Construction and Characterization of *Aspergillus niger* Expression Vectors

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ABSTRACT

Construction and Characterization of
Aspergillus niger expression vectors

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I constructed and characterized the expression of a set of integrating *Aspergillus niger* expression vectors. In all these vectors, the *E. coli* GUS gene was used as a reporter and the glucoamylase gene (*glaA*) of *A. niger* was used to drive gene expression. Four glucoamylase-GUS fusions each with a different portion of the glucoamylase gene were generated using a PCR based strategy. GUS activity was assayed in *A. niger* transformants expressing these fusions. Although the GUS mRNA levels as detected on RNA blots remained relatively unchanged, glucuronidase activity decreased as the length of the *glaA* coding region increased in the fusion genes. The GUS activity of the fusion proteins produced were more sensitive to high concentrations of SDS than GUS expressed in *Helix pomatia*. These results suggest that the GUS activity of the glucoamylase-GUS chimeras is unstable. I also obtained results which confirmed that selecting for the *amdS* gene is effective in isolating transformants harbouring many copies of the plasmid. A combination of RNA and DNA blot analysis was used to show that both copy number and the site of integration dramatically affected GUS expression.

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ABBREVIATIONS

aa	amino acid
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
hr	hour
kb	kilobases
min	minute
mRNA	messenger ribonucleic acid
PMSF	phenylmethylsulfonyl fluoride
PNPG	4-nitrophenyl-ß-D-glucuronide
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
X-gluc	5-bromo-4-chloro-3-indolyl ß-D-glucuronide
µg	microgram
µl	microliter

1. INTRODUCTION

The name *Aspergillus* was first introduced by Micheli (1729, cf Raper and Fennel, 1965) for the moulds with a characteristic pattern of conidiophores and sporeheads. The fungi that belong to this genus are worldwide in distribution and are found on almost any type of substrate including foods, textiles, leathers, decaying vegetation, *etc*. (Raper and Fennel, 1965).

Aspergillus is an organism of considerable importance for a variety of biotechnological industries. The black Aspergilli (*A. niger*) are especially widely used in industry for the production of organic acids such as gallic acid, citric acid and gluconic acid (Lockwood, 1975), for the production of industrial enzymes such as amylase, glucoamylase, cellulase, hemicellulase, pectinase, glucose oxidase and catalase (Underkofler, 1976) and food fermentations practised in the orient (Wood, 1977).

The black Aspergilli are called *Aspergillus niger* because of the characteristic pigmentation of the conidial heads. The pigment production is greatly influenced by the presence of minute quantities of copper in the substratum and this has been used to estimate copper concentrations (Mudler, 1948). This easily recognizable fungus is found in all parts of the world. *Aspergillus awamori* is a variety within the species *A. niger*.

The life cycle of *A. niger* consists of the sequence: hyphae --- airborne conidiospores --- hyphae. Septate hyphae forms mycelia which develop into the conidial apparatus during growth and produce

conidia at maturity. The dry conidia are easily spread by air. The conidia germinate under suitable conditions (such as ample nutrients, favourable temperature, *etc.*) and grow into hyphae. No sexual cycle is known for *A. niger*.

With the development of molecular biology in recent years, filamentous fungi, particularly several species of *Aspergillus*, have been widely used in industrial microbiology for the production of heterologous proteins. For example, biologically active human tissue plasminogen activator has been secreted into the growth medium by *Aspergillus nidulans* (Upshall *et al.*, 1987), calf chymosin has been secreted by *A. oryzae* (Tsuchiya *et al.*, 1994), and porcine pancreatic prophospholipase A_2 by *A. niger* (Roberts *et al.*, 1992). Moreover commercial quantities of hen egg-white lysozyme (1 g/l) (Jeenes *et al.*, 1993) and human lactoferrin (2 g/l) (Ward *et al.*, 1995) have been demonstrated to be secreted by *A. niger* and *A. awamori* respectively.

There are several advantages for using these fungi as hosts for the production of heterologous proteins (Saunders *et al.*, 1989). The fermentation processes and downstream processing are well established. These organisms, because they have a long history of use in the production of food products, are Generally Regarded As Safe (GRAS) by regulatory authorities. They are capable of secreting a wide range of proteins including heterologous proteins from organisms as distantly related as mammals. Since they are eukaryotes, they can glycosylate specific proteins.

In addition to the general attributes of filamentous fungi

summarized above, *Aspergillus niger* has the advantage of being able to secrete proteins more efficiently than others, e.g., some strains can secrete more than 30 grams per liter of glucoamylase (Ward, 1989; Dunn-Coleman *et al.*, 1991).

