Proc. Int'l Symp. Microorganisms and Health
April 28~29, 2000, Seoul, Korea

Apolar growth of *Neurospora crassa* leads to increased secretion of extracellular proteins

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Abstract

Protein secretion in filamentous fungi has been shown to be restricted to actively growing hyphal tips. To determine whether an increase in the amount of growing surface area of a fungus can lead to an increase in the amount of protein secretion, we examined secretion in a temperature-sensitive *Neurospora crassa mcb* mutant that shows a loss of growth polarity when incubated at restrictive-temperature. Incubation of the *mcb* mutant at restrictive-temperature results in a three- to five-fold increase in the level of extracellular protein and a 20-fold increase in carboxymethyl cellulase activity relative to a wild-type strain. A mutation in the *cr-1* gene has been shown previously to suppress the apolar growth phenotype of the *mcb* mutant, and we find that the level of extracellular protein produced by a *mcb*; *cr-1* double mutant was reduced to that of the wild-type control. Immunolocalization of a secreted endoglucanase revealed that proteins are secreted mainly at hyphal tips in hyphae exhibiting polar growth and over the entire surface area of bulbous regions of hyphae that are produced following a shift of the *mcb* mutant to restrictive-temperature. These results support the hypothesis that secretion of extracellular protein by a filamentous fungus can be significantly increased by mutations that alter growth polarity.

Introduction

Because of their ability to secrete large quantities of proteins, filamentous fungi have been utilized to produce enzymes industrially for several decades. With the development of gene-transfer systems, filamentous fungi are considered to be attractive expression systems for the production of non-fungal as well as fungal proteins (Gouka et al., 1997; Punt et al., 1994). Unfortunately, little is known about the molecular mechanisms underlying protein secretion in filamentous fungi. Analysis of protein secretion in *Aspergillus niger* and *Phanerochaete chrysosporium* revealed that proteins exit exclusively from hyphae at actively growing tips, suggesting that protein secretion and hyphal tip growth are coupled processes (Moukha et al., 1993; Wösten et al., 1991).

A major question concerning secretion of fungal proteins is how they pass through the cell wall, which acts as a significant barrier to passive diffusion (Chang and Trevithick, 1974; Trevithick and Metzenberg, 1966). In the "bulk-flow" model, proteins are proposed to pass through the cell wall by being carried outward by a flow of "plastic" cell wall material (Sietsma et al., 1995; Wessels, 1993). Proteins secreted at the very tip of a growing hypha are displaced to the outside of the wall and ultimately into the growth medium as new cell wall material is constantly being secreted behind it and the hyphal tip grows outward. In contrast, protein secreted within the hyphal tip extension zone but slightly distal to the tip will be retained within the inner layers of the wall and not secreted into the medium.

Considering that protein secretion occurs primarily at actively growing tips, it is possible that protein secretion is occasionally limited by the amount of growing surface area (i.e. the number of growing tips) per unit length of hypha. Therefore, we proposed that mutations resulting in either a general loss of growth polarity or an increase in the number of hyphal tips per unit length of hypha may lead to increased secretion of extracellular proteins. Recently, we examined a conditional *Neurospora crassa* mutant that is defective in growth

polarity when grown at restrictive-temperature. The *N. crassa mcb* mutant forms defined hyphae at 25°C, but it shows a complete loss of growth polarity when hyphal filaments are transferred to 37°C (Bruno et al., 1996). In this study, we examined protein secretion from the *N. crassa mcb* mutant relative to a wild-type control strain. The results support our hypothesis that mutations resulting in an increase in the amount of growing surface area can lead to significant increases in protein secretion relative to a wild-type strain.

Materials and Methods

Strains and media.

The wild-type *N. crassa* strain used in this study was 74-OR23-1A (FGSC 987) and was obtained from the Fungal Genetics Stock Center (FGSC), Department of Microbiology, University of Kansas Medical Center, Kansas City, KS. The *mcb* (FGSC 7094) and *cr-1* (B123) (FGSC 4008) strains were also obtained from the FGSC. The construction of a *mcb*; *cr-1* double mutant has been described (Bruno et al., 1996). The medium used for protein secretion assays was Vogel's minimal medium supplemented with 1% sucrose and 0.5% CMC (Davis and de Serres, 1970).

Measurement of total extracellular proteins and CMCase activity.

Fresh conidia (1.25×10^8) were inoculated into 50 ml of liquid medium and grown for 18 to 90 h at either 25°C or 37°C. Ampicillin (50 μg/ml) and chloroamphenicol (170 μg/ml) were added to each culture to prevent bacterial contamination during long incubations. Supernatants were collected at various time points during growth by filtration through a Whatman GF/D filter (Whatman International Ltd., Maidstone, England). Before collecting supernatant, phenylmethylsulfonyl fluoride (PMSF) and leupeptin were added to final concentrations of 2 mM and 0.2 mg/ml, respectively, to inhibit degradation of proteins. The culture wet weights were determined by squeezing out excess liquid from the hyphal masses prior to weighing. Protein concentrations within supernatants were determined by the Bradford assay using the Bio-Rad Protein Assay System according to the manufacturer's instructions (Bio-Rad laboratories, Richmond, CA). Bovine serum albumin was used as the protein standard.

