Purification and properties of cyanide hydratase from *Fusarium lateritium* and analysis of the corresponding *chy1* gene

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The filamentous fungus *Fusarium lateritium* is cyanide tolerant, due, at least in part, to the induction by cyanide of the enzyme formamide hydrolyase (EC 4.2.1.66). This enzyme, more commonly known as cyanide hydratase, catalyses the hydration of cyanide to formamide. The enzyme was purified from *F. lateritium* and showed a subunit molecular mass of 43 kDa (as judged by SDS-PAGE), while the native protein appeared to form aggregates of up to 1217 kDa (as judged by gel-filtration and non-denaturing PAGE). mRNA samples from cultures grown with and without cyanide were *in vitro* translated and immunoprecipitated. This demonstrated that, in this species, the gene encoding the enzyme designated *chy1*, is cyanide inducible. Differential screening was used to isolate a cyanide hydratase cDNA clone which was subsequently used to obtain the corresponding genomic clone. A fragment of the cDNA clone encoding all but the first seven amino acids of the protein was expressed in *E. coli* using the expression vector pGEX-2T. Features of *F. lateritium* cyanide hydratase together with an analysis of the nucleotide sequence encoding this enzyme are presented.

Introduction

Despite the extreme toxicity of cyanide, many micro-organisms can grow in its presence either by developing a cyanide-insensitive respiratory system and/or degrading the cyanide (reviewed by Knowles, 1976, 1988). In at least one micro-organism, *Pseudomonas fluorescens*, cyanide can be used as the sole nitrogen source (Harris & Knowles, 1983). Many fungi, particularly those which are pathogens of cyanogenic plants, are cyanide tolerant, and this is, at least in part, due to the presence of the enzyme formamide hydrolyase (EC 4.2.1.66), more commonly known as cyanide hydratase (Fry & Millar, 1972). Cyanide hydratase activity appears to be correlated with the ability of some fungi to infect cyanogenic plants despite the release of hydrogen cyanide into the infection site in potentially toxic concentrations (Fry & Munch, 1975; Fry & Evans, 1977). In one study, fungal pathogens of cyanogenic plants were found to contain

various levels of cyanide hydratase activity (Fry & Evans, 1977). Enzyme activity in those species tested was inducible, although in some species low constitutive levels of cyanide hydratase activity were also seen. In general, lower levels of cyanide hydratase activity were seen in those species not regarded as pathogens of cyanogenic plants.

Cyanide hydratase enzyme has been studied in two phytopathogenic fungi: *Gloeocercospora sorghi* and *Stemphylium loti* (Fry & Millar, 1972; Fry & Munch, 1975). The enzymes appear similar, giving optimal activity in the range pH 7 to pH 9. The *G. sorghi* enzyme has a K_m of approximately 30 mM-hydrogen cyanide and an estimated molecular mass of $2\text{--}10 \times 10^6$ Da.

The work presented here describes the purification and biochemical analysis of the cyanide hydratase enzyme and the cloning and sequencing of the cyanide hydratase gene from *Fusarium lateritium* Nees (Commonwealth Mycological Institute 300533). This species is used in the production of the cyanide-degrading product CYCLEAR by ICI Biological Products.

Methods

Microbial strains, vectors and recombinant DNA. These are listed in Table 1.

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession nos M99045 (genomic sequence) and M99046 (cDNA sequence).

Table 1. Microbial strains, vectors and recombinant DNA used in this study

	Comment	Origin or reference
<i>F. lateritium</i>	Wild-type Nees (CMI 300533)	ICI Biological Products, Billingham, Cleveland, UK
<i>E. coli</i> Y1088	Used in cDNA library construction	Young & Davis (1983b)
<i>E. coli</i> NM514	Used in genomic library construction	Murray (1983)
<i>E. coli</i> JM83	Host for pUC plasmids/recombinants	Vieira & Messing (1982)
<i>E. coli</i> XL-1 Blue	Host for expression studies	Bullock <i>et al.</i> (1987)
λ NM1149	λ b538 <i>srl</i> λ 3 ⁰ <i>limm</i> ⁴³⁴ <i>shindIII</i> 6 ⁰ <i>red</i> ⁺ <i>gam</i> ⁺	Murray (1983)
λ gt11	<i>lac</i> -5 (<i>shindIII</i> 12-3) <i>srl</i> λ 3 ⁰ <i>nin</i> -5 <i>cI</i> 857 <i>srl</i> λ 5 ⁰ <i>Sam</i> 100	Young & Davis (1983a)
pUC9	Ap ^r <i>lacZ'</i>	Vieira & Messing (1982)
pUC18	Ap ^r <i>lacZ'</i>	Yanisch-Perron <i>et al.</i> (1985)
pCH1	Cyanide hydratase genomic clone	This study
pMC211	Cyanide hydratase cDNA clone	This study
pMC311	Cyanide hydratase cDNA clone (full length)	This study
PMC312	Truncated cDNA clone for expression studies	This study
pGEX:2T	Bacterial plasmid expression vector	Pharmacia
pGEX312	<i>chyl</i> cDNA correct orientation for expression	This study
pGEX313	<i>chyl</i> cDNA incorrect orientation for expression	This study