Although *A. niger* has several advantages as an organism for the production of heterologous proteins, its utility is limited because the genetics and molecular biology of this organism are still in their infancy. There are presently few cloned genes from *A. niger*, therefore there are limited numbers of promoters one can use for the expression of heterologous proteins (Buxton *et al.*, 1985; Gwynne *et al.*, 1987; Buxton *et al.*, 1989; Fungal genetics newsletter #38, 1991). There are few selectable markers available in *A. niger* strains for the introduction of exogenous DNA encoding heterologous proteins (*argB*, *pyrG*, Ble^R and *amdS*) (Austin *et al.*, 1990; Buxton *et al.*, 1985; Gwynne *et al.*, 1987; Buxton *et al.*, 1989; Fungal genetics newsletter #38, 1991; Kelly and Hynes, 1985). As well, it is relatively difficult to transform *A. niger*.

Most work that has been done for the production of proteins in *Aspergillus niger* is with the glucoamylase gene (*glaA*), therefore, I will review the work done on the *glaA* gene first.

1.1 The glucoamylase gene (*glaA*) of *Aspergillus niger*

1.1.1 Glucoamylases of *A. niger*

Glucoamylases comprise a class of extracellular enzymes, secreted by a variety of organisms, which progressively hydrolyze

starch and related poly- and oligo- saccharides to yield glucose (Fogarty and Kelly, 1979). The glucoamylase [α-(1-4), (1-6)-D-glucan glucohydrolase] of *Aspergillus niger* possesses both α(1-4) and α(1-6) debranching activities (Ueda *et al.*, 1974), and has been widely used in the starch processing industries (Norman, 1979). The glucose produced can directly be used for applications in the processing and preparation of foods.

Glucoamylase from *Aspergillus* sp. (EC 3.2.1.3) exists in two forms (Figure 1). Glucoamylase 1 (G1, 616 aa) has a large catalytic domain (aa 1-440), a linker region (aa 441-512), and a starch binding domain (aa 513 - 616) (Svensson *et al.*, 1982; 1986). Glucoamylase 2 (G2, 510 aa) lacks the starch binding domain (Svensson *et al.*, 1982).

The amino terminal portion of the two forms of glucoamylases is responsible for the hydrolysis of substrates. Through selected deletions, it was found that this region (aa 1-470) facilitates secretion and increases thermostability, but does not bind starch granules (Evans *et al.*, 1990). The linker regior is rich in serine and threonine residues. This region may stabilize the enzyme and protect it against proteolytic attack (Manjunath *et al.*, 1983). However, up to 30 aa from the C-terminal end of G2 can be deleted with little effect on the activity, thermostability or secretion of the enzyme (Evans *et al.*, 1990). The function of the starch binding domain is to allow the enzyme to absorb onto and therefore digest native starch granules. It has been shown that this region shares significant amino acid sequence homology with the starch-binding

domains of other carbohydrases (Svensson *et al.*, 1989). G2 does not have the starch binding domain and therefore is unable to absorb onto and degrade starch granules (Ueda, 1981). This phenomenon has been used to purify G1 from G2 using starch as an absorbent (Ueda *et al.*, 1974). Both G1 and G2 are active towards soluble substrates but G1 shows increased activity towards insoluble forms. The two enzymes have the same pH optima (pH=5.0) when starch is used as substrate, temperature stability at elevated temperatures and immunological cross-reactivity (Lineback et *al.*, 1969).

1.1.2 Structure of the *glaA* gene

The *glaA* gene of *A. awamori* was isolated by screening genomic libraries using the cDNA to the glucoamylase mRNA as a probe (Nunberg *et al.*, 1984). All positive clones contained a 3.4 kb *Eco*RI fragment encoding the *glaA* gene. DNA blot hybridization analysis using the cloned *glaA* gene as a probe indicated that there is only one copy of this gene in the *A. awamori* genome.

There are multiple transcription initiation sites within a region -73 to -52 nucleotides from the translation start site (ATG), as determined by primer extension and nuclease S1 mapping analyses (Nunberg *et al.*, 1984). A Goldberg-Hogness TATA box (TATAAAT) was found 31 bp upstream of the first transcription initiation site, a CCAAT box (CAAT) was found 62 bp upstream of the TATA box. The polyadenylation site is 116 bp after the stop codon of G1.

Comparison between the *glaA* gene sequence and the G1 cDNA

sequence revealed the presence of 4 introns, all of these are small and range from 55 to 75 bp in length (Nunberg *et al.*, 1984). It was shown that the G1 mRNA contains a 169 nucleotide intervening sequence that can be spliced out to generate a G2 mRNA. The removal of the intervening sequence from the G1 mRNA changes the reading frame and results in the addition of 8 aa at position 526 in G1 before a translation stop (Boel *et al.*, 1984a).

The precursors of the 2 forms of glucoamylases are 640 aa (G1) and 534 aa (G2) in length, both of them contain a putative signal peptide of 18 aa followed by a short peptide of 6 aa (Figure 1). The apparent molecular weight is 71000 dalton for G1 and 61000 dalton for G2. In their secreted form, G1 and G2 are 616 aa (Svensson *et al.*, 1983) and 510 aa (Svensson *et al.*, 1982; Smiley *et al.*, 1971) in length respectively. *In vitro* translation experiments indicated that the two forms of glucoamylases were expressed in close to equal molar amounts (Boel *et al.*, 1984a).