For carboxymethyl cellulase (CMCase) assays, 0.25 ml of culture supernatant was added to 0.25 ml of 2% CMC in 50 mM sodium citrate buffer (pH 4.8) and the mixture was incubated for 30 min at 50°C. The reaction was stopped by adding one ml of 3,5-dinitrosalicylic acid (DNS) reagent followed by boiling for 5 min (Miller, 1959). After cooling, the absorbance at 540 nm was determined relative to a control. One unit of CMCase was defined as the amount of enzyme required to release one μmol of glucose during the 30 min incubation at 50°C. CMCase assays and protein determinations were conducted on two or more independently grown cultures of each strain examined.

SDS-PAGE, immunobloting, and CMCase activity staining following SDS-PAGE

The number and sizes of secreted proteins were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Extracellular proteins were concentrated by precipitation from supernatants by adding trichloroactetic acid (TCA) to a final concentration of 10% (v/v). The protein contained within 4 ml of supernatant was loaded onto 10% SDS-polyacrylamide gels (Sambrook et al., 1989).

Immunobloting was performed using ProtoBlot® Western Blot AP Systems (Promega, Madison, WI) (Harlow and Lane, 1988). Polyclonal antibodies against *Humicola insolens* endoglucanase (obtained from Dr. Randy Berka, Novo Nordisk Biotech, Inc., Davis, CA) were used at a 1:2000 dilution.

CMCase activity following SDS-PAGE was determined using the protocol described by Blanco and Paster (1993) with a few modifications. 0.2% CMC was included in SDS-PAGE gels before polymerization. To renature proteins following electrophoresis, gels were rinsed with distilled water briefly and then soaked in 2.5% Triton X-100 for 30 min with gentle

shaking. Gels were then incubated in 100 mM sodium citrate buffer (pH 4.8) for 4 hours at 50°C. After washing with distilled water, gels were stained with 0.1% Congo red for 30 min and washed with 1 M NaCl until CMCase bands became visible (i.e. yellowish bands in a red background). The contrast between the bands and the background was improved by gradually adding HCl to decrease the pH of the gel immersed in the 1 M NaCl solution.

Indirect immunofluorescence and microscopy.

Hyphal morphologies of the wild-type strain, and the *mcb* and *mcb; cr-1* mutants were examined through an Olympus BH-2 microscope at 40X magnification.

The localization of CMCase secretion from hyphae was examined using indirect immunofluorescence as described except that hyphae were not subjected to enzymatic digestion to permeabilize the cell wall (Tinsley et al., 1996). In brief, hyphae were grown overnight on small pieces (0.5 x 0.5 cm) of cellulose filter (GN-6 membrane filter, Gelman Sciences, Ann Arbor, MI) lying on either sucrose minimal agar plates or CMC minimal agar plates. For temperature-shift experiments, hyphae were first grown overnight on filters lying on sucrose minimal plates at 25°C. The filters were then transferred to prewarmed sucrose minimal or CMC minimal agar plates and grown at 37°C for an additional 6 h. Quick-freezing and fixation of hyphae was performed as described (Tinsley et al., 1996). The primary antibody was polyclonal anti-*H. insolens* endoglucanase antibodies used at a 1:500 dilution. The secondary antibody was sheep anti-rabbit IgG conjugated to Cy3 (Sigma, St. Louis, MO). Cy3 was observed using a rhodamine filter set.

Examination of nuclei was conducted by staining quick-frozen, low-temperature-fixed hyphae with 1 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) as described (Tinsley et al., 1996). Samples examined by indirect immunofluorescence or stained with DAPI were observed using an Olympus BX-50 microscope with the appropriate epi-illumination filter cube using an 1.3 N.A. 100X oil immersion objective lens. Pictures were taken with Kodak Technical Pan film (ASA 100).

Results

N. crassa mcb secretes more protein than wild-type

Conidia of *N. crassa* wild-type germinated and produced smooth-walled hyphae (Figure 1A-B). However, conidia of the *N. crassa mcb* mutant germinated and gave rise to hyphae with swollen tips when incubated at 25°C in CMC and sucrose supplemented Vogel's liquid minimal medium (Figure 1D). The initial swollen tips and bulbous hyphae in turn generated smaller hyphal tips that developed into typical fungal hyphae. After 66 h of growth at 25°C, most of the hyphae of the *mcb* strain were like that of wild-type even though some swollen hyphae were still present (Figure 1E vs. 1B). When *mcb* hyphae incubated at 25°C for 18 h were shifted to 37°C and then grown for an additional 48 h, tips became swollen and some parts of pre-existing hyphae became bulbous (Figure 1F). Even though some of the *mcb* hyphae incubated at 37°C looked like wild-type, they were approximately three times thicker than wild-type hyphae (Figure 1B vs. 1F).

During the growth of the cultures, hyphal wet weights were determined to follow the accumulation of biomass, and the levels of protein in the culture medium were measured to compare the amount of secretion from the *mcb* mutant with the wild-type control strain (Figure 2A and 2B). The wild-type strain grew slightly better at 25°C than at 37°C (Figure 2A). The level of secreted protein was also greater at 25°C than at 37°C (Figure 2B). Consistent with our hypothesis, the *mcb* mutant secreted more extracellular protein under all culture conditions than the wild-type control strain (Figure 2B). In Figure 3A, the levels of protein secreted into the media were normalized to total biomass for the cultures harvested at 66 h. At this time point, the *mcb* mutant had produced about two-fold more extracellular protein than the wild-type strain when both cultures were grown at 25°C and about seven-fold more extracellular protein when both cultures were grown for 18 h at 25°C followed by 48 h at 37°C (Figure 3A). The

best incubation temperature for secretion of extracellular proteins was 25°C for the wild-type strain, while it was 37°C for the *mcb* mutant (Figures 2 and 3).