Cyanide hydratase enzyme assay. Samples (5–100 μ l) were incubated in 1 ml 120 mM-NaCN/100 mM-Tris/HCl buffer, (pH 8.5, 20 °C) for 15 min. The reaction was stopped with 2 ml 2.3 M-hydroxylamine and samples incubated for 7 min at 20 °C. After cooling on ice, 1 ml 4 M-HCl was added followed by 1 ml 1.23 M-FeCl₃/75 mM-HCl. Absorbance at 540 nm was used to estimate formamide concentration by means of a standard curve (Snell & Snell, 1954). Activity is expressed in μ mol min⁻¹ (U).

Enzyme purification. Culture was collected over a 48 h period from a laboratory chemostat (growth conditions as described by Richardson & Clarke, 1987) and maintained at 4 °C. Cells were harvested by vacuum filtration through muslin, and were then washed and resuspended in 100 mM-Tris/HCl buffer (pH 8.5) containing 10 mM-EDTA. Batches of cell suspension were mixed with glass beads (50 ml; 1 mm diam.) and disrupted in a bead mill (3 min) at 4 °C. The beads were removed and washed by decanting. The combined crude extract was centrifuged (17000 g, 15 min, 4 °C) to obtain the crude supernatant. Crude supernatant was stirred with 120 g DEAE-cellulose (equilibrated with buffer, as above) for 75 min at 4 °C. The gel was recovered by vacuum filtration, washed with buffer, packed into a column (5 $\times$ 16 cm) and eluted with a salt gradient, 0–4 M-NaCl in buffer. Active fractions were pooled to yield the DEAE eluate. The DEAE eluate was brought to 40% saturation with ammonium sulphate by addition of crystals. The supernatant was recovered by centrifugation (as above) and brought to 60% saturation. The pellet was recovered by centrifugation (as above) and resuspended in buffer. This was desalted using a Sephadex G-25 column eluted with 20 mM-Tris/HCl buffer (pH 8.5) containing 10 mM-EDTA. The desalted 60% ammonium sulphate fraction was loaded onto an anion-exchange column (LKB FPLC system Mono Q, HR 16/10 column) and eluted with a 0–0.4 M-NaCl gradient. The active fractions were pooled and loaded onto a Sephacryl S-400 column (1.6 $\times$ 46 cm, bed volume 92.5 ml) and eluted with buffer (as above, 5 ml h⁻¹). The column was calibrated using the following standards: blue dextran (mol. mass 2 $\times$ 10⁶ Da); ribonuclease; ovalbumin; albumin; aldolase; catalase; ferritin; thyroglobulin, (Pharmacia gel-filtration calibration kit).

Gel electrophoresis. PAGE with and without SDS was carried out using a Pharmacia Phast system using standard development and staining methods. Molecular mass standards were Pharmacia high or low molecular mass kits. SDS-PAGE for *in vitro* translation, immunoblotting and immunoprecipitation was done using 16 $\times$ 20 cm

12.5% (w/v) polyacrylamide slab gels and Sigma Delta VII-L molecular mass markers. Immunoblotting was done using standard methods as described in Harlow & Lane (1988).

RNA analysis. Total RNA was prepared by the method of Chambers & Russo (1986) except that 5 g of frozen mycelia were used as the starting material. mRNA was purified by two cycles of oligo-dT-cellulose chromatography according to Maniatis *et al.* (1982). Samples were quantified by absorbance at 260 nm. Purified mRNA (2.5 μ g) was *in vitro* translated by the method of Pelham & Jackson (1976) using a rabbit reticulocyte lysate (Amersham) and L-[³⁵S]methionine (Amersham). Samples (5 μ l) were removed and solubilized in SDS-PAGE sample loading buffer (30 μ l) [60 mM-Tris/HCl pH 6.8, 5% (w/v) SDS, 1% (w/v) dithiothreitol, 20% (v/v) glycerol, 0.001% bromophenol blue]. Samples were heat denatured at 100 °C before loading onto a 12.5% SDS-PAGE gel via a 5% stacking gel. Gels were dried then exposed to X-ray film (Fuji RX) for periods of 12–72 h.

Immunoprecipitation of *in vitro* translated proteins was done using the method described by Jagus (1987). Anti-cyanide hydratase antiserum was raised in rabbits and supplied by ICI Pharmaceuticals Division, Alderley Park, Cheshire, UK.

cDNA library construction. cDNA was prepared using mRNA isolated from *F. lateritium* culture obtained 57 min post-cyanide induction using a cDNA synthesis kit (Pharmacia) that uses a method adapted from Gubler & Hoffman (1983). *Eco*RI adaptors were ligated to the blunt-ended cDNA for ligation into *Eco*RI-restricted λ gt11 DNA (Young & Davis, 1983a). Phage were packaged *in vitro* according to Maniatis *et al.* (1982). The cDNA library contained 2 $\times$ 10⁵ recombinant phage and was amplified in *E. coli* Y1088 (Young & Davis, 1983b) using the plate lysate method (Maniatis *et al.*, 1982).

