CURRENT MICROBIOLOGY Vol. 45 (2002), pp. 303–306
DOI: 10.1007/s00284-002-3618-8

Current Microbiology
An International Journal

News & Notes

Conservation of *XYN11A* and *XYN11B* Xylanase Genes in *Bipolaris sorghicola, Cochliobolus sativus, Cochliobolus heterostrophus,* and *Cochliobolus spicifer*

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Received: 12 July 2001 / Accepted: 6 March 2002

Abstract. Two types of xylanase gene, *XYN11A* (*XYL1*) and *XYN11B* (*XYL2*), were amplified by PCR and partially sequenced in four phytopathogenic species of the ascomycete fungal genus *Cochliobolus* (anamorph genus *Bipolaris*). Three of the species, *C. heterostrophus* (*B. maydis*), *C. sativus* (*B. sorokiniana*), and *Bipolaris sorghicola* (no teleomorph known), are interrelated; the fourth, *C. spicifer* (*B. spicifera*), was found, through analysis of the 5.8S RNA and internal transcribed spacer (ITS) sequences of its ribosomal DNA, to be more distantly related to the other three. Isolates from all four species contain orthologous *XYN11A* and *XYN11B* genes, but a set of laboratory strains of *C. heterostrophus* gave no product corresponding to the *XYN11B* gene. The patterns of evolution of the two xylanase genes and ribosomal DNA sequences are mutually consistent; the results indicate that the two genes were present in the common ancestor of all *Cochliobolus* species and are evolving independently of each other.

Hemicelluloses of plant cell walls provide an important nutrient source for many microorganisms; cell walls of gramineous plants, in particular, have a high content of glucuronoarabinoxylans [4]. Breakdown of these polymers by microorganisms requires several enzymes, among which multiple endoxylanases are likely to have a major role [11, 14]. Differences in their activities could be important for their ability to use different substrates and for tissue and host specificity of pathogens. Although many xylanase genes from diverse microorganisms have been described, relatively little is known about patterns of xylanase gene evolution in groups of related species. The aim of the work reported here was to investigate the occurrence and evolution of xylanase genes in the genus *Cochliobolus* (anamorph genera *Bipolaris* and *Curvularia*). This genus includes several pathogens of gramineous plants. Three xylanase genes, called *XYL1, XYL2,* and *XYL3*, have been characterized in the maize pathogen *C. carbonum* [1, 2]. *XYL1* and *XYL2* code for typical members of glycoside (glycosyl) hydrolase family 11, whereas *XYL3* codes for an unusual enzyme that resembles family 11 members but does not have their standard amino acid "signature" [8]. The only other xylanase gene described in a *Cochliobolus* species is an ortholog of the *XYL2* gene of *C. carbonum* in the wheat and barley pathogen *C. sativus* [5]. The *XYL2* genes of *C. carbonum* and *C. sativus* are unusual in containing two introns; most family 11 xylanase genes, including *XYL1*, contain only one.

In accordance with current principles for naming glycoside hydrolases [9], we refer here to the products of the *XYL1* and *XYL2* genes as Xyn11A and Xyn11B and to the genes as *XYN11A* and *XYN11B*, respectively. We describe orthologs of the *XYN11A* (*XYL1*) and *XYN11B* (*XYL2*) genes of *C. carbonum* in four species: *Cochliobolus sativus*, the maize pathogen *Cochliobolus heterostrophus*, the sorghum pathogen *Bipolaris sorghicola* (no teleomorph reported), and *Cochliobolus spicifer*. *C. spicifer* is an occasionally serious pathogen of wheat and barely in warm climates [23] and an opportunistic human pathogen causing phaeohyphomycosis [12]. We have also examined the relationship of *C. spicifer* to other

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Cochliobolus species; this has not been reported previously.