N. crassa mcb secretes more CMCase than wild-type

Because CMC was used as a carbon source to induce production of extracellular proteins, we reasoned that the major secreted protein(s) may be CMCase. As expected, the level of CMCase activity from the *mcb* mutant was found to be three-fold more than from the wild-type control strain when grown at 25°C and more than 20-fold higher when both strains were shifted to 37°C (Figure 2C and 3B). The large increase in the levels of CMCase activity from the *mcb* mutant relative to the wild-type strain, when the two strains were incubated at 37°C, was due in part to a five-fold reduction in CMCase production from the wild-type strain and a two-fold increase in CMCase production from the *mcb* mutant (Figure 3B).

Because hyphae were cultured in CMC media, which induces production of CMCase, one or more of the major extracellular proteins may represent cellulolytic enzymes. Extracellular proteins resolved by SDS-PAGE were renatured and assayed for CMCase activity. As shown in Figure 4B, the 150, 130, and 50 kDa proteins showed CMCase activity. Four different endoglucanases (46.5, 37.3, 30, and 21 kDa), which are all glycosylated, have been identified previously in the *cell-1* mutant of *N. crassa* (Yazdi et al., 1990a; Yazdi et al., 1990b). Polyclonal antibodies against *H. insolens* endoglucanase cross-reacted with a number of proteins including the 50 kDa *N. crassa* protein (Figure 4C). This evidence suggested that the 50 kDa secreted protein of the wild-type and *mcb* strains was a CMCase and may be equivalent to the 46.5 kDa endoglucanase identified previously from the *N. crassa cell-1* mutant.

A _cr-1_ mutation suppresses increased production of extracellular protein from the _mcb_ mutant

An adenylate cyclase mutation, *cr-1*, has been shown to be a suppressor of *mcb* (Bruno et al., 1996). We suspected that apolar growth of hyphae in *mcb* is the main reason for higher extracellular protein production, and therefore, we predicted that the level of extracellular proteins should be decreased in a *mcb*; *cr-1* double mutant. Hyphal morphology of *mcb*; *cr-1* grown at 25°C or of a culture shifted to 37°C were indistinguishable from that of wild-type (Figure 1C). As predicted, the levels of extracellular protein from the *mcb*; *cr-1* double mutant and the *cr-1* control strain were reduced relative to the *mcb* mutant and similar to that of wild-type (Figure 3A). In addition, CMCase activity was much reduced in the *mcb*; *cr-1* double mutant compared to the *mcb* mutant (Figure 3B). These results indicate that the *cr-1* mutation suppresses not only the effects of the *mcb* mutation on hyphal growth, but also the effects of *mcb* on protein secretion and secretion of CMCase activity.

Determining the site of protein secretion from _mcb_ hyphae

As mentioned above, it has been determined that proteins are secreted at actively growing tips in filamentous fungi (Moukha et al., 1993; Wösten et al., 1991). Because antibodies to *H. insolens* endoglucanase cross-react with a number of secreted proteins of *N. crassa* including one of the endoglucanases (Figure 4C), they provided an excellent tool to examine the sites of protein secretion from *mcb* hyphae. Staining of a broken hypha of the *mcb* mutant, in which the cytoplasmic region of the hypha was well preserved (note wild-type nuclear morphology as seen following DAPI stain; Figure 5F), only showed strong staining of its wall (Figure 5E). This result illustrates that these antibodies recognize specifically cell wall regions where secreted proteins would be associated, and the antibodies do not appreciably recognize proteins in the cytoplasm.

Tip regions were the predominately stained areas of hyphae of a wild-type strain grown on either a sucrose or CMC supplemented agar medium (Figure 5A and 5B). In contrast, tip and distal regions of hyphae were stained in the *mcb* mutant (Figure 5C and 5D). Growth of the *mcb* mutant on a CMC supplemented agar medium resulted in a much stronger signal compared to *mcb* grown on a sucrose supplemented agar medium (Figure 5C vs. 5D). (Figure 5C and 5D pictures were taken at equivalent lengths of exposure to demonstrate actual difference in signal.) In addition, the intensity of staining was much higher in *mcb* than in wild-type

(compare Figure 5B and 5D; note that the photographic exposure time was 5 sec for the wild-type hypha and 1 sec for the *mcb* hyphae). For *mcb* hyphae grown at 25°C on a CMC supplemented agar medium and then shifted to 37°C for 6 h, the staining signal was so bright that it was hard to photograph and capture the outline of the hyphae. We observed that regions of the membrane surrounding the hyphae as well as cell wall regions were highly stained, appearing as an intense haze (Figure 5G). The tips of leading hyphae and newly formed hyphae were swollen and highly stained (Figure 5H and 5I). Also, the bulbous regions located in the middle of hyphae were brightly stained (Figure 5J). These results indicate that the sites for protein secretion from the *mcb* mutant are not limited to tip regions as in a wild-type strain. All regions of hyphae growing in an apolar fashion secrete very high levels of extracellular protein.