Genomic library construction. Total *F. lateritium* DNA was prepared by the method of Raeder & Broda (1985) using approximately 0.2 g of frozen mycelia. Samples of DNA were restricted to completion with *Hind*III for cloning into the bacteriophage λ insertion vector λ NM1149 (Murray, 1983). Phage were packaged *in vitro* as before and recombinants were selected by infection of *E. coli* NM514 (Murray, 1983). The gene library containing approximately 1 $\times$ 10⁶ recombinant phage.

Differential screening. Single-stranded cDNA probes were prepared from mRNA from *F. lateritium* which had been exposed to cyanide for 57 min, and also from *F. lateritium* which had not been exposed to cyanide. These probes were termed induced and uninduced probes

respectively. Probes were prepared essentially by the method used for the production of 1st strand cDNA described in the Pharmacia kit. Specific activities of cDNA probes were typically $1-2 \times 10^7$ c.p.m. per μg of mRNA reverse transcribed. Probes were denatured before use by heating at 95°C for 3 min.

For differential screening, four nitrocellulose filters (Schleicher and Schuell) were prepared from each plate containing recombinant phage (Benton & Davis, 1977). Filters were prehybridized for at least 1 h in prehybridization buffer [$6 \times \text{SSC}$, 0.1% SDS, 0.25% dried milk powder (Marvel)] at 65°C . Duplicate filters from each plate were hybridized overnight at 65°C with induced and uninduced cDNA probes in hybridization buffer ($3 \times \text{SSC}$, 0.1% SDS, 0.02% Ficoll, 0.02% polyvinylpyrrolidone). Filters were washed in three changes of wash buffer ($2 \times \text{SSC}$, 0.1% SDS), preheated at 65°C . Exposure of filters to X-ray film was from overnight to 72 h with two Cronex Lightening Plus intensifying screens.

Oligonucleotide probing. The oligonucleotide solution used was a mixture of 256 different oligonucleotides (20-mers) synthesized to ensure all possible sequences of the heptapeptide Ala Phe Pro Glu Val Trp Ile (the last base of Ile was omitted). This sequence was determined from random sequencing of a partial hydrolysis of pure cyanide hydratase. The oligonucleotide mixture was supplied by ICI Pharmaceuticals Division at a concentration of $67.2 \mu\text{g ml}^{-1}$ as judged by absorbance at 260 nm. The oligonucleotides were end-labelled with [^{32}P]dATP (Amersham) using T4 polynucleotide kinase according to Maniatis *et al.* (1982) and gave specific activities of 2×10^8 c.p.m. per μg DNA.

Filters were pre-hybridized at 65°C for 3 h in $6 \times \text{NET}$ (90 mM-Tris/HCl, pH 8.3, 0.9 M-NaCl, 6 mM-EDTA), 0.1% SDS, $5 \times \text{Denhardt's}$ solution, 100 mg calf thymus DNA ml^{-1} . This solution was replaced with $6 \times \text{NET}$, 0.1% SDS, $5 \times \text{Denhardt's}$ solution and 6 ng end-labelled oligonucleotide ml^{-1} . A 1 ml vol. of hybridization solution was used per filter and hybridization was at 44°C for 2 h. The hybridization temperature was calculated from Wallace & Miyada (1987). Filters were washed in $6 \times \text{SSC}$, 0.1% SDS at 4°C with four changes of buffer for 5 min each then for 5 min at the hybridization temperature. Filters were exposed to X-ray film overnight.

Expression studies. The expression vector pGEX-2T was used for expression in *E. coli* (Smith & Johnson, 1988). In pGEX-2T, sequences to be expressed are fused to the glutathione *S*-transferase gene linked to the strong *tac* promoter. Crude extracts for protein analysis were prepared by growing cultures to mid-exponential phase before adding IPTG to 1 mM. The cultures were then grown for a further 2 h at 37°C . A 1.5 ml vol. of culture was pelleted by centrifugation in a microfuge for 3 min; the pellet was then resuspended in 50 μl SDS-PAGE sample buffer and boiled for 2 min. Samples (15 μl) were run on SDS-PAGE gels.

DNA manipulations. Standard methods were used for Southern blotting, nick-translation, phage and plasmid DNA isolations, gel electrophoresis, subcloning and restriction analysis (Maniatis *et al.*, 1982). DNA sequencing was by the dideoxy chain termination method of Sanger *et al.* (1977) adapted for double-stranded DNA by Chen & Seeburg (1985). Non-standard sequencing primers were synthesized on an Applied Biosystems 381A DNA synthesiser at the Department of Biological Sciences, University of Durham, UK.

