Genetics and Molecular Biology, 23, 3, 685-688 (2000)

Short Communication

Cloning of a chitinase gene from *Ewingella americana*, a pathogen of the cultivated mushroom, *Agaricus bisporus*

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Abstract

We have isolated a gene encoding a chitinase (EC 3.2.1.14) from *Ewingella americana*, a recently described pathogen of the mushroom *Agaricus bisporus*. This gene, designated *chi*A (EMBL/Genbank/DDBJ accession number X90562), was cloned by expression screening of a plasmid-based *E. americana Hin*dIII genomic library in *Escherichia coli* using remazol brilliant violet-stained carboxymethylated chitin incorporated into selective medium. The *chi*A gene has a 918-bp ORF, terminated by a TAA codon, with a calculated polypeptide size of 33.2 kDa, likely corresponding to a previously purified and characterised 33-kDa endochitinase from *E. americana.* The deduced amino acid sequence shares 33% identity with chitinase II from *Aeromonas* sp. No. 10S-24 and 7.8% identity with a chitinase from *Saccharopolyspora erythraeus*. Homology to other chitinase sequences was otherwise low. The peptide sequence deduced from *chi*A lacks a typical *N*-terminal signal sequence and also lacks the chitin binding and type III fibronectin homology units common to many bacterial chitinases. The possibility that this chitinase is not primarily adapted for the environmental mineralisation of pre-formed chitin, but rather for the breakdown of nascent chitin, is discussed in the context of mushroom disease.

INTRODUCTION

Ewingella americana has been shown to be associated with a browning disorder of the stipe of the mushroom *Agaricus bisporus*, called internal stipe necrosis (Inglis *et al.*, 1996). Chitin is a vital component of the cell walls of the majority of fungi (Cabib, 1987). In the stipe cells of *A. bisporus*, chitin is present in a diffuse form that has been shown to be highly susceptible to the action of endochitinases (Mol and Wessels, 1990; Mol *et al.*, 1990). This structure is thought to be necessary during the rapid extension of the stipe during fructification. Strains of *E. americana* infecting mushroom stipes are chitinolytic, although these bacteria were unable to grow on chitin as a sole source of carbon (Inglis and Peberdy, 1996a). Degradation of chitin by this organism is caused by a single, constitutively produced 33-kDa endochitinase, which has been purified from *E. americana* strain PI98 (Inglis and Peberdy, 1996b). It was hypothesized, therefore, that this chitinase may be implicated in the pathogenesis of internal stipe necrosis.

The majority of bacterial chitinase genes have been cloned by screening plasmid-based genomic libraries in *Escherichia coli* plated on media incorporating colloidal chitin. Such an approach has proven to be effective in cloning chitinases from Enterobacteriaceae such as *S. marcescens* (Fuchs *et al.*, 1986) and other gram-negative bacteria such as *Aeromonas hydrophila* (Roffey and Pemberton, 1990) and *Vibrio vulnificus* (Wortman *et al.*, 1986). Molecular genetics would allow us to understand more about the significance of chitinase in mushroom disease, and therefore, an attempt was made to clone this gene from *E. americana* strain PI98.

MATERIAL AND METHODS

Strains

E. coli DH5α was used for propagation of all recombinant plasmids. The chitinolytic mushroom-derived strain of *E. americana* PI98 (Deposited in the UK National Collection of Plant Pathogenic Bacteria as NCPPB 3905) was used as the source of genomic DNA for library construction.

Library construction and screening of clones

Genomic DNA (1 μg) from *E. americana* strain PI98 was digested to completion with *Hin*dIII and the products ligated to *Hin*dIII digested pBluescript II sk+ (Stratagene; 0.5 μg). Ligation products were then used to transform *E. coli*, which was plated on LB agar supplemented with 50 μg/ml ampicillin, 20 μg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) and 0.2 mM IPTG (isopropyl-β-D-thiogalactoside) and incubated overnight. White recom-