Discussion

Filamentous fungi have been utilized extensively for the large scale production of secreted fungal proteins and more recently the production of nonfungal proteins. Efficient production of secreted protein is often limited by "bottlenecks" in the secretion pathway (for review see Gouka et al., 1997). Some of the factors that have been shown to limit production of a specific product include: low mRNA stability; improper folding of protein in the ER; and proteolytic degradation of products. Because protein secretion is restricted primarily to growing hyphal tips, we have proposed that an additional "bottleneck" may be a limitation in the amount of growing surface area (i.e. hyphal tips) at which proteins may exit hyphae. In this study, we have shown that the loss of growth polarity observed with the *N. crassa mcb* mutant is accompanied by an increase in the amount of secreted protein relative to a wild-type control strain. The *mcb* mutant secretes three- to five-fold more protein and 20-fold more CMCase activity than wild-type (Figures 2 and 3). Immunolocalization of *N. crassa* endoglucanase, using antibodies raised against *H. insolens* endoglucanase, showed that proteins are highly secreted at swollen apical tips and bulbous hyphae of the *mcb* mutant (Figure 5). It is likely that the increased level of extracellular proteins from *mcb* results primarily from increased growing surface area that in turn is due to a loss in growth polarity.

In the "bulk-flow" model, proteins are proposed to pass through the cell wall at actively growing hyphal tips by being carried outward along with a flow of "plastic" cell wall material (Sietsma et al., 1995; Wessels, 1993). This implies that protein release at hyphal tips is a passive activity of cell wall extension. In our time course examination of protein secretion, extracellular proteins were produced initially during late logarithmic phase and the majority of the extracellular protein was secreted while the hyphae were in stationary phase (Figure 2). These results indicate that the level of protein secretion is not likely to be linked with hyphal growth rate and that high level secretion can occur even when hyphae are growing slowly. Because protein secretion as described in the "bulk-flow" model is dependent on displacement of newly secreted protein by the outward flow of nascent wall polymers, one might predict that secretion is at least partly dependent on the rate of hyphal extension. While it is clear that protein secretion is localized primarily to sites of wall growth, the apparent lack of dependency of the level of protein secretion on hyphal extension rate suggests that there may be a more active mechanism involved in the passage of protein through cell wall than simple outward displacement by plastic wall material.

Regions of *mcb* hyphae that have lost growth polarity have cell walls that are very thick (Bruno et al., 1996). Walls in mature regions of *N. crassa* hyphae are approximately 150 nm thick, while at hyphal tips the wall is approximately 50 nm thick (Hunsley and Kay, 1976). In regions of *mcb* hyphae that have become bulbous, the hyphal wall may be more than 1 μm thick (Bruno et al., 1996). Interestingly, this does not appear to be a barrier to secretion as one might predict from the "bulk-flow" model (Figure 5). In this model, the ability of proteins to exit at hyphal tips is due not only to displacement by the flow of nascent wall material, but also to the relatively thin wall at hyphal tips which is predicted to be more porous. The ability of proteins

to readily pass through the very thick walls of the *mcb* mutant again suggests that there may be a more active mechanism by which proteins pass through the wall.

The *mcb* gene encodes a regulatory subunit of PKA, and we have proposed that when the *mcb* mutant is incubated at restrictive-temperature the defective regulatory subunit releases from the catalytic subunit resulting in increased PKA activity (Bruno et al., 1996). It is unclear how PKA activity regulates growth polarity, but it has been shown that cAMP and the PKA pathway also play a role in controlling the cell wall growth pattern of two additional fungi, *Ustilago maydis* and *Magnaporthe grisea* (Gold et al., 1994; Mitchell and Dean, 1995). These results suggest that protein secretion can be increased in many if not all fungi by increasing PKA activity. The ability to increase protein secretion from filamentous fungi by altering growth polarity has the potential to significantly increase the production yields of many industrially important fungi. This could be achieved by the introduction of specific mutations that alter polar growth, as with the *mcb* mutant, or by the addition of compounds to the growth medium that increase PKA activity and lead to a loss of growth polarity.

Acknowledgments

This work was supported by funds from Novo Nordisk Biotech, Inc. and from the National Science Foundation (Grant BIR-9217251). We thank Peter Minke for assistance with microscopy and image processing, and Dr. Randy Berka of Novo Nordisk Biotech, Inc. for providing us with polyclonal antibodies against *H. insolens* endoglucanase.

References

Blanco, A. and Pastor, F.I.J. (1993) Characterization of cellulase-free xylanases from the newly isolated *Bacillus* sp. strain BP-23. *Can. J. Microbiol.* **39:** 1162-1166.

Bruno, K. S., Tinsley, J. H., Minke, P. F., and Plamann, M. (1996) Loss of growth polarity and mislocalization of septa in a *Neurospora* mutant altered in the regulatory subunit of cAMP-dependent protein kinase. *EMBO J.* **15:** 5772-5782.

Chang, P. L. Y., and Trevithick, J. R. (1974) How important is secretion of exoenzymes through apical cell walls of fungi? *Arch. Microbiol.* **101:** 281-293.

Davis, R. H., and de Serres, F. J. (1970) Genetic and microbiological research techniques for *Neurospora crassa*. *Methods Enzymol.* **27A:** 79-143.

Gold, S., Duncan, G., Barrett, K., and Kronstad, J. (1994) cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. *Genes Dev.* **8:** 2805-2816.

Gouka, R. J., Punt, P. J., and van den Hondel, C. A. M. J. J. (1997) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl. Microbiol. Biotechnol.* **47:** 1-11.

Harlow, E., and Lane, D. (1988) Antibodies: a laboratory manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Hunsley, D., and Kay, D. (1976) Wall structure of the *Neurospora* hyphal apex: immunofluorescent localization of wall surface antigens. *J. Gen. Microbiol.* **95:** 233-248.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.

Miller, G. L. (1959) Use of dinitrosalicylic acid for determination of reducing sugar. *Anal. Chem.* **31:** 426-428.