Materials and Methods

Sources of DNA. *Cochliobolus sativus* isolates were from Scottish barley, obtained from J. Chard, Scottish Agricultural Science Agency, except that isolate YB1 was isolated in this laboratory from a Yemeni barley seed with black point disease [7]. *C. heterostrophus* isolate IMI146543 was from the International Mycological Institute (now part of CABI Bioscience), Egham, Surrey, UK, and near-isogenic *C. heterostrophus* laboratory strains B30.A3.R.1, B30.A3.R.65, and B30.A3.R.85 were from C.R. Bronson, Department of Plant Pathology, Iowa State University [20]. *Bipolaris sorghicola* was International Mycological Institute isolate IMI146555. The *C. spicifer* isolate, YW1, was isolated in this laboratory from a Yemeni wheat seed with black point disease [7].

DNA preparation and analysis. Fungi were grown in liquid CM medium [22]. Total DNA was extracted as described previously [5, 22]. Xylanase genes were amplified by PCR with primers described previously [5]; ribosomal internal transcribed spacer (ITS) regions and the 5.8S rRNA gene of *C. spicifer* were amplified by PCR by using standard fungal primers ITS4 and ITS5 [21]. Amplification was carried out with the Expand High Fidelity PCR Kit (Roche Diagnostics). PCR products were either subjected to direct sequencing or cloned in the pGEM®-T Easy (Promega) or pCR®-Blunt (Invitrogen) vectors. Nucleotide sequences were determined for both strands at the Molecular Biology Facility of the University of Newcastle.

Sequences were analyzed with programs in the EMBOSS package [15] and were aligned by using CLUSTAL [19]; alignments were adjusted manually with the aid of the SEAVIEW multiple sequence alignment editor [6]. For investigation of the relatedness of *C. spicifer* to other *Cochliobolus* species, the *C. spicifer* ITS/5.8S rDNA sequence was aligned to ITS/5.8S rDNA sequences for *Cochliobolus* and related genera [3] obtained from TreeBASE (http://herbaria.harvard.edu/treebase/; matrix accession number M523). Phylogenetic analysis was carried out by using PAUP* version 4.0b4a [17], and trees were drawn with the NJplot program [13]. The Kishino-Hasegawa test [10, 18] was used to compare support for different trees.

Results and Discussion

Phylogeny of *C. spicifer*. In order to determine the taxonomic position of *C. spicifer*, the sequence of its rDNA internal transcribed spacers and 5.8S rRNA gene was determined (EMBL accession number AJ303084) and fitted into the tree reported for *Cochliobolus* and related species by Berbee et al. [3]. *Cochliobolus* divides naturally into two groups; *C. sativus*, *C. heterostrophus*, and *B. sorokiniana* belong to group 1 [3]. Figure 1 shows that *C. spicifer* is a member of a cluster of species in group 2 of the genus. This cluster has 90% bootstrap support in parsimony analysis and 95% bootstrap support in distance analysis with the neighbor-joining method (not shown). The finding that *C. spicifer* belongs to group 2 is consistent with its morphological characteristics, notably the characteristics of its conidia [16]. The most serious pathogens in the genus *Cochliobolus* are in group 1 [3], but *C. spicifer* shares an ability to attack plants with several other group 2 species [16]. In being an opportunistic human pathogen, it resembles two of its close relatives, *C. australiensis* and *C. hawaiiensis* [12].