1.1.3 Regulation of the *glaA* gene

The *glaA* gene of *A. awamori* is regulated by carbon source (Nunberg *et al.*, 1984; Fowler *et al.*, 1990). Upon growth on starch or maltose, the *glaA* expression was maximumly induced. When glucose or sucrose was used as the carbon source, the glucoamylase activity detected in the growth media dropped to approximately 1/3 of the level observed when using starch. Also, little or no glucoamylase activity was found when glycerol or xylose was used as the carbon source.

Figure 1. Functional domains of the two forms of glucoamylases from *A. niger*. The precursors of the two forms of glucoamylases are 640 aa (G1) and 534 aa (G2) in length, both of them contain a putative signal peptide (SIG) of 18 aa followed by a short peptide of 6 aa. In their secreted form, G1 is 616 aa long and has a catalytic domain (aa 1-440), a linker region (441-512), and a starch binding domain (SBD, aa 513-616); G2 is 510 aa long and does not have the starch binding domain. Numbers shown in the figure correspond to the amino acid number in the glucoamylase precursors. G2 is generated by an alternative mRNA splicing event as indicated in the figure.


G1:
SIG
CATALYTIC DOMAIN
LINKER
SBD
1
19
25
465
537
640


G2:
SIG
CATALYTIC DOMAIN
LINKER
1
19
25
465
526
527
534

RNA blot analysis as well as *in vitro* translation followed by immunoprecipitation experiments demonstrated similar changes in the glucoamylase mRNA levels present in cultures grown on different carbon sources. These results showed that the regulation of the *gla*A gene is exerted primarily at the level of transcription (Nunberg *et al.*, 1984; Fowler *et al.*, 1990).

1.1.4 Regulatory segments in the promoter region of the *gla*A gene

Functional elements in the promoter region of the *gla*A gene have been defined by both Fowler *et al.* (1990) and Verdoes *et al.* (1994a).

In the studies carried out by Fowler *et al.* (1990), a series of *gla*A 5′ deletion plasmids were made. These deletion constructs were transformed into an *A. niger* strain whose native *gla*A gene has been disrupted. The transformants were grown on different carbon sources and assayed for glucoamylase activity. The effects of each deletion on the expression of the *gla*A gene was used to determine the upstream limits of regulatory sequences responsible for *gla*A expression. Their results showed that there are two functional elements within the upstream region of the *gla*A gene. Element I is between 562 and 318 bp upstream of the translation start site. It is responsible for high level expression of the *gla*A gene. Element II is within 224 bp of the translation start point. It is required for transcription initiation and provides basal level expression.

A more extensive study was done by Verdoes *et al.* (1994a). They used both titration and deletion analysis to characterize the

promoter region of the *glaA* gene. In their study a reporter strain containing a fusion of the *glaA* promoter of *A. niger* with the *E. coli* GUS gene integrated at the *pyrG* locus was used. Their studies showed that the introduction of multiple copies of the *glaA* gene promoter caused a decrease in the expression of GUS. This titration effect revealed the presence of at least one positive regulatory factor. Under different inducing conditions (maltose, maltodextrin or glucose), similar titration effects were seen which indicated the *glaA* gene expression under these conditions may be controlled by the same *trans*-acting factor. It was observed that the titration effect is more obvious in glucose grown cultures than in maltose or maltodextrin grown cultures, which implied that the amount of the regulatory proteins under these conditions were more abundant than in glucose grown cultures.

Xylose alone did not induce *glaA* expression, and when mixed with other inducing carbon sources (maltose, maltodextrin or glucose), the expression levels were lowered (compared to using the other carbon sources alone), which indicated that a repressor dependent mechanism may also be involved in *glaA* regulation. The repression with xylose was not influenced by the introduction of multiple copies of the *glaA* gene.

Deletion analysis showed that sequences between -815 and -517 bp were responsible for high level of expression while sequences within 517 bp upstream of the translation start site were needed for initiation of transcription and therefore contained at least one binding site for the putative regulatory protein.

The regulatory segments determined by these two groups are not completely the same, which may be due to the different strategies employed as well as the different conditions for the assays.

1.2 Production of proteins in *Aspergillus*

1.2.1 Strain improvement strategies

Aspergilli are the source of a number of industrial enzymes. Before these enzymes can be commercially exploited, it is generally necessary to undergo the process of strain improvement to increase production yields for the desired enzyme.