Mitchell, T. K., and Dean, R. A. (1995) The cAMP-dependent protein kinase subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* **7:** 1869-1878.

Moukha, S. M., Wšsten, H. A. B., Asther, M., and Wessels, J. G. H. (1993) In situ localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using sandwhiched mode of culture. *J. Gen. Microbiol.* **139:** 969-978.

Punt, P. J., Veldhuisen, G., and van den Hondel, C. A. (1994) Protein targeting and secretion in filamentous fungi. *Antonie van Leeuwenhoek* **65:** 211-216.

Sambrook, J., Fritsch, E. H., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edit. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Sietsma, J. H., Wšsten, H. A. B., and Wessels, J. G. H. (1995) Cell wall growth and protein secretion in fungi. *Can. J. Bot.* **73 (Suppl. 1):** 388-395.

Tinsley, J. H., Minke, P. F., Bruno, K. S., and Plamann, M. (1996) p150^{Glued}, the largest subunit of the dynactin complex, is nonessential in *Neurospora* but required for nuclear distribution. *Mol. Biol. Cell* **7:** 731-742.

Trevithick, J. R., and Metzenberg, R. L. (1966) Molecular sieving by *Neurospora* cell walls during secretion of invertase isozymes. *J. Bacteriol.* **92:** 1010-1015.

Wessels, J. G. H. (1993) Wall growth, protein excretion and morphogenesis in fungi. *New. Phytol.* **123:** 397-413.

Wšsten, H. A. B., Moukha, S. M., Sietsma, J. H., and Wessels, J. G. H. (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. *J. Gen. Microbiol.* **137:** 2017-2023.

Yazdi, M. T., Radford, A., Keen, J. N., and Woodward, J. R. (1990a) Cellulase production by *Neurospora crassa*: purification and characterization of the cellulolytic enzymes. *Enzyme Microb. Technol.* **12:** 120-123.

Yazdi, M. T., Woodward, J. R., and Radford, A. (1990b) The cellulase complex of *Neurospora crassa*: activity, stability and release. *J. Gen. Microbiol.* **136:** 1313-1319.

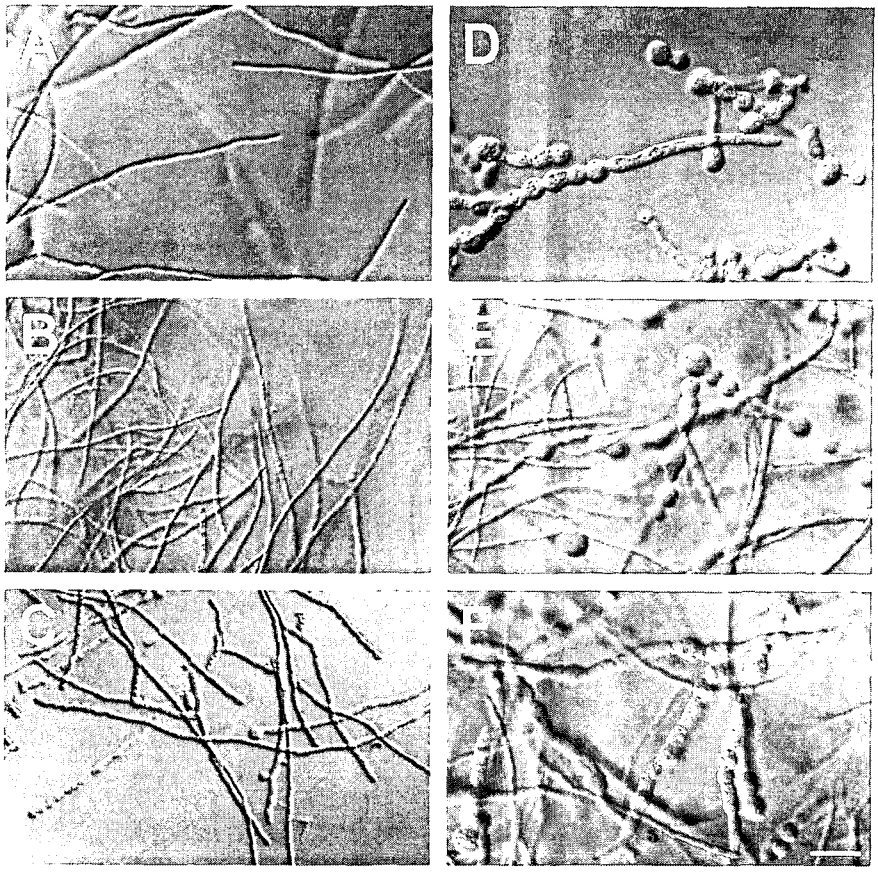

Figure 1. Hyphal morphologies of *N. crassa* wild-type, and *mcb* and *mcb*; *cr-1* mu
Hyphae of wild-type (A and B), *mcb* (D, E, and F), and *mcb*; *cr-1* (C) were gr
Vogel's minimal liquid media with 0.5% CMC and 1% sucrose as carbon
Photographs of cultures A, C, and D were taken after 18 h of growth at 25℃,
and E after 66 h of growth at 25℃. In F, *mcb* hyphae were grown for 18 h a
then shifted to 37℃ for 48 h. Bar indicates 50 μm.