Results and Discussion

Induction of cyanide hydratase activity

Cyanide hydratase activity was induced by addition of sodium or potassium cyanide to continuously grown culture (Richardson & Clarke, 1987). In a typical

fermentation, *F. lateritium* was grown at a dilution rate of 0.08 h^{-1} and a cell dry wt of 12.0 g l^{-1} . No activity was detected in cells before exposure to cyanide, [i.e. $< 0.02 \text{ U (mg dry wt)}^{-1}$]. Sodium cyanide (5 M) was added to the culture at 0.85 mM h^{-1} for 50 min. It should be noted that the considerable 'dead volume' of the addition system resulted in the first indication of cyanide in the fermenter at 37 min (detection of hydrogen cyanide by exhaust gas analyser). Cyanide addition was ceased in response to this observation. The RNA preparations from which cyanide hydratase mRNA was isolated (see below) were made from mycelium taken at 57 min. Cyanide hydratase activity was detected after 71 min. After the initial detection of activity, the cyanide addition rate was increased in steps. The maximum steady state activity obtained was $102.5 \text{ U (mg dry wt)}^{-1}$ obtained over a 36 h period during which $6.0-6.4 \text{ mM h}^{-1}$ of cyanide was added.

Enzyme purification

The results of a typical purification are shown in Table 2. The culture used in this experiment was obtained by induction under conditions similar to those described above but on a smaller scale. The starting material was 15.8 l containing 74.9 g dry wt of mycelium having an activity of $19.6 \text{ U (mg dry wt)}^{-1}$. The purified enzyme eluted from the gel-filtration column as a single peak and formed one major band on SDS-PAGE. The purification factor of 19.8 (Table 2) indicates that the enzyme formed 5% of the soluble protein in these cells. This suggests that in the maximally induced cells described above [activity $102.5 \text{ U (mg dry wt)}^{-1}$] around 25% of the soluble protein was cyanide hydratase (the soluble protein was 22% of the total dry wt).

Enzyme properties

The K_m for cyanide was found to be 43 mM. Rapid inactivation by cyanide was observed at concentrations over 150 mM. Formamide was found not to be a substrate and did not inhibit the hydration of cyanide. The pH profile showed an optimum at pH 8.5, the decline at high

Table 2. Purification of cyanide hydratase from *F. lateritium*

Fraction	Specific activity (U mg^{-1})	Purification factor
Crude lysate	55.9	1
17000 g supernatant	87.8	1.5
DEAE-bound fraction	354	6.3
$(\text{NH}_4)_2\text{SO}_4$ pellet	667	11.9
Ion-exchange peak	1102	19.7
Gel-filtration peak	1109	19.8

Fig. 1. *In vitro* translation and immunoprecipitation. Lanes 1 and 2 show duplicate *in vitro* translations of mRNA isolated from uninduced *F. lateritium* culture. Lane 3 shows immunoprecipitation of products from uninduced *in vitro* translations with anti-cyanide hydratase antiserum (see text for details). Lanes 4, 5 and 6 are as for lanes 1, 2 and 3 except that mRNA isolated from cyanide-induced cultures was used for *in vitro* translation.

pH following the titration curve for cyanide, (pK 9.2). These properties are similar to those described previously for the enzyme from *S. loti* and *G. sorghi* (Fry & Millar, 1972; Fry & Munch, 1975; Fry & Myers, 1982) and from *F. solani* (Shimizu & Taguchi, 1969). The purified enzyme described above exhibited a subunit molecular mass of 43 kDa on SDS-PAGE electrophoresis. This is in reasonable agreement with the estimate of 40 kDa reported for *F. solani*, although a second strain was reported to contain an enzyme with a subunit molecular mass of only 17 kDa (Shimizu & Taguchi, 1969).

The gel-filtration elution profile obtained for purified enzyme indicated the native molecular mass to be very high. Non-denaturing PAGE revealed several proteins of molecular mass 300–400 kDa, despite the apparent purity on denaturing gels. This was explored further

using a calibrated gel-filtration column and gel electrophoresis. In order to extract material as rapidly as possible, dried mycelium was obtained from the CYCLEAR production plant and ground using a domestic coffee grinder. The powder was extracted using 100 mM-Tris/HCl (pH 8.5) buffer in a Potter–Elvehjem homogenizer. The supernatant obtained by centrifugation (17000 g, 15 min) contained 25.8 U (mg dry mycelium)⁻¹ and was analysed by gel-filtration as described above. The peak fraction was eluted at a volume corresponding to a molecular mass of 313 kDa but the activity was distributed over a range of 272–1217 kDa. The crude supernatant and the gel-filtration peak were examined by PAGE in the presence and absence of SDS. The SDS gel (not shown) indicated the subunit molecular mass to be as described above, both in the crude extract and in the column eluate. The native enzyme after gel-filtration behaves as predicted by the gel-filtration elution profile (not shown). The crude supernatant, however, shows much higher molecular masses, a significant proportion of the protein failing to enter the resolving gel (not shown). Dissection of the gels and prolonged incubation in the standard assay system indicated the regularly spaced bands at the top of the gel to be active cyanide hydratase; however, the assay was not sensitive enough to be more than a qualitative test. No activity was detected in the rest of the gel.