Traditional methods for strain improvement include mutagenesis and genetic recombination. Strain improvement through mutagenesis involves two steps. First, the strain has to be mutagenized by exposure to mutagenic chemicals or UV light. Random mutations of various kinds would be generated as a result of the exposure. Subsequently, the mutagenized strain with desirable traits has to be identified by analysis of a large number of putative mutant strains. The development of simple screening procedures for the identification of strains with improved characteristics has facilitated strain improvement strategies to a certain extent. One such procedure done on plates has been used by Mattern *et al.* (1992) to identify *A. niger* strains deficient in extracellular proteases. A number of strains have been isolated using this approach which showed higher levels of production for several enzymes, including proteases in *A. oryzae* and *A. sojae* (Hara *et*

al., 1992), and glucoamylase in *A. niger* (Bonatelli *et al.*, 1991) and *A. awamori* (Vialta and Bonatelli, 1988). However, additional mutations could also be generated in the selected strains during mutagenesis which may be a drawback in the commercial exploitation of these strains.

As an alternative to multiple rounds of mutagenesis, strain improvement could also be done through genetic recombination to combine several favourable characteristics in one strain. Although many species of *Aspergillus* do not have a sexual cycle, genetic recombination can be carried out in diploids formed by protoplast fusion or hyphal fusion. Using this approach, a higher production level of proteins has been achieved, including protease production in *A. oryzae* and *A. sojae* (Hara *et al.*, 1992) and glucoamylase in *A. niger* (Ball *et al.*, 1978). A disadvantage of this method is that it is primarily limited to the same (or closely related) species.

As a completely new strategy for strain improvement, genetic engineering emerges with the development of molecular biology in recent years. The availability of recombinant DNA technology has allowed one to rapidly alter the genetic makeup of an organism. It is possible to introduce additional gene copies encoding the protein of interest, therefore increasing the yield for the protein encoded by the amplified gene. This strategy has made it possible to produce heterologous proteins in *Aspergillus* (Jeenes *et al.*, 1993; Broekhuijsen *et al.*, 1993) in addition to accelerating the rate of strain improvement. Most recent work that has been done for the production of proteins in *Aspergillus* has used this strategy.

In conjunction with traditional strain improvement methods, genetic engineering has enabled human lactoferrin to be produced in excess of 2 g/l in *Aspergillus awamori* (Ward *et al.*, 1995).

1.2.2 Production of native proteins

For the production of native proteins, genes encoding proteins of interest are introduced into the host (strain) of the same species through transformation. Transformants harbouring the introduced genes are selected then assayed for the yield of production. The selection markers used could be auxotrophic markers (*e.g. pyrG*), resistance markers (*e.g.* Ble^R, which confers Bleomycin resistance) or a dominant nutritional marker (*amdS*) (Finkelstein, 1991). *amdS* selection is particularly useful because it can be used to identify strains carrying many copies of the introduced genes (Kelly and Hynes, 1985). It has been shown that *A. niger* transformants that are able to grow using either acetamide or acrylamide as the sole nitrogen source generally contain more copies of the *amdS* gene than those that can only use acetamide as the sole nitrogen source. Also, retardation of growth occurs for strains carrying even more copies of the *amdS* gene in liquid media containing ω-amino acids (β-alanine or γ-amino butyric acids) as the sole nitrogen source (Kelly and Hynes, 1985).

Using the approach outlined above, glucoamylase (Finkelstein *et al.*, 1989; Verdoes *et al.*, 1993), catalase (Fowler *et al.*, 1993) and glucose oxidase (Whittington *et al.*, 1990) have been overproduced in *A. niger*. Using the same strategy, phytase

(Piddington *et al.*, 1993) and xylanase (Van Gorcom *et al.*, 1991) have been produced in *A. awamori*. Some of these are produced in high amounts (*e.g.* glucoamylase at 3.1 g/l before further optimization (Finkelstein *et al.*, 1989)).

Further attempts to improve the production yield using retransformation or genetic recombination may not always be successful. In the case of glucoamylase, Verdoes *et al.* (1993; 1994b) using *amdS* selection obtained *A. niger* transf mants carrying up to 200 copies of the *glaA* gene, but the production only increased about 20-fold (0.9 g/l). Introduction of additional copies of *glaA* by genetic recombination in diploids produced by using strains carrying many copies of *glaA* on different chromosomes did not result in a significant increase in the production of glucoamylase. In some strains frequent loss of the *glaA* copies were observed. The same was observed for retransformation of strains harbouring multiple copies of the *glaA* gene. Contrary to what was hoped for, these manipulations only produced a decrease in glucoamylase production. The newly introduced genes were shown to be heavily rearranged.

Another approach that can be used to further increase the production of proteins in multi-copy strains is medium development and controlled fermentation. It has been shown that the growth medium used can critically influence the yield of the desired product (Smith and Wood, 1991). Since many industrial fermentations are carried out in 100,000-liter vessels, well defined fermentation conditions is of great importance. By altering the medium and using

controlled large scale fermentation, Finkelstein *et al.* (1989) further increased glucoamylase production from 3.1 g/l to 9.0 g/l in *A. niger*.

1.2.3 Production of heterologous proteins

One useful aspect of molecular biology is that it has made it possible to express foreign proteins in an organism. The efficient production of foreign or heterologous proteins in filamentous fungi, however, is often more difficult compared to homologous protein production. The problems associated with expressing heterologous proteins include synthesis, glycosylation and secretion. In addition degradation by proteases present in the culture medium can also adversely affect expression (Cohen, 1977; Thompson, 1991). As a result of these problems, yields of heterologous proteins have generally been low compared to those of homologous proteins (Van den Hondel *et al.*, 1992).