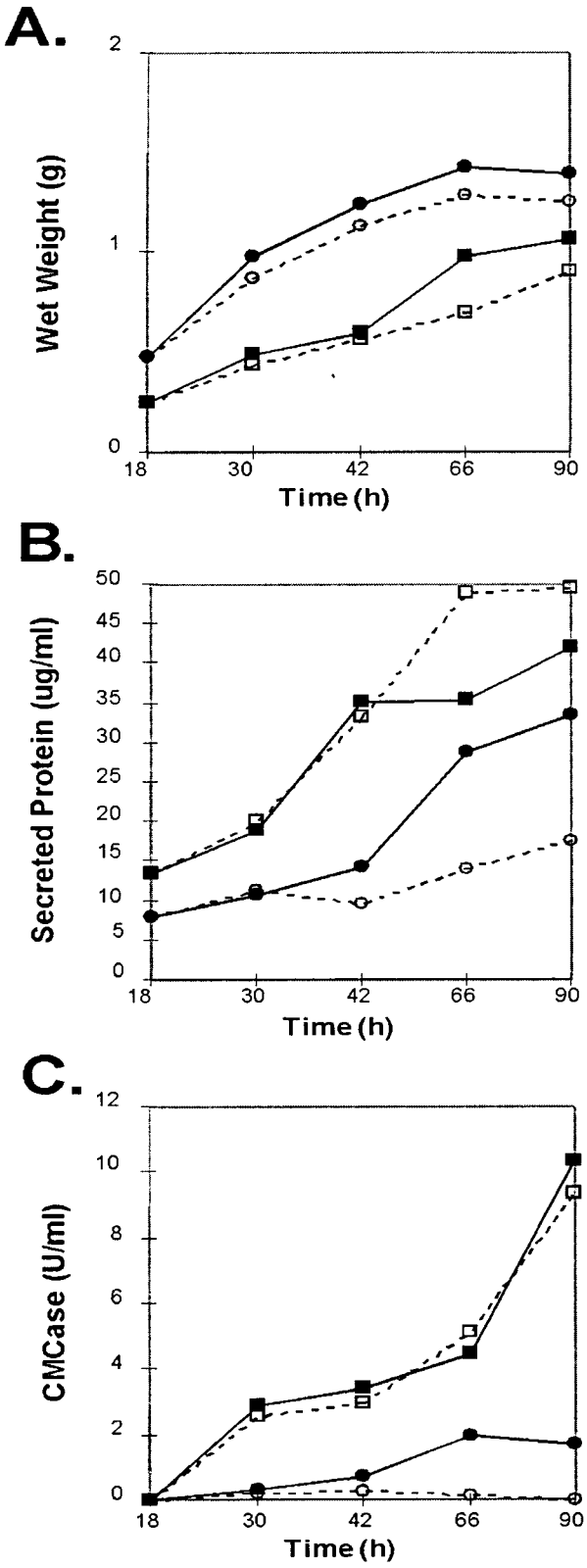

Figure 2. Growth, protein secretio CMCase activity of wild-type and cultures.

Culture wet weights were determined to follow biomass increase of the cultures (A). The levels of extracellular protein (B) and CMCase activity (C) were determined at various times for wild-type and *mcb* cultures grown in CMC and sucrose supplemented liquid minimal media. The secreted protein values are shown as μg/ml of culture medium. The CMCase activity values are shown as units of CMCase activity per ml of culture medium. Symbols: filled circles, wild-type grown at 25℃; open circles, wild-type shifted to 37℃; filled squares, *mcb* grown at 25℃; and open squares, *mcb* shifted to 37℃. The wild-type and *mcb* temperature-shifted cultures were incubated at 25℃ for 18 h followed by an additional 12, 24, 48 or 72 h at 37℃. The total incubation time of each culture is indicated on the x-axis.

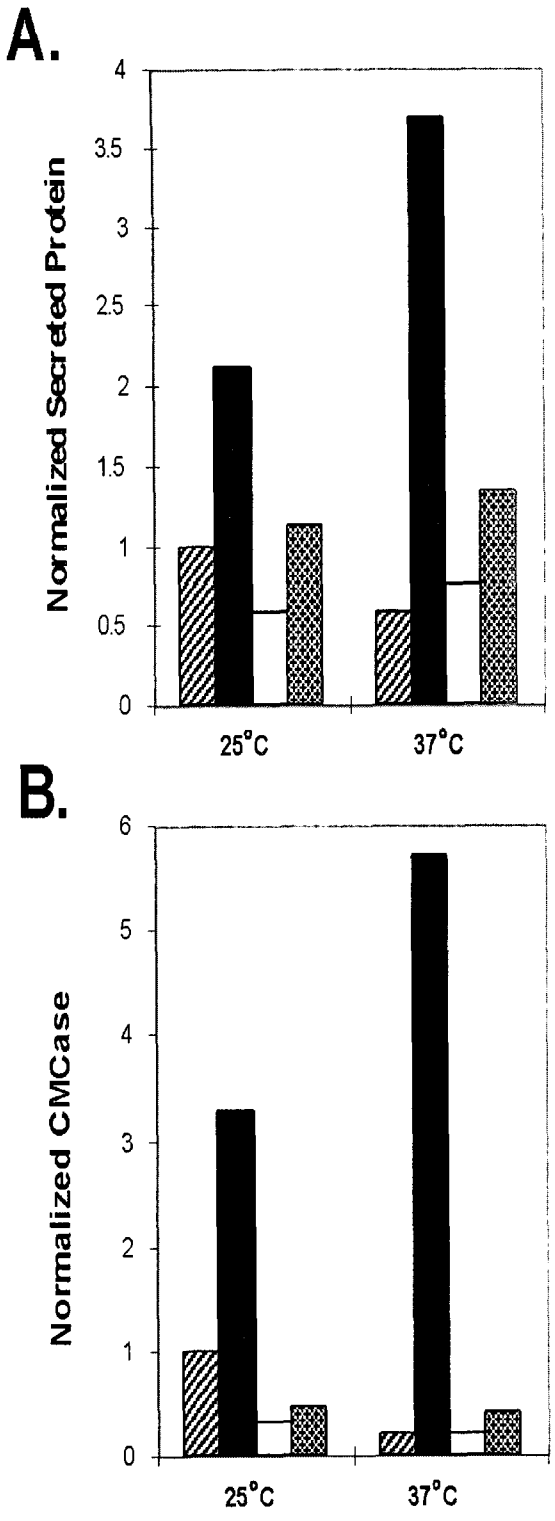

Figure 3. Normalized protein se and CMCase activity.