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     ctgggctacgaggggcacgtggtctcttccgcgctctgttcgccttccacgaaatgaccc   60

     tgccagatagctacggcgacccggtaaaccagttctgcgatcagcccgattttcaccgcc   120

     gcgcgaccgctttgcgctttcacgccctaatggcggctatgatctggtggcggcgatgat   180

     tgagcagggcgcgcaccacgtcttcgccccgcgttcaccagtaatgggataaacatttaa   240

     aactatctaactgttttatatcgttttaattttgatgctgttgtgccagcggtgctacta   300

     ccctgatcgaatgcatcaaaaaaggttgtaggagaagatgactttgccgccggagaaggc   360

     cccggcgtaaacgttcttacctacataaaaaggatataaaacATGGCTACTTTATATACC   420
  1                                                  M  A  T  L  Y  T

     CCTTACATTGATGTGTCCCTGAATGCGCTGTGGAGTGACTGGCAGAACTACCCTAAAGGT   480
  7  P  Y  I  D  V  S  L  N  A  L  W  S  D  W  Q  N  Y  P  K  G

     CGCCCAAACCCAATTTACTCTCAACAGGCGATTTCTTACGGCGTTGATGGTCTGGTGCTG   540
 27  R  P  N  P  I  Y  S  Q  Q  A  I  S  Y  G  V  D  G  L  V  L

     GGCTTCCTGACACTTTCCCCGTCGAATAAAGCCTGCTGGGCCGCCTCCGACGCCATGCCG   600
 47  G  F  L  T  L  S  P  S  N  K  A  C  W  A  A  S  D  A  M  P

     CTGGAATGGGCGCTGCCGCTGGCAAACGAGCTGAACGCGGCAAACCGTCAGGTAATTGTC   660
 67  L  E  W  A  L  P  L  A  N  E  L  N  A  A  N  R  Q  V  I  V

     TCCTTCGGCGGCGCGTCTAACGCCGATATCTCCACCAAGTTCACCGTTGACCAGCTGGTA   720
 87  S  F  G  G  A  S  N  A  D  I  S  T  K  F  T  V  D  Q  L  V

     CAGACTTACACCGACGTTGTGCAGAAGTTTAAAGCCAAGCAGTTGGACTTCGACTTGGAA   780
107  Q  T  Y  T  D  V  V  Q  K  F  K  A  K  Q  L  D  F  D  L  E

     AACGGTCAGTACGACTACAACAAAATCAGCTCAGCCCTGGCGATTTTCCAGAAGAGCAAC   840
127  N  G  Q  Y  D  Y  N  K  I  S  S  A  L  A  I  F  Q  K  S  N

     CCATCGGTTCGCATCAGCTTTACGCTGCCAACCATGCCAGCAGGTTTGGTCACTGAAGGT   900
147  P  S  V  R  I  S  F  T  L  P  T  M  P  A  G  L  V  T  E  G

     CTGAACATCATTCAGAGCGCCAAAAATGCCGGTGTGAAGTTCAGCGTAAACGGTATGGCG   960
167  L  N  I  I  Q  S  A  K  N  A  G  V  K  F  S  V  N  G  M  A

     ATGGACTATAACGACGGCGTTTCCAGCCAAGATATGGGTAAAGCAGCGACCAGCGCGGCA   1020
187  M  D  Y  N  D  G  V  S  S  Q  D  M  G  K  A  A  T  S  A  A

     ACCAGCATCAAAACCCAGCTGGGCAAACTTTATCCGGGCCAGACTGACGCACAGCTTTAC   1080
207  T  S  I  K  T  Q  L  G  K  L  Y  P  G  Q  T  D  A  Q  L  Y

     CCACTGGTAGCCATCACCCCGATGATCGGTCTGAACGACGACACCACCGAGATGTTCAAA   1140
227  P  L  V  A  I  T  P  M  I  G  L  N  D  D  T  T  E  M  F  K

     CTGCCAGACGCCACCACCGTTGCCACTTTCGCCAAGCAGAAAAACATGGCGTTTATCGGT   1200
247  L  P  D  A  T  T  V  A  T  F  A  K  Q  K  N  M  A  F  I  G

     AGCTGGTCGTTTAACCGCGACAACCCATCGAGCTACACCTACGTTGATTTGCAGACCAGC   1260
267  S  W  S  F  N  R  D  N  P  S  S  Y  T  Y  V  D  L  Q  T  S