Various strategies have been employed to improve the production of heterologous proteins in *Aspergillus*. For proper synthesis, promoters of strongly expressed *Aspergillus* genes, like the promoters from the constitutively expressed gene encoding glyceraldehyde-3-phosphate dehydrogenase and the *glaA* gene, are usually used for the production of heterologous proteins. Since the fermentation conditions have been optimized for the production process for glucoamylase in industry, the use of the *glaA* promoter is of great importance because this may allow high level production of the desired heterologous protein under the optimized culture

conditions.

To have the desired protein secreted, different secretion signals were used, including that of the heterologous protein itself, or the 24 aa signal sequence of the *glaA* gene. Although the heterologous signal peptide worked at times, *i.e.*, the heterologous protein was found in the medium (Jeenes *et al.*, 1993; Tsuchiya *et al.*, 1994), it was found that the *glaA* signal sequence was more efficient (Carrez *et al.*, 1990). In some instances, the secretion signal of the heterologous protein was simply not functional (Roberts *et al.*, 1992; Broekhuijsen *et al.*, 1993). Even using the glucoamylase signal sequence may not result in the heterologous protein being secreted (Broekhuijsen *et al.*, 1993).

Further improvement of secretion was achieved by fusing part of the glucoamylase protein before the protein of interest. It was hypothesized that including part of an efficiently secreted *Aspergillus* protein would improve expression because the protein would be more efficiently processed during passage along the secretion pathway. It was known that glucoamylase has several functional domains (catalytic, linker and starch binding domains, Svensson *et al.*, 1982; 1986), therefore, to allow for correct folding, the protein of interest was usually fused after the linker region of glucoamylase. This was because the linker region separates the catalytic and starch binding domains in glucoamylase. It was therefore hypothesized that fusion after the linker would permit independent folding of the catalytic domain of glucoamylase and the heterologous protein. On the other hand, fusion with the

entire glucoamylase may fail in efficient secretion and/or processing due to spatial interference. Results supporting this idea were obtained by Tsuchiya *et al.* (1994), who fused glucoamylase of *A. oryzae* (aa 1-603, lacking only 9 aa at the C-terminus) to the cDNA encoding prochymosin and found this fusion was only secreted as efficient as a construct without any glucoamylase sequence, whereas fusion to glucoamylase lacking the starch binding domain (aa 1-511) resulted in a 5-fold increase in chymosin production. Note, all of these fusions were expressed under the control of the *gla*A promoter. In contrast, Ward *et al.* (1990) found that a prochymosin fusion gene with the full length *A. awamori gla*A gene improved the production yield of chymosin.

To obtain the heterologous protein in a mature and functional form, processing of the fusion protein is required. This can be achieved by the introduction of a KEX2 processing site (Broekhuijsen *et al.*, 1993). In some cases, the extracellular proteases could process the fusion proteins (Roberts *et al.*, 1992). Also, the fusion protein may be processed by itself (autocatalytically) as in the case of chymosin (Ward *et al.*, 1990; Tsuchiya *et al.*, 1994).

There is a protease in *Aspergillus* with specificity like the KEX2 protease from *Sacchromyces cerevisiae* (Contreras *et al.*, 1991). Several studies have placed KEX2 sites at the fusion junction between glucoamylase and the heterologous protein of interest. In all cases where this strategy was used, the fusion proteins were correctly processed at the KEX2 site generating

mature, biologically active heterologous proteins in the media (Carrez *et al.*, 1990; Jeenes *et al.*, 1993; Broekhuijsen *et al.*, 1993; Ward *et al.*, 1995). In cases where the KEX2 site was not introduced, the secreted fusion protein may not be processed, as in the case of the hIL-6 (human interleukin-6) fusion with glucoamylase (aa 1-514) of *A. niger* (Broekhuijsen *et al.*, 1993), in which case the hIL-6 was not functional (or having very little activity) in the fused form, although substantially more fused protein was produced compared to a similar construct where KEX2 was present.

The glucoamylase-porcine pancreatic prophospholipase A2 fusion protein (Roberts *et al.*, 1992) was secreted in young cultures as a fusion protein. In older cultures, it was correctly processed from the glucoamylase portion to yield active phospholipase, although 2 other forms of phospholipases were processed from the fusion protein which included 2 aa (Ser-Arg) from the propeptide. These 2 forms of phospholipases differed slightly in charge, but had indistinguishable size on SDS-PAGE.

The fusion protein may also be processed autocatalytically. It was shown by immunoblot analysis that chymosin was processed by this mechanism from the fused glucoamylase protein (Tsuchiya *et al.*, 1994).