The native molecular mass of the enzyme from *S. loti* was not determined, although it was observed to elute in the void volume of a Sephadex G-200 column (exclusion limit 600 kDa) (Fry & Millar, 1972). The *G. sorghi* enzyme was later reported to be between 2000 and 10000 kDa (Fry & Munch, 1975). The results reported here indicate that for *F. lateritium* the enzyme is also aggregated into what must approach a particulate structure *in vivo*. It exhibits, however, a tendency to dissociate not shown previously. The failure of protein dissociated during gel-filtration to reassociate when analysed by PAGE indicates the process to be irreversible. The observations that the aggregates are active and do not reassociate suggest that they do reflect a genuine *in vivo* behaviour. This behaviour is similar to that reported for several other enzymes in related areas of metabolism. The β -glycosidase of *Hevea brasiliensis* breaks down the cyanogenic glycoside linamarin, and exhibits a reversible dissociation into aggregates of up to 1700 kDa (Selmar *et al.*, 1987). The nitrilase from *F. oxysporum* was reported to form active oligomers of between 4 and 14 subunits, with molecular masses up to

Fig. 2 (facing page). Nucleotide sequence of the cyanide hydratase gene and predicted protein sequence of the cyanide hydratase enzyme. Putative 5' and 3' control sequences are shown in capitals as is the coding region. The boxed regions spanning the second intron indicate the peptide sequence used in the production of the oligonucleotide.

-198 acgttcggcgctctcggattaccacgctggttggtgtac -161

-160 tgtatcagtttgacagtcagtggtttatacCAAATtcttctgtctgtatctggTATAAAtatctagctctgccgcatcca -81

-80 aatcatcaatatcacattcatcatcacacagaaacactacaatcaaacaatcccaacagttctctttatcaaccttcac -1

1 ATG GCT ATC ACC AAG TAC AAG GCC GCC GCT GTC ACC TCC GAG CCT GGA gtgagttctctcaccac 64
 1 M A I T K Y K A A A V T S E P G 16

65 tacaacaccgatgcattttactgacgatatttag TGG TTC GAC CTC GAG GGC GGC GTT CGC AAG ACC 130
 17 W F D L E G G V R K T 27

131 ATT GAC TTC ATC AAC GAG GCT GGT GAA GCT GGA TGC AAG TTC GTT GCC TTT CCC GAA Ggt 190
 28 I D F I N E A G E A G C K F V A F P E V 47

191 aagtttttttcacattttaatgaatttacaatatctaatacccaacag TTT TGG ATT CCT GGA TAT CCC TAC 261
 48 W I P G Y P Y 54

262 TGG ATG TGG AAG GTT ACC TAC CTC CAG TCT CTC CCT ATG CTC AAG AGG TAC CGT GAG AAC 321
 55 W M W K V T Y L Q S L P M L K R Y R E N 74

322 TCC ATG GCT GTC GAC TCT GAG GAG ATG CGC CGC ATC CGA CGC GCT GCT CGC GAT AAC CAG 381
 75 S M A V D S E E M R R I R R A A R D N Q 94

382 ATC TTT GTC TCT CTG GGA TTC TCC GAG ATT GAC CAC GCC ACT CTT TAC CTT TCC CAG GTT 441
 95 I F V S L G F S E I D H A T L Y L S Q V 114

442 CTG ATC GGT CCT GAT GGT GCC GTC ATC AAC CAC CGC CGC AAG ATC AAG CCC ACT CAT GTT 501
 115 L I G P D G A V I N H R R K I K P T H V 134

502 GAG AAG CTC GTT TAC GGT GAT GGA TCC GGT GAC ACC TTC ATG TCT GTC AGT GAG ACT GAG 561
 135 E K L V Y G D G S G D T F M S V S E T E 154

562 ATT GGC CGC GTC GGT CAG CTC AAC TGC TGG GAG AAC ATG AAC CCC TTC CTC AAG TCT CTC 621
 155 I G R V G Q L N C W E N M N P F L K S L 174

622 AAC GTC TCT GCT GGT GAG CAG GTT CAC GTT GCT GCA TGG CCT GTC TAC CCT GGC AAG GAG 681
 175 N V S A G E Q V H V A A W P V Y P G K E 194

682 CGC CAG GTT CAC CCC GAC CCT GCT ACC AAC TAT GCC GAT CCA GCC TCT GAC CTG GTT ACT 741
 195 R Q V H P D P A T N Y A D P A S D L V T 214

742 CCT GAG TAT GCT ATC GAG ACT GGC ACG TGG ACT CTT GCT CCC TTC CAG CGT CTC TCG GTT 801
 215 P E Y A I E T G T W T L A P F Q R L S V 234