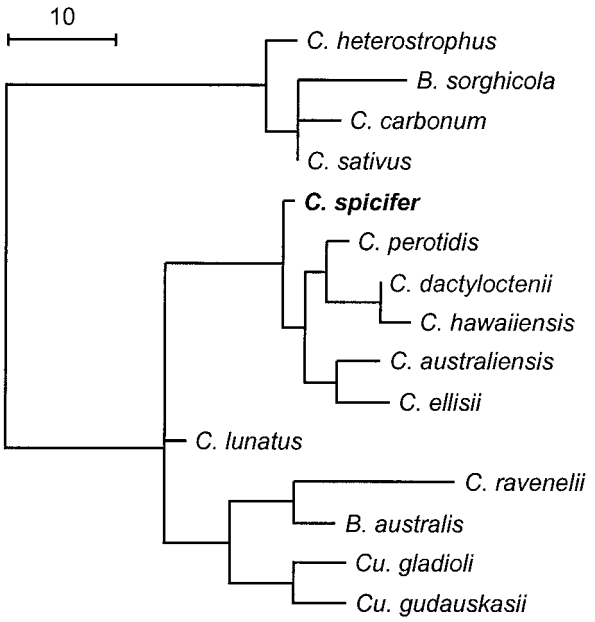

Fig. 1. Fast parsimony bootstrap tree for selected *Cochliobolus* (*C.*), *Bipolaris* (*B.*), and *Curvularia* (*Cu.*) species showing position of *C. spicifer*. The tree was constructed by using all taxa in TreeBASE alignment M523; then, all except those studied here and close relatives of *C. spicifer* were removed. Bootstrap values are not shown because they would pertain to the complete tree. The scale represents 10 nucleotide substitutions.

Xylanase genes in *Cochliobolus* (*Bipolaris*) isolates and species. A region of approximately 530 bp of the *XYN11B* gene, including its two introns, can be amplified by PCR with primers corresponding to sequences that are nearly identical in *C. sativus XYN11B*, *C. carbonum XYN11B*, and *C. carbonum XYN11A*, and with primer annealing at 62°C [5]. Figure 2 shows that when the annealing temperature is reduced to 52°C, two DNA fragments, about 530 and 470 bp in length, are amplified from DNA of *C. sativus*, *B. sorghicola*, and *C. heterostrophus* isolate IMI146543. DNA of *C. heterostrophus* laboratory strains B30.A3.R.1, B30.A3.R.65, and B30.A3.R.85 gives only the smaller band. DNA from other *C. sativus* isolates and from *C. spicifer* gives two bands (not shown).

DNA sequencing showed that the longer products (452–455 nucleotides between the primers) correspond to the *XYN11B* (*XYL2*) genes of *C. carbonum* and *C. sativus*, and the smaller products (395–398 nucleotides between the primers) correspond to the *XYN11A* (*XYL1*)

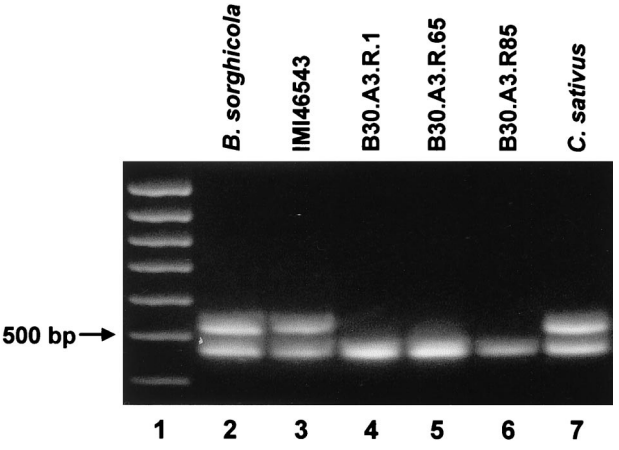

Fig. 2. PCR amplification products with xylanase gene primers from DNA of *Cochliobolus* (*Bipolaris*) species, analyzed by agarose gel electrophoresis. Lane 1, 100-bp ladder; lane 2, *Bipolaris sorokiniana*; lanes 3–6, *C. heterostrophus* isolate IMI146543 and strains B30.A3.R.1, B30.A3.R.65, and B30.A3.R85; lane 7, *C. sativus* isolate SB5.