The fusion plasmids were transformed into *Aspergillus* strains and transformants were selected for their ability to produce the fusion proteins. One interesting discovery is that the expression of the glucoamylase protein without the starch binding domain

results in an active enzyme, even in a fused form. This has been used to select transformants expressing the highest amount of the fusion protein (Broekhuijsen *et al.*, 1993). It supports the hypothesis that fusion after the hinge region permits independent folding of both the catalytic domain of glucoamylase and the heterologous protein in the fused form.

One advantage of using filamentous fungi as hosts for protein expression is that they glycosylate proteins in a manner similar to that of animal cells. Because protein glycosylation may affect enzyme activity, this aspect has drawn some attention. Ward *et al.* (1995) have shown that the core linkage and extent of glycosylation of recombinant lactoferrin secreted from *A. awamori* were similar to the human breast milk lactoferrin. Although there were differences in specific carbohydrate residues, the biological activity of recombinant lactoferrin was not affected. On the other hand, it was also shown that recombinant human interleukin-6 secreted by *A. nidulans* was not N-glycosylated, although 2 potential N-glycosylation sites were present (Carrez *et al.*, 1990). In a few cases where overglycosylation was observed, this type of modification did not affect the activity of the recombinant protein (Boel and Huge-Jensen, 1989; Ward and Kodama, 1991; Joutsjoki *et al.*, 1993).

Aspergillus niger is known to secrete a number of proteases (Jeenes *et al.*, 1991; Van Noort *et al.*, 1991). The major protease is aspergillopepsin A. It is responsible for 80-85% of the total extracellular protease activity and is inhibited by pepstatin. In

addition, there is a second protease called aspergillopepsin B, it is much less sensitive to inhibition by pepstatin. The presence of these proteases in the culture medium may lead to a situation in which a heterologous protein is highly expressed and efficiently secreted but recovered in low yields because of proteolytic degradation. Although some proteins were less sensitive to proteases secreted by *A. niger* (like hen egg-white lysozyme, Jeenes *et al.*, 1993), there is still a need for solutions to this problem. For this purpose, several strategies were devised. The first of these was to isolate protease-deficient strains, for example, by UV-mutagenesis or gene disruption as described by Mattern *et al.* (1992). The isolated protease-deficient strains were shown to dramatically improve the production yields for a number of heterologous proteins (Broekhuijsen *et al.*, 1993; Roberts *et al.*, 1992). The second approach was to protect the secreted heterologous proteins from proteolysis. Ammonium addition to the medium is known to repress protease secretion (Cohen, 1977) and this has been used to elevate the production level of chymosin (Tsuchiya *et al.*, 1994). A third method which might be proven useful is to express the protein of interest in a fused form with glucoamylase but not to insert a KEX2 site in the fusion junction, as in the case of production of human interleukin-6 (Broekhuijsen *et al.*, 1993). Although the heterologous protein may not be active in the fused form, being part of the homologous enzyme glucoamylase in a fusion protein might offer some protection against proteolysis. As shown by immunoblot analysis, this fused form was produced in

substantially higher amounts than the unfused form in which there was a KEX2 site at the fusion junction (Broekhuijsen *et al.*, 1993). However, to obtain heterologous protein in a mature, active form, subsequent treatment *in vitro* with protease or chemical methods is needed.

A further increase in the production of some proteins was achieved by the use of a different medium. Using soy medium (for the production of hen egg-white lysozyme, Jeenes *et al.*, 1993) or wheat-bran solid state culture (for chymosin, Tsuchiya *et al.*, 1994), resulted in 10-fold and 500-fold increases in production of the desired proteins respectively.

A number of assays have been used to quartify the expression of heterologous proteins. Gene expression can also be assayed using immunoblotting and RNA blotting.

By applying the above described strategies, very high level production of heterologous proteins in *Aspergillus* have been achieved, *e.g.*, 1 g/l of hen egg-white lysozyme in *A. niger* (Jeenes *et al.*, 1993) and 2 g/l human lactoferrin in *A. awamori* (Ward *et al.*, 1995). Future research in this area is expected to enable heterologous proteins to be produced at the very high levels obtained with homologous proteins like glucoamylase.

1.3 Rationale for the present project

Since using *A. niger* as a host for the production of heterologous proteins appears to be a promising field of research, we intended to explore the potential for improving the utility of

this organism. For this purpose, it was necessary to characterize the expression of the *gla*A gene of *A. niger* and that was why the present project was initiated.

1.3.1 Reporter gene GUS

Characterization of the *A. niger gla*A gene expression in the present work was done using GUS as the reporter gene. The GUS reporter gene system developed by Jefferson *et al.* (1986) uses the *E. coli* ß-glucuronidase gene (GUS) as a gene fusion marker for analysis of gene expression. It has been proven to be both sensitive and versatile.