In order to compare secretion from the various strains relative to accumulated biomass, the levels of secreted protein and CMCase activity were determined per gram of hyphal wet weight. The cultures shown were either incubated for 66 h at 25℃ or for 18 h at 25℃ followed by an additional 48 h at 37℃. Relative values are presented with the wild-type strain incubated at 25℃ for 66 h set at one for both normalized secreted protein and normalized CMCase activity. The graph bars represent the following strains: diagonally striped bar, wild-type; filled bar, *mcb*; shaded bar *mcb*; *cr-1*; and hatched bar, *cr-1*.

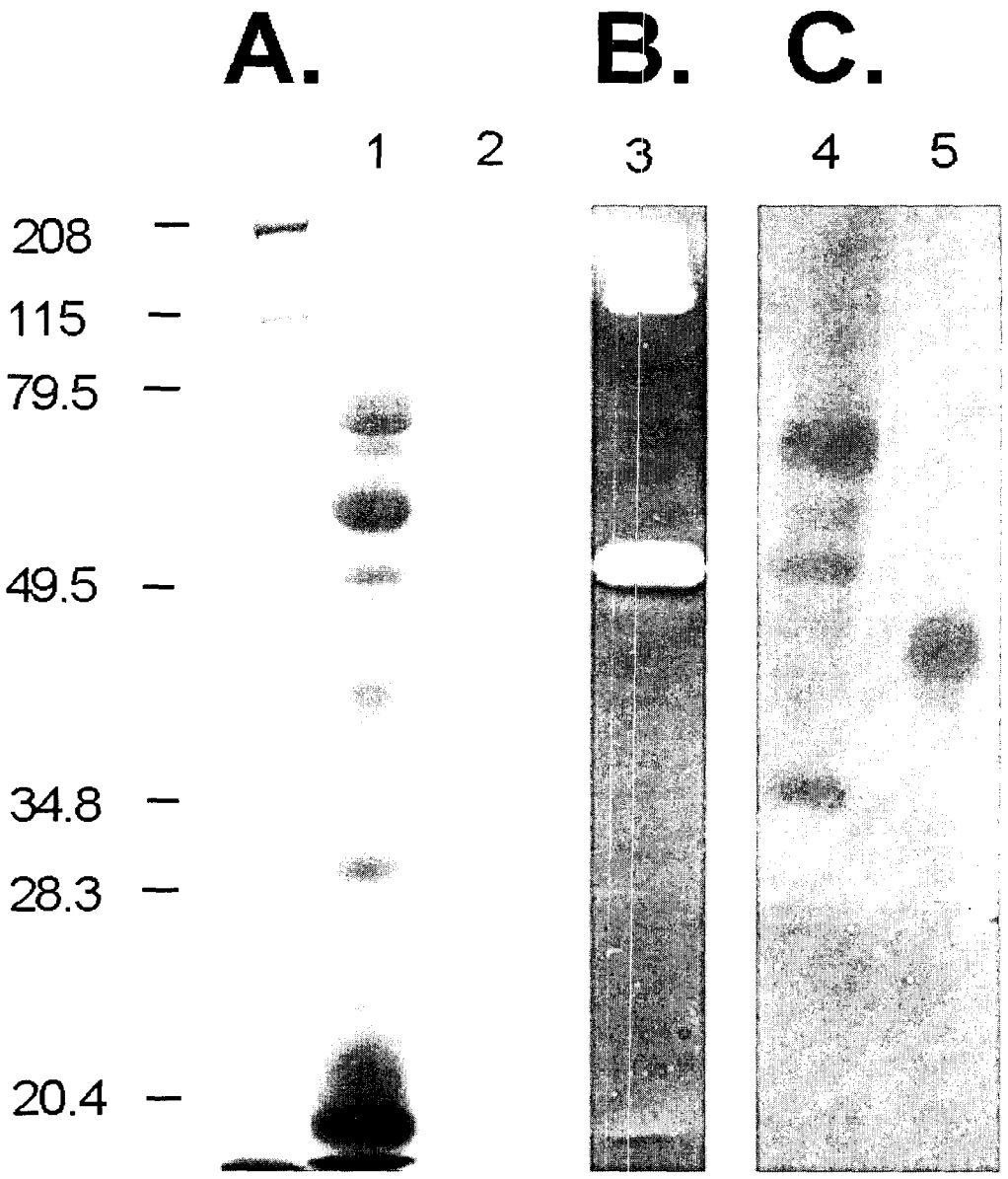

Figure 4. Antibodies to *H. insolens* endoglucanase cross-react with a *N. crassa* endoglucanase.