     AGTAACCCGGCGCAGAAAGCCTCCGGTGATTATGCGAAGACGTTTTTAGCGGGTATTCGT   1320
287  S  N  P  A  Q  K  A  S  G  D  Y  A  K  T  F  L  A  G  I  R

     TAAggggaaatagtgggcagggaggcccacagctcctgtttagatctttaaattcaaaaa   1380
307  *
     ccgtgggctcgccgcccacacttaggttttttaaattcaaaaccgtgggctcgccgccca   1440
       >>>>>>     <<<<<<                        >>>>>>     <<<<<<
     cacttaggtcttttaaattcaaaaccgtgggctcgccgcccacacttaggtctttaaatt   1500
     <                          >>>>>>     <<<<<<
     caaaaccgtgggctcgccgcccacactggcccaaaggtttaaacgaaacccttggaat   1560
             >>>>>>     <<<<<<
     cctgcgtttttttgttgttgtaagatcgaggcttcgctgggttgggatggacatgtttca   1620

     gcagcttctatg                                                   1632
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Figure 1 - Nucleotide and deduced amino-acid sequences of the *E. americana chi*A gene. The translated region of the gene is depicted in upper case letters. Four direct repeats downstream of the TAA stop codon (marked with an asterisk) are underlined and a palindromic sequence within the direct repeats is indicated by chevrons. This sequence is deposited in EMBL/Genbank/DDBJ with accession number X90562.

binant colonies were screened for chitinase production by overnight incubation on LB agar supplemented with ampicillin and sterile remazol brilliant violet-stained carboxymethylated chitin (Wirth and Wolf, 1990) (CM-chitin-RBV, 5 mg/ml).

Subcloning and sequence analysis

Direct plating of primary transformants was found to be possible on this medium and was used to select chitinase-positive subclones. Chain termination sequencing of both strands of clone pPI1 was carried out using the Sequenase 2.0 kit (Amersham) and [α-^{35}S]dATP as label. DNA fragments were subcloned and regions lacking suitable restriction sites were sequenced using primers synthesized according to previously determined sequence data. Sequence data were compared to published chitinase sequences using FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) searches of the EMBL and SWISSPROT databases, respectively. More detailed analysis was carried out with the Wisconsin Genetics Computer Group GCG suite of programs and the Staden suite and Clustal W v.1.6 (Thompson *et al.*, 1994). All programs were used with default settings.

RESULTS AND DISCUSSION

A chitinase-positive clone containing an insert of 10 kb was selected from the *E. americana* PI98 *Hin*dIII genomic library. This insert was subcloned to a 2.2-kb *Bam*HI-*Xho*I fragment (pPI1) that produced large clearing zones on CM-chitin-RBV after overnight incubation. Sequencing of pPI1 revealed a 918-bp ORF with an ATG start codon and TAA stop (Figure 1). A putative Shine-Dalgano sequence (AGGA) identified 9 nucleotides in the 5' direction from the ATG codon, which is consistent with the general range (7 to 11 nucleotides) found in prokaryotes (Gold *et al.*, 1981) . Motifs exactly matching those of *E. coli* consensus promoters could not be found upstream of the Shine-Dalgano site. No other chitinase-like ORFs were detected in the sequence of pPI1.

Sequences downstream of the TAA stop codon demonstrated a highly unusual structure of four direct repeats. Within this repeated motif was a palindromic element, potentially forming a stem-loop structure. However, there is no extensive poly-thymidine element immediately following such structures, which would be typical of bacterial *Rho*-independent transcriptional terminators. The first direct repeat contains an additional adenosine, when compared to subsequent repeats. The spacer bases between repeats were also found to vary slightly; so that the first spacer reads TT, the second, CT and the third, C. The significance of this structure to chitinase or downstream gene regulation warrants further investigation. Sequences downstream from the TAA stop, and translated in all three reading frames, showed no similarity to any published chitinase sequence.