The natural habitat of *E. coli* is the gut, which is a rich source of glucuronic acid compounds. These compounds are taken up by *E. coli*, cleaved by ß-glucuronidase and the glucuronic acid residue released is used as a carbon source. The utilization of these compounds by *E. coli* is part of a process called enteroheptic circulation. This circulation is one of the major pathways of detoxification of endogenous and xenobiotic organic compounds in vertebrates (Dutton, 1966; 1980). The gene encoding ß-glucuronidase is *gus*A (formerly *uid*A), it is one of the 3 structural genes of the GUS operon in *E. coli* (Novel and Novel, 1973).

ß-glucuronidase (EC 3.2.1.31) is a hydrolase that catalyses the cleavage of a wide variety of ß-glucuronides (Stoeber, 1961). It is a tetrameric glycoprotein composed of 4 identical subunits. The monomers are synthesized as precursors of M_r=75,000-82,000 and are proteolytically processed at the C-terminus. The whole enzyme

is stable to many detergents and a wide variety of ionic strength conditions. It is very stable at 37°C and is highly resistant to high temperature denaturation. For example, human ß-glucuronidase is almost completely stable to heating at 65°C for 90 min (Gallagher, 1992). *E. coli* ß-glucuronidase has a broad pH optimum (pH 5.0-7.5), it is half as active at pH 4.3 and pH 8.5 as at its pH optimum of 7.0 (Jefferson *et al.*, 1986). It is known that ß-glucuronidase is inhibited by certain divalent cations (Cu^{2+}, Zn^{2+}, *etc.*), glycerol and potassium iodide (Gallagher, 1992).

The advantages for using GUS as a reporter gene include: i) It is easy to quantify by fluorometry, spectrophotometry and allows for histochemical localization. ii) It has very low background in a variety of tissues and organisms, including *A. niger* (Roberts *et al.*, 1989). iii) Due to the extensive analysis of mammalian glucuronidases (Paigen, 1979), many substrates are commercially available. iv) ß-glucuronidase is capable of tolerating large amino terminal additions (Gallagher, 1992) and can be translocated across membranes (Jefferson *et al.*, 1987), thus allowing the study of translocation and processing events involved in protein transport. The GUS gene used in this work has been mutagenized to eliminate a N-linked glycosylation site, so that it could be used in secretory or vacuolar targeting studies because its enzymatic activity could be retained when it enters the endoplasmic reticulum (Farrell and Beachy, 1990).

1.3.2 Outline of this thesis

This thesis describes the construction of a series of *A. niger* expression plasmids and their characterization. For this purpose, different portions of the glucoamylase protein were fused in front of the GUS gene. These fusion constructs were transformed into *A. niger* and their ability to express GUS was assayed to determine the optimum length of the glucoamylase portion of the fusion protein. A zymogram was also used to characterize the expression of the fusion proteins. DNA and RNA blot analysis were performed to correlate the GUS expression with its copy number and RNA level in transformants. In addition, GUS assays were done under various conditions to find out the best conditions for the production of the fusion proteins.

2. MATERIALS AND METHODS

2.1 Growth and transformation of *Aspergillus niger*

2.1.1 Media

The media used for *A. niger* were practically the same as those used for *Aspergillus nidulans* (Kafer, 1977) and they are outlined below. Agar (or agarose) was added to 1.5% final concentration for plates and 0.7% for overlayers.

A. Minimal Medium (MM)

Minimal Medium contained per liter: 20 X nitrate salts, 50 ml; $MgSO_4$, 0.52 g; trace elements, 1 ml; D-glucose, 10 g. The 20 X nitrate salts used in the media comprised 1.41 M $NaNO_3$, 0.14 M KCl, 0.12 M KH_2PO_4, 0.12 M K_2HPO_4. The Trace Elements contained per 100 ml: $ZnSO_4 \cdot 7H_2O$, 2.2 g; H_3BO_3, 1.1 g; $MnCl_2 \cdot 4H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $CoCl_2 \cdot 5H_2O$, 0.16 g; $CuSO_4 \cdot 5H_2O$, 0.16 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.11 g; Na_4EDTA, 5.0 g; with the pH adjusted to 6.5 with KOH.

B. Complete Medium (CM)

The Complete Medium contained per liter in addition to the components in the minimal medium the following constituents: peptone, 2 g; yeast extract, 1 g; casamino acids, 1 g; and 1 ml of vitamin solution which comprised 0.01% of biotin, pyridoxin, thiamine, riboflavin, *p*-aminobenzoic acid, and nicotinic acid.

C. Media used for transformation using the *pyr* selection (section 2.1.3)

The *pyr* selection medium is simply MM with sucrose (205 g per liter) added. The *pyr* survival medium, in addition, had 10 mM uracil and 5 mM uridine.

D. Media used for acetamide transformation

The Acetamide Selection Medium contained per liter: 20 X potassium salts (0.14 M KCl, 0.12 M KH_2PO_4, 0.12 M K_2HPO_4), 50 ml; $MgSO_4$, 0.52 g; trace elements, 1 ml; CsCl, 2.1 g; sucrose, 205 g; D-glucose, 10 g; and 1 M acetamide, 10 ml. Agarose which contained less contaminants than agar was used in place of agar for plates and overlayers to reduce background growth. As well, CsCl was used to minimize the background. In case of the *pyr*$^-$ strain RS31, uracil and uridine were added to 10 mM and 5 mM final concentration respectively to both the acetamide selection and survival media.