The *mcb* mutant was incubated for 66 h at 25°C and proteins contained within 4 ml of the culture medium were TCA precipitated and resolved on a 10% SDS-polyacrylamide gel. The resolved proteins were then processed in three ways. (A) Coomassie blue staining of secreted proteins from the *mcb* mutant (lane 1). Purified *H. insolens* endoglucanase (1 μg) was loaded in lane 2. (B) A zymogram (i.e. a CMCase activity stain) of secreted proteins from the *mcb* mutant (lane 3). (C) Western analysis of secreted proteins from the *mcb* mutant using an antibody raised against a *H. insolens* endoglucanase (lane 4), and 0.1 μg of purified *H. insolens* endoglucanase (lane 5). Molecular mass standards (kDa) are shown on the left side of the gel.

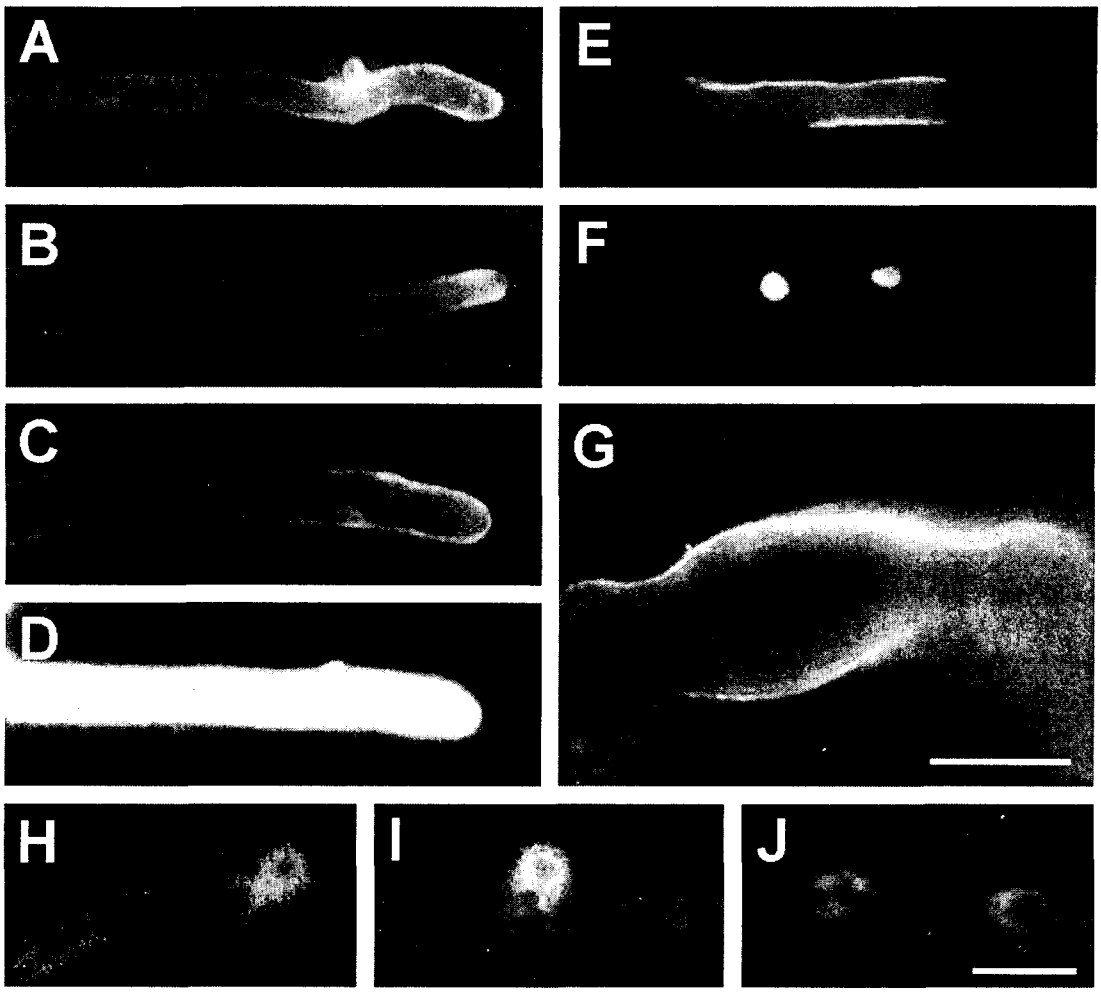

Figure 5. Localization of the sites of protein secretion in *N. crassa* hyphae.
For A-F, the various strains were grown overnight on sucrose minimal agar media and small pieces of agar, containing mature hyphae, were transferred to the middle of filter papers (0.5 x 0.5 cm) overlaid on sucrose or CMC supplemented minimal agar media and incubated overnight at 25℃. The filters were processed for indirect immunofluorescence to visualize the sites at which extracellular proteins exit *N. crassa* hyphae. (A) Wild-type grown on a sucrose minimal agar medium. (B) Wild-type grown on a CMC supplemented minimal agar medium. (C) *mcb* grown on a sucrose minimal agar medium. (D) *mcb* grown on CMC supplemented minimal agar medium. Photographs (C) and (D) were taken at equivalent exposure to demonstrate actual difference in signal. (E) Hyphal fragment of *mcb* grown on CMC supplemented minimal plates. (F) Nuclei contained with the hyphal fragment (E) are observed by DAPI staining. For G-J, *mcb* hyphae were grown on CMC supplemented minimal plates overnight at 25℃ and then shifted to 37℃ and incubated for 6 h. Pictures A-G were taken at the 100X magnification and bar indicate 10 μm. Pictures H-J were taken at 40X magnification and bar indicates 50 μm.