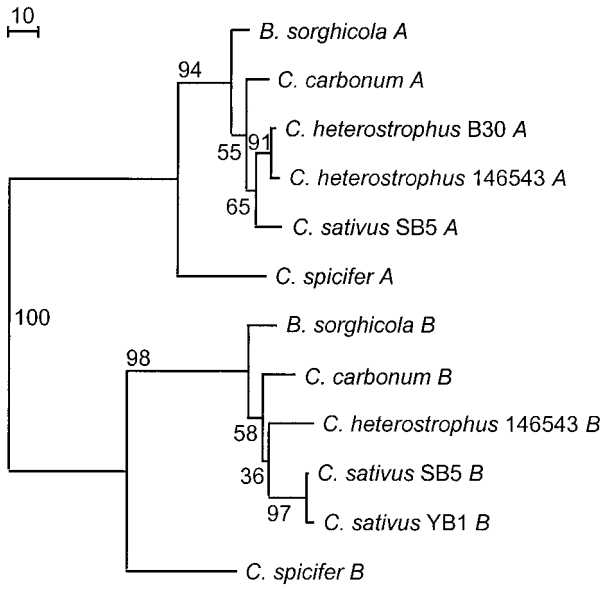

Fig. 3. Phylogenetic tree of *Cochliobolus XYN11A* and *XYN11B* genes, generated using parsimony analysis. The scale represents 10 nucleotide substitutions. Numbers on branches are bootstrap values. The alignment used to calculate the trees is available on request.

gene of *C. carbonum*.[1] Sequences of each type are similar throughout their lengths, implying that the genes have corresponding introns. All putative intron sequences begin with GT and end with AG, as expected. The most variation among sequences, including all variation in length, is in the intron regions. The *XYN11B* sequences from isolates SB2 and SB5 of *C. sativus* are identical, whereas the sequence from isolate YB1 differs from these at four positions. The *XYN11A* sequences from the three near-isogenic laboratory strains of *C. heterostrophus* are identical but differ at five positions from the *C. heterostrophus* isolate 146543 sequence.

The DNA sequences were translated, with the expected introns removed. The Xyn11A and Xyn11B amino acid sequences are the same length, but the two sequences in any one species are only about 67% identical. In contrast, sequences of each type differ from each other at no more than 6 positions out of 114. Amino acid differences between sequences of the same type are generally conservative, whereas several of those between types are nonconservative. Thus, there are likely to be functional differences between the types.

Since all the *Cochliobolus* species tested possess orthologs of the *XYN11A* and *XYN11B* genes first described in *C. carbonum*, both types of gene predate the genus *Cochliobolus* and have been conserved in this genus. PCR with DNA of a set of near-isogenic laboratory strains of *C. heterostrophus* gave only a *XYN11A* product. Because this could have been owing to a mutation in one of the sequences corresponding to the primers, despite their high conservation, we attempted to amplify DNA of *C. heterostrophus* strain B30.A3.R85 with an alternative, *XYN11B*-specific primer pair (RT-PCR primers [5]). In this case, too, no product was obtained, suggesting that the laboratory strains of *C. heterostrophus* have lost their *XYN11B* gene. Since these strains are pathogenic, the *XYN11B* gene is apparently dispensable for pathogenicity in *C. heterostrophus*. Similarly, deliberate disruption of either the *XYN11A* or the *XYN11B* gene in *C. carbonum* does not affect the pathogenicity [1, 2]. These genes are present in both strong and weak pathogens of different hosts and were probably present in the common ancestor of all *Cochliobolus* species. Thus, it is likely that the fundamental role of both gene products is in saprotrophic growth.

Evolution of *Cochliobolus* xylanase genes. Because of the similarity of their amino acid sequences, evolution of xylanase genes in *Cochliobolus* was analyzed at the nucleotide sequence level. Figure 3 shows the tree generated by parsimony analysis with bootstrap replication for all the *XYN11A* and *XYN11B* sequences, including those from *C. carbonum* [1, 2]. This tree shows identical patterns for divergence of the *XYN11A* and *XYN11B* genes. Where more than one *XYN11A* or *XYN11B* sequence was determined for a single species, the se-

[1] Accession numbers for the new sequences are: *XYN11A*, AJ297244 (*C. heterostrophus* IMI146543); AJ297246 (*C. heterostrophus* B30.A3.R1); AJ297248 (*C. sativus* SB5); AJ297245 (*B. sorghicola*); AJ297247 (*C. spicifer*); *XYN11B*, AJ303046 (*C. heterostrophus* IMI146543), AJ303047 (*C. sativus* YB1); AJ303048 (*B. sorghicola*); AJ303049 (*C. spicifer*).

quences cluster together. Bootstrap analysis gives 100% support to the separation of *XYN11A* and *XYN11B* genes and >90% support to separation of each of the *C. spicifer* sequences from the others. Support for the pattern shown within species group 1 is relatively weak.