802 GAG GGT CTG AAG ATC AAC ACT CCT GAG GGT GTT GAG CCC GAG ACC GAC CCC TCG GTT TAC 861
 235 E G L K I N T P E G V E P E T D P S V Y 254

862 AAC GGT CAT GCC CGT ATC TAC CGA CCC GAC GGT AGC CTT GTT GTC AAG CCT GAG AAA GAC 921
 255 N G H A R I Y R P D G S L V V K P E K D 274

922 TTC GAT GGT CTT CTA TTT GTT GAC ATC GAT CTA AAC GAG TGC CAT CTG ACC AAG GTC CTC 981
 275 F D G L L F V D I D L N E C H L T K V L 294

982 GCT GAC TTT GCT GGC CAC TAC ATG CGC CCT GAT CTC ATT CGC CTT CTG GTC GAC ACT CGC 1041
 295 A D F A G H Y M R P D L I R L L V D T R 314

1042 CGC AAG AAA CTC ATC ACT GAG GCT GAC CCT AAC GGC TCC ATT GCT ACC TAC AGT ACT CGC 1101
 315 R K K L I T E A D P N G S I A T Y S T R 334

1102 CAG AGA CTT GGA TTG GAC AAG CCT CTT TCG AAG AAG GAG GGA GAT GAG ACA ACT GAC GTG 1161
 335 Q R L G L D K P L S K K E G D E T T D V 354

1162 CTC TGA atgagacttgacgaggttgggggttatcaccgccaaggatgatttcatctgatgatggatggaacttgtaaga 1239
 355 L • 356

1240 tagAAATAtgtgtatagaaattgAAATAtatcgaggaatcataaagtta 1289

550 kDa (Goldlust & Bohak, 1989). In this case, the dissociation was apparently irreversible as for cyanide hydratase, although the initial state was not so highly aggregated.

Molecular cloning of cyanide-inducible genes

Cyanide hydratase activity is detectable only in cultures of *F. lateritium* which have been exposed to cyanide. This suggests that expression of the gene encoding cyanide hydratase may be inducible. Differential screening of a *F. lateritium* cDNA library was the method chosen to obtain a cyanide hydratase cDNA clone, utilizing cDNA probes prepared from mRNA isolated from cyanide-induced and uninduced cultures. The validity of this approach was tested by *in vitro* translation of mRNA from uninduced and induced culture followed by the immunoprecipitation of the translation products with an antibody specific for cyanide hydratase. Fig. 1 illustrates immunoprecipitations of *in vitro* translation products of uninduced mRNA and induced mRNA from the 57 min post-cyanide-induction time point. Close examination of the autoradiograph indicates that a number of proteins are apparently induced by cyanide, but the most obvious of these is a protein of approximate molecular mass 43 kDa, which is precipitated by the cyanide-hydratase-specific antiserum. These results confirm that expression of the *F. lateritium* cyanide hydratase gene is inducible by cyanide, and so justifies the differential screening approach taken to isolate a cyanide hydratase cDNA clone.

Differential screening

A cDNA library containing 2×10^5 recombinants was generated in λ gt11 using mRNA isolated 57 min post-cyanide induction. A sample of the cDNA library corresponding to 10000 recombinant phage was differentially screened as described. Five differentially hybridizing clones were obtained that were found to give a reproducible hybridization pattern following subsequent rounds of screening. Initially, two clones were used for further study; purification and analysis of the remaining clones is currently under way. The *Eco*RI inserts from the two λ gt11 phage were subcloned into pUC18 to give pMC111 and pMC211. *Eco*RI digestion of pMC111 and pMC211 showed cDNA insert sizes of 0.68 kb and 1.2 kb respectively.

Identification of the cyanide hydratase gene (chy1)

A Southern blot of *Eco*RI digests of pMC111 and pMC211 was probed with the degenerate end-labelled oligonucleotide derived from a partial sequence of the cyanide hydratase protein. The oligonucleotide was

found to hybridize strongly to the pMC211 cDNA insert (data not shown). Subsequent repeat experiments in which the blot was washed 4×15 min at room temperature followed by a 1×15 min wash at 52°C (8°C above the calculated $T_d - 2^\circ\text{C}$ temperature) gave autoradiographs which showed no difference in the intensity of the signal obtained with the pMC211 cDNA insert. The fact that pMC211, already shown to represent a cyanide-inducible cDNA by virtue of its differential hybridization pattern, also hybridized to an oligonucleotide probe derived from the cyanide hydratase protein sequence, led to the conclusion that pMC211 was in a cyanide hydratase cDNA clone.

Isolation of a cyanide hydratase genomic clone

A total of 25000 clones from the *F. lateritium* genomic library was screened using the purified cDNA insert from pMC211. Eight strongly hybridizing clones were obtained and DNA was isolated from each of these clones. Each clone was shown to contain the same 9.0 kb *Hind*III fragment. The 9.0 kb *Hind*III fragment was subcloned into pUC9 to give pCH1.