The *chi*A gene would code for a polypeptide of 306 amino acids with a molecular weight of 33.2 kDa, corresponding closely to a 33-kDa chitinase previously purified from *E. americana* (Inglis and Peberdy, 1996b). The *E. americana* chitinase is most homologous to chitinase II from *Aeromonas* sp. (Ueda *et al.*, 1994; 33% identity) and the chitinase from *Saccharopolyspora erythraeus* (Kamei *et al.*, 1989; 7.8% identity), but otherwise displays very low overall similarity to other chitinases. The characteristic aspartic and glutamic acid-containing catalytic motifs of chitinase-like proteins, thought to promote the acid hydrolysis of glycosidic bonds (Henrissat, 1990; Gilkes *et al.*, 1991), however, are also present in the *E. americana* sequence.

Analysis with the GCG *Signal Scan* program did not predict the presence of a typical N-terminal signal peptide (von Heijne, 1983) in the *E. americana* chitinase. This is also true of chitinase B of *Serratia marcescens*-BLJ200, which is secreted into the periplasm *in vivo*, but remains in the cytoplasm when expressed in *E. coli* (Bruberg *et al.*, 1994). The *E. americana* enzyme, however, appears to be readily secreted in *E. coli*, as indicated by the overnight production of large clearing zones on chitin agar.

Most bacterial chitinases appear to possess several distinct domains. These include the catalytic domain, at least one chitin-binding domain, and in some instances, one or more copies of the type III homology unit of fibronectin (Watanabe *et al.*, 1993) . By comparison, only the catalytic domain is present in the *E. americana* chitinase. The chitin-binding domains of chitinases promote affinity towards insoluble forms of chitin. Deletion of this domain in the chitinase A1 of *Bacillus circulans* reduced by half the activity against colloidal chitin (Watanabe *et al.*, 1994). Deletion of the type III units from this enzyme did not affect colloidal chitin binding, although it did reduce activity by about half. The hydrolysis of soluble carboxymethylated chitin was unaffected, indicating that the function of the type III units is to promote efficient hydrolysis of insoluble chitin once the enzyme becomes bound via the binding domain.

It has been demonstrated that the chitin in elongating mushroom stipe hyphae is diffuse and not well crystallized, leading to a high susceptibility to degradation by chitinases (Mol and Wessels, 1990) . The simple domain structure of the *E. americana* chitinase may correlate with a presumed role as a virulence factor in mushroom disease, since a chitin-binding domain and type III fibronectin homology units in the *E. americana* chitinase are probably not important for the efficient hydrolysis of chitin prevalent in the mushroom stipe.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of the BBSRC and Middlebrook Mushrooms for provision of a PhD studentship for P.W.I. We would also like to thank Dr. Cléria Valadares-Inglis and Dr. Cas Kramer for invaluable discussions and help during this work.

RESUMO

O gene que codifica uma quitinase (EC 3.2.1.14) foi isolado de *Ewingella americana*, recentemente descrita como patógeno do cogumelo *Agaricus bisporus*. Este gene, denominado *chi*A (EMBL/Genebank/DDBJ número de acesso X9061), foi clonado e selecionado a partir de livraria genômica construída por digestão do DNA de *E. americana* com *Hin*dIII e ligação em plasmídio de expressão em *E. coli*, utilizando meio seletivo contendo quitina carboximetilada, corada com "remazol brilliant violet" para seleção de clones. O gene *chi*A apresenta uma ORF de 918 bp, código terminador TAA, tendo o tamanho do polipeptídeo sido calculado como 33,2 kDa, o qual corresponde ao tamanho de 33 kDa da endoquitinase previamente purificada de *E. americana*. A seqüência deduzida de aminoácidos apresenta 33% de identidade com a quitinase II de *Aeromonas* sp. No. 10S-24 e 7,8% de identidade com quitinase de *Saccharopolyspora erythraeus*. Baixa homologia com outras quitinases foi observada. A seqüência deduzida de aminoácidos de *chi*A não apresenta sinal típico de *N*-terminal e também não apresenta típico sítio de ligação com quitina nem unidades de homologia à fibronectina do tipo III, comuns a muitas quitinases bacterianas. Existe a possibilidade de que esta quitinase não seja primariamente adaptada para mineralização de quitina pré-formada no ambiente, sendo discutida a digestão e quebra de quitina nascente, no contexto de doenças de cogumelos.

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(Received May 18, 2000)