₂₅	0.61	31
G ₃₂₆	0.44	16

Site positions are given in reference to *S. sp. Mg1* ChiJ. Posterior probabilities and Bayes factors are determined using random effects likelihood method.

Finally, the co-evolving N₁₄₂ – A₁₄₄ pair was predicted to be physically close to site A₁₅₂ and regions V and VI in the homology model (Figure 2A and B). In three cases, co-evolving sites were also evolving under positive selection (Figure 3).

Comparison between chiJ and fungal orthologs

Fungal species of the genera *Hypocrea* and *B. ochroleuca* were previously shown to possess an ortholog to *chiJ* (*chi18-15/chit36* and *ech37* respectively), acquired through HGT from its actinobacterial origin [7,10,11]. An alignment of ChiJ (excluding the first 36 amino acids) from *S. coelicolor* and *S. sp. Mg1*, *chi18-15* from *H. jecorina*, *CHIT36* from *H. atroviridis* and *Ech37* from *B. ochroleuca* shows that no insertions or deletions have occurred since the HGT event (data not shown). However, when comparing regions exhibiting high amino acid diversity from the RCA analysis in the current paper with a previous analysis on the fungal (*Hypocrea*) ChiJ orthologs [10], several differences were observed. ChiJ regions I and IV both exhibited high amino acid variation between *Streptomyces* species and contained rapidly evolving sites, while these regions were highly

Table 2 Co-evolving sites in ChiJ

Amino acid position 1	Amino acid position 2	Posterior probability
N ₁₄₂	A ₁₄₄	0.85
A ₁₅₂	Q ₂₀₀	0.59
T ₁₈₈	Q ₂₀₀	0.55
A ₁₉₉	G ₃₂₅	0.58
A ₂₀₃	H ₃₁₀	0.68

Site positions are given in reference to *S. sp. Mg1* ChiJ. Posterior probabilities for site 1 and site 2 to be conditionally dependent, determined by Spidermonkey/BGM, is given.

conserved in the fungal counterparts (Figure 3). The same trend was observed for ChiJ regions VI and VII (Figure 3). On the other hand, one region (region A) in the fungal *chi18-15/CHIT36* protein alignment was highly variable between fungal species at the amino acid level, but conserved between bacterial ChiJ orthologs (Figure 3).

Discussion

Adaptive evolution of chitinases have previously been shown for plant chitinases involved in defence against fungal pathogens [12,13], and in fungal chitinases involved in mycoparasitic interactions [10]. The common theme between these examples is that parasite – host interaction seem to exert selection for rapid modification of the involved enzymes in an arms race fashion. Enzyme modification may thus partly account for variation in virulence or disease resistance in the parasite and host, respectively.

The biological function of ChiJ is suggested to include degradation of exogenous chitin for nutrient acquisition and aggressive interactions with soil fungi. Evolution of ChiJ may be influenced by the presence of several additional chitinases in certain *Streptomyces* species [6]. Conservation of gene duplications may be favoured if high levels of a specific chitinase is needed. However, the substantial divergence between ChiA, ChiB and ChiJ that is evident from our phylogenetic analysis suggest that these paralogous proteins represent isozymes with different functional properties that may act synergistically in chitin degradation. Presence of a fungal *chiJ* ortholog in both *Hypocrea spp.* and in *B. ochroleuca* suggests that the HGT event preceded the split of these species. These species belong to the same order (*Hypocreales*) but different families, so the HGT event cannot be considered to be recent. *B. ochroleuca* and certain *Hypocrea* species are mycoparasitic, and thus *Ech37/CHIT36* are suggested to participate in degradation of the cell wall of antagonistic fungal species [36,37]. Expression data show that *ech37* in *B. ochroleuca* is indeed induced during interaction with *Botryotinia fuckeliana* on strawberry leaves [37], while *chit36* in *T. asperellum* is induced during plate confrontation with *Rhizoctonia solani* [38].

No region of high amino acid diversity or sites showing signs of adaptive evolution constitute parts of the catalytic cleft of ChiJ. This suggests that adaptation for changes in substrate-specificity between different *Streptomyces* species is not the primary selection force for ChiJ evolution. This conclusion is further supported by the localization of all residues predicted to be important for catalysis and substrate-binding to conserved regions. In addition, *Hypocrea spp.* *Chi18-15/CHIT36* interspecific sequence variability is also reported to localize to other parts of the enzyme than the catalytic

cleft [10]. This may indicate that the primary substrate of ChiJ and Chi18-15/CHIT36 are of similar composition, presumably fungal cell wall material.