Sequence analysis of the cyanide hydratase gene

The cDNA clone pMC211 was sequenced, revealing an open reading frame (ORF) of 1014 bp encoding a peptide of molecular mass 35 kDa with no obvious methionine translation initiation codon. Therefore, the cDNA library was rescreened to isolate a full length clone. This clone, pMC311, contains an extra 84 bp at the 5' end of the gene but is a little shorter at the 3' end. The ATG codon at position +1 (Fig. 2) is taken as the translation start point as it marks the beginning of a 1068 bp ORF encoding a protein 355 amino acids long with a predicted molecular mass of 39.9 kDa. The predicted size of the cyanide hydratase protein is in good agreement with the size of 43 kDa estimated by biochemical determinations of the purified enzyme.

The sequence of a region of approximately 1.5 kb of the genomic clone, corresponding to the coding region plus 198 bp of 5' untranslated sequence, has been determined (Fig. 2) to confirm the delimitation of the coding region. This sequence shows that there is an in-frame stop codon at position -105 and from that point downstream to the presumptive translation start codon, there are no further in-frame ATG codons. The sequence of the genomic clone reveals the presence of two introns in the *chy1* gene, both near the 5' end of the gene. The boundary sequences and the internal sequences of both introns are in good agreement with the consensus sequences proposed for fungal introns (Gurr *et al.*, 1987). A putative promoter region has been identified and the

Fig. 3. SDS-PAGE of (a) crude cell extracts (Coomassie-blue-stained) and (b) the corresponding Western blot showing expression of cyanide hydratase as a glutathione *S*-transferase fusion protein in *E. coli*. Lanes are as follows: 1, molecular mass markers; 2, cyanide-induced *F. lateritium*; 3, *E. coli* XL1-Blue; 4, pGEX2T; 5, pGEX312 (correct orientation); 6, pGEX313 (reverse orientation); 7, molecular mass markers.

CAAT and TATA sequences are indicated in Fig. 2. A sequence, CAACCTT, bearing strong homology to the proposed ribosome binding site (CCACCTT) of the *Neurospora crassa am* gene (Kinnaird & Fincham, 1983) is found at -5 in *cyh1*.

The sequence of the 3' region of pMC211 shows that no polyadenylated tail is present in this clone, although the mRNA used to construct the library was purified by two cycles of oligo-dT-cellulose chromatography and cDNA synthesis was primed using oligo-dT. It can only be concluded that the polyA tail was lost during the cloning process. The sequence of the 3' untranslated region of *cyh1* contains two putative polyadenylation signal sequences (Proudfoot & Brownlee, 1976), both of which have the sequence AAATA.

Recently, cyanide hydratase has been purified and the gene cloned from the phytopathogenic fungus *Gloeocercospora sorghi* (Wang *et al.*, 1992; Wang & Van Etten, 1992). The enzyme is very similar in properties to the enzyme from *F. lateritium*. The genes are approximately 65% homologous and the proteins are 75% identical. Interestingly, the *G. sorghi* gene does not have any introns.

Expression of cyanide hydratase in *E. coli*

A cyanide hydratase cDNA clone, pMC312, was identified which contains an ORF for all but the first seven amino acids of cyanide hydratase. In order to confirm the identity of the protein encoded by the cloned *cyh1* gene, the 1090 bp fragment of pMC312 was fused in-frame with the glutathione *S*-transferase (GST) gene in the expression vector pGEX-2T (Smith & Johnson, 1988) to give pGEX-312. Insertion of the fragment in the

reverse orientation gave pGEX-313. Analysis of crude cell extracts of *E. coli* containing these plasmids (Fig. 3) indicates that pGEX-312 leads to the production of a novel protein of approximately 66 kDa while pGEX-313 leads to the production of a protein of approximately 28 kDa (the GST protein itself is 26 kDa). The 66 kDa protein produced by pGEX-312 is the expected size for the GST-CYH fusion protein. Immunoblotting of this gel with the cyanide hydratase antiserum confirms that this protein reacts strongly with the antiserum while no reaction is found in the control extracts. No detectable cyanide hydratase enzyme activity was observed in extracts of *E. coli* strains expressing the GST-CYH fusion protein.