The consistency among the patterns of divergence of the *XYN11A* and *XYN11B* genes and the ITS/5.8S rDNA sequences was evaluated. The 15 unrooted trees possible for five species were compared by using the *XYN11A* and *XYN11B* sequences both separately and concatenated to form a single sequence. Which tree is best depends on which sequence is used, but the differences are not statistically significant, according to the Kishino-Hasegawa test. Berbee et al. [3] studied *Cochliobolus* evolution by using rDNA and glyceraldehyde-3-phosphate dehydrogenase (*GPD*) gene sequences. They could not establish unambiguously the order of species divergence within group 1; this implies that this group evolved through a rapid radiation of species [3]. The results for the xylanase sequences, which provide better discrimination than ITS/5.8S rDNA sequences and similar discrimination to *GPD* sequences, support this view. The data are consistent with the arrangements of species in both Figs. 1 and 3 and with the hypothesis that the *XYN11A* and *XYN11B* genes have evolved independently of each other and together with the organisms that contain them.

ACKNOWLEDGMENTS

We thank C.R. Bronson, Iowa State University, for laboratory strains of *C. heterostrophus*; J. Chard, Scottish Agricultural Science Agency, for *C. sativus* isolates from Scottish barley; M.L. Berbee, University of British Columbia, for a preprint [3]; A.A. Hassan for Yemeni isolates; and S.E. Durham and K. Unwin for their contributions to determining sequences. This work benefited from the use of the UK Human Genome Mapping Project Resource Centre computing facilities, Hinxton, UK.

Literature Cited

1. Apel PC, Panaccione DG, Holden FR, Walton JD (1993) Cloning and targeted gene disruption of *XYL1*, a β1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol Plant-Microbe Interact 6:467–473
2. Apel-Birkhold PC, Walton JD (1996) Cloning, disruption, and expression of two endo-β1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. Appl Environ Microbiol 62:4129–4135
3. Berbee ML, Pirseyedi M, Hubbard S (1999) *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. Mycologia 91:964–977
4. Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47:445–476
5. Emami K, Hack E (2001) Characterisation of a xylanase gene from *Cochliobolus sativus* and its expression. Mycol Res 105:352–359
6. Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12:543–548
7. Hassan AA (1999) The interactions between Yemeni wheat and barley cultivars and isolates of *Cochliobolus sativus* and the role of root exudates and toxins in susceptibility and pathogenicity. PhD thesis, University of Newcastle upon Tyne
8. Henrissat B, Bairoch A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788
9. Henrissat B, Teeri TT, Warren RAJ (1998) A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants. FEBS Lett 425:352–354
10. Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J Mol Evol 31:151–160
11. Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456
12. McGinnis MR, Rinaldi MG, Winn RE (1986) Emerging agents of phaeohyphomycosis: pathogenic species of *Bipolaris* and *Exserohilum*. J Clin Microbiol 24:250–259
13. Perrière G, Gouy M (1996) WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78:364–369
14. Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13:101–131
15. Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16:276–277
16. Sivanesan A (1987) Graminicolous species of Bipolaris, Curvularia, Drechslera, Exserohilum and their Teleomorphs. Wallingford, UK: C.A.B. International
17. Swofford DL (2000). PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4.0b4a. Sunderland, MA: Sinauer Associates
18. Swofford DL, Olsen GJ, Waddell PJ, Hillis DM (1996) Phylogenetic inference. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics, 2nd edn. Sunderland, MA: Sinauer Associates, pp 407–514
19. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
20. Tzeng TH, Lyngolm LK, Ford CF, Bronson CR (1992) A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. Genetics 130:81–96
21. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. San Diego, CA: Academic Press, pp 315–322
22. Yoder OC (1988) *Cochliobolus heterostrophus*, cause of Southern corn leaf blight. Adv Plant Pathol 6:93–112
23. Zillinsky FC (1983) Common diseases of small grain cereals: a guide to identification. Mexico, D.F.: CIMMYT