Instead, regions with high amino acid diversity and sites under positive selection in ChiJ coincided with coil regions that connect certain α -helices and β -strands in the family 18 chitinase TIM barrel structure. This result is not surprising as the basic architecture of the TIM barrel is highly conserved between species, while surface loops may vary considerably due to low selective constraints which results in accumulation of neutral mutations. However, some of these surface loops will also be determinants of enzyme properties and interactions with other proteins, and may thus be targets for diversifying selection. Therefore, a major challenge in studies of enzyme evolution is to determine if the observed sequence diversity between orthologs is due to low selective constraints or due to positive selection. Identification of positive selection in a gene strongly implies that these changes are the result from diversifying selection and that the involved sites are connected to protein function.

In the current work, regions I, II, IV, V, VI and VII all contain sites evolving under positive selection. Hence, the most valid interpretation is that the high amino acid diversity between *Streptomyces* species in these regions is the result from diversifying selection and may influence functional properties of ChiJ. The localization of the variable parts to certain coil-regions predicts changes in enzyme architecture between species. Another observation is that two pairs of co-evolving sites are located on opposite sides of the enzyme, indicating that they participate in the same functional domain, i.e. slightly deleterious mutations in the substrate side of ChiJ may be compensated by other mutations on the products side, or vice versa. The surface-exposure of all seven regions of high amino acid diversity may suggest that interactions with environmental factors are the cause of the observed interspecific variability. No single factor can be identified at this stage, but may include interactions with chitinase inhibitors that are produced from soil fungi [39], plants [40], or from *Streptomyces* itself [41]. ChiJ may also interact and evolve together with chitin-binding proteins that enhance the efficiency of the degradation process [42,43]. A comparison of the enzymatic activities of *S. coelicolor* ChiA, ChiC, ChiD and ChiF showed that both pH optima and hydrolytic activity against soluble or crystalline chitin differed substantially between the enzymes [6]. Sites evolving under positive selection in ChiJ are candidates for targeted mutagenesis to elucidate mutational effects on enzyme properties.

The comparison between ChiJ and its fungal orthologs identified two regions that display high amino acid diversity in combination with sites evolving under positive selection in *Streptomyces* but not in fungi (regions I

and IV), and one region of high sequence diversity in the fungal orthologs only (region A). This difference in amino acid conservation/diversity patterns is a hallmark of selection for type 1 functional divergence [44]; if the observed diversity in these regions is due to low selective constraints we would expect a similar level of diversity in both bacterial and fungal orthologs. Regions I and IV may thus represent areas on the ChiJ surface that contain structural features that specifically interact with environmental factors, such as inhibitors or chitin-binding helper proteins, present in the ecological niche of *Streptomyces* but not *Hypocrea*. Similarly, region A with high amino acid diversity between fungal orthologs may represent adaptations after the HGT event towards fungal preferences. In fact, this particular region was previously identified in *Hypocrea* chi18-15/CHIT36 to contain sites evolving under positive selection [10]. Furthermore, that study also shows discrete differences in amino acid conservation pattern between *Hypocrea* clades in this region; species in clades *Rufa* and *Pachybasioides* display high interspecific amino acid diversity in this region compared to a conserved interspecific pattern in other clades [10].

In summary, we show that several surface-exposed regions of *Streptomyces* chitinase ChiJ evolves rapidly and that this divergence is driven by selection for adaptive modifications. This shows that ChiJ function, whether it be interactions with soil fungi or nutrient acquisition, is important in the ecological niche of *Streptomyces* spp. We also identify three regions that display signs of type 1 functional divergence, where unique adaptations in the bacterial and fungal taxa are driven by positive selection. These results are noteworthy as it shows that different regions of the ChiJ orthologs have evolved under different selective constraints following the HGT event. The identified regions may thus influence functional properties of ChiJ.

Availability of supporting data

The data set supporting the results of this article is included within the article (and its additional files).

Additional files

Additional file 1: Protein structures used for structural alignment.

Additional file 2: Alignment of *Streptomyces* sp. ChiJ sequences.

Additional file 3: Predicted catalytic and substrate binding residues in ChiJ.

Abbreviations

HGT: Horizontal gene transfer; NCBI: National center for biotechnological information; PDB: Protein data bank; RCA: Reverse conservation analysis; REL: Random effects maximum likelihood; TIM: Triosephosphate isomerase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WU carried out homology modelling studies and helped to draft the manuscript. MK conceived of the study, carried out studies of molecular evolution and wrote the manuscript. Both authors read and approved the final manuscript.

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