Homology of cyanide hydratase to other proteins

The predicted protein sequence of cyanide hydratase was compared to the NBRF Data Bank using Microgenie software. This revealed that the cyanide hydratase protein has remarkable homology to the bromoxynil-specific nitrilase from *Klebsiella pneumoniae* (Stalker *et al.*, 1988), showing 35% identity over the entire length of the proteins (Fig. 4). Recently, the sequence of a nitrilase from *Alcaligenes faecalis* JM3 has been published (Kobayashi *et al.*, 1993) and this is approximately 35% homologous to both cyanide hydratase and the *Klebsiella* nitrilase. The alignment of the three sequences is shown in Fig. 4. Interestingly, the cysteine residue at position 163 in the *Alcaligenes* nitrilase, which has been shown to be required for activity (Kobayashi *et al.*, 1993), is conserved in both cyanide hydratase and the bromoxynil nitrilase. Site-directed mutagenesis will be used to establish the importance of this residue in cyanide

CH	MAITKY-KAAAVTSEPGWFDLEGGVRKTIDFINEAGEAGCKF-VAFPEVWIPGYP-YWMW	57
BXN	MDTTF--KAAAVQAEFVWMDAAATADKTVTLVAKAAAAGAQLLVAFPELWIPGYPGFMML	58
JM3	MQTRKIVRAAAVQAASPNYDLATGVDKTIELARQARDEGCDL-IVFGETWLPGYP-FHVW	58
	* .**** . . * ** . . * * . . * * * .**** . .	
CH	KVTYLQSLPMLKRYRENSMAVDSEEMR-RIRRAARDNQIFVSLGFSEIDHATLYLSQVLI	116
BXN	THNQETLFPFIKRYKQAIADGPEIEEKIRCAAQEHNIALSFGYSERAGRTLMSQMLI	118
JM3	LGAPAWSLKYSARYYANSLSLDSAEPQ-RIAQAARTLGIFIALGYSESSGGSYLQGLI	117
	* . * * . * . * ** . * . . . * . * . * . * . * . * .	
CH	GPDGAVINHRRKIKPTH-VEKLVYGDGSGDTFMSVSETEIGRVGQLNCWENMNPFLKSLN	175
BXN	DADGITKIRRRKLKPTRRFERELFGEEDGSDLLQVAQTSVGRVGALNCAENLQSLNKFAL	178
JM3	DDKGQMLWSRRKLKPTH-VERTVFGEYARDLI-VSDTELGRVGALCCWEHLSPLSKYAL	175
	. * * * * * * *	
CH	VSAGEQVHVAAWFVYPGKERQVHPDPATNYADPASDLVTPEYAIETGTWTLAPFQRLSVE	235
BXN	AAEGEQIHISAWP-----FTLGSPVLVGDSIGAINQVYAAETGTFVLMSTQVVGPT	229
JM3	YSQHEAIIHAAWPSFS-----LYSEQAHALSQVYMAASQIYSVEGQCFTIAASSVVTQE	229
	* . * . . * * . *	
CH	GLKINTPEGVEPETDPSVYNGHARIYRDPG-SLVVKPEKDFDGLLFVDIDLNECHLTKVL	294
BXN	GIAAFEIEDRYNNPNQYLGCGYARIYGPDMQLKSKSLSPTEEGIVYAEIDLMSLEAAKYS	289
JM3	TLDMLEV-GEHNASLLKVGCGSSMIFAPDGRTLAPYLPDAEGLIADLNMEEIFAKAI	289
	 * . * . * . * * *	
CH	ADFAGHYMRPDLIRLLVDTRRKLITEADPNNGSIATYSTQRQL-GLDKPLSKKEGDETTD	353
BXN	LDPTGHYSRDPVFSVSINRQRQPAVSEVIDSNGDEDPRAACEPDEGDREVVISTA---IG	346
JM3	NDPVGHYSKPEATRLVLDLGHREPMTRVHKSQVIEEAPEPHVQSTAAPVAVSQTQSDST	349
	* *** . * .	
CH	VL-----	355
BXN	VLPYRCGHS	355
JM3	LLVQ--EPS	356
	*	

Fig. 4. Comparison of amino acid sequence of *F. lateritium* cyanide hydratase (CH) with the *K. pneumoniae* bromoxynil-specific nitrilase (BXN) and the nitrilase from *Alcaligenes faecalis* JM3 (JM3). The sequences were aligned using the Clustal program available through the SERC Daresbury Laboratory computer system. Identical amino acids are indicated by (*), similar changes are marked by (.).

hydratase. The similarity of cyanide hydratase to nitrilases is a little surprising, as one may anticipate a greater degree of similarity to the functionally similar nitrile hydratases. This may be explained by the reaction scheme previously proposed (Zervos & Cordes, 1971), in which nitriles may be converted via a thioimide intermediate to either amide or acid depending on conditions. However, no homology was seen to the nitrile hydratases from *Rhodococcus* sp. 774 (Kobayashi *et al.*, 1991) and *Myrothecium verrucaria* (Endo & Watanabe, 1989; Maier-Greiner *et al.*, 1991). There are no reports in the literature of cyanide being used by either nitrilases or nitrile hydratases. The similarity of cyanide hydratase to the bromoxynil nitrilase does not appear to reflect the structural similarities seen between cyanide hydratase and the *F. oxysporum* nitrilase (see above), as the *K. pneumoniae* enzyme does not form

aggregates. No nitrilase activity was found in crude extracts of *F. lateritium* prepared from cultures with a high level of cyanide hydratase activity (P. D. Turner, unpublished).

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