The Acetamide Survival Medium contained per liter: 20 X potassium salts, 50 ml; $MgSO_4$, 0.52 g; trace elements, 1 ml; sucrose, 205 g; D-glucose, 10 g; proline, 1.15 g. Proline was used as the single nitrogen source for the protoplasts to determine their survival rates. Agarose was used instead of agar for plates and overlayers.

2.1.2 Culturing conditions for *A. niger*

Stock plates were prepared by adding about $1x10^7$ conidia to CM molten agar overlayers, the overlayers were then poured onto CM

plates (diameter: 10 cm) and allowed to solidify. The plates were incubated at 30°C for 5 - 6 days to generate large quantities of conidia. The conidia were harvested from stock plates as follows. About 5 ml sterile saline/Tween [0.5% NaCl, 0.002% Tween 80] were poured onto a stock plate, the conidia on the stock plate were mixed with the added saline/Tween with a glass spreader. The suspension was transferred to a vial and the concentration of conidia in the suspension was determined using a hemocytometer. The suspended conidia were stored at 4°C until use. *A. niger* liquid cultures were started from conidia at a concentration of $1x10^6$ conidia/ml liquid culture and grown at 30°C.

2.1.3 Transformation of *A. niger*

The *A. niger* strain used for transformation was the uracil requiring strain RS31 (*csp*A1; (III) *pyr*G5; Goosen *et al.*, 1987). Approximately $4x10^8$ six-day-old conidia were inoculated in 400 ml CM and grown at 30°C, 150 rpm for 16 hr. The mycelia were harvested by filtration through Miracloth (InterSciences Inc.) and blot-dried. The dried mycelia were digested with Novozyme 234 (InterSpex Products, Inc.) at a concentration of 10 mg/ml in lytic buffer [0.7 M NaCl, 0.2 M $CaCl_2$, pH 5.8] for about 2 hr at 30°C. When 30% - 50% of the cells seen under the microscope (400x magnification) turned into protoplasts, the digestion mixture was filtered. The filtrate was spun in a table-top centrifuge at 3000 rpm, 4°C for 10 min to collect the protoplasts. The protoplasts were washed with 10 ml of 0.7 M KCl, centrifuged again as above, and washed with 10 ml of SC

[1 M Sorbitol, 50 mM CaC_2]. After another round of centrifugation, the protoplasts were resuspended in SC at a concentration of $1x10^8$ protoplasts/ml. Aliquots of 200μl of the suspension were kept on ice for transformations.

A maximum of 10 μl DNA (about 10 μg) and 50 μl PEG buffer [25% PEG 8000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5] were added to each 200 μl aliquot and mixed well. The mixture was kept on ice for 20 min. Then 2 ml PEG buffer was added and the mixture was left at room temperature for 5 min. Four milliliters of SC was added and the mixture was centrifuged at 3000 rpm and room temperature for 10 min. The pellet was resuspended in SC to 500 μl. Aliquots of the resuspended protoplasts were plated using 4 ml molten agar overlayers at 45°C on selection plates.

Two kinds of transformation selections were used in this work, the *pyr* selection and the acetamide selection. For *pyr* selection (selecting for *pyrG*$^+$ phenotypes) the auxotrophic *pyr*$^-$ strain RS31 was used. The plasmid carrying the *pyrG* gene was pPYRG (Wilson *et al.*, 1988). Cotransformation of pPYRG with the fusion plasmids (section 3.1.2.1) was used for this selection. The acetamide selection selects for acetamidase encoded by the *amdS* gene which enables *A. niger* cells to utilize acetamide as a nitrogen source. The *amdS* gene was subcloned to a reporter gene plasmid to avoid cotransformation (section 3.1.1.2). Transformants were purified by streaking onto fresh selection plates, then spreaded onto CM plates to generate large quantities of conidia for further analyses.

Designation of transformants was as follows.

(1) The *pyrG*$^+$ transformants were designated as:

when cotransformed with pGGC16: "A", e.g.: A1-4

with pGL-GUS: "B", e.g.: B2-3

with pG2E-GUS: "C", e.g.: C1-17

with pG1E-GUS: "D", e.g.: D4-6

when transformed with only pPYRG: "E".

(2) The *pyrG*$^-$ acetamide transformants were called "Ac" followed by a number, e.g., "Ac16" means #16 of such transformants.

2.2 Plasmid construction

2.2.1 Manipulation and isolation of plasmid DNA

The methods for DNA manipulations, *i.e.*, digestion, modification, synthesis, and ligation were conducted according to standard protocols described in Sambrook *et al.* (1989). Enzymes and buffers used for DNA manipulations were obtained from Bio/Can Scientific, Boehringer Mannheim, or New England Biolabs. Restriction enzymes used were usually about 3-fold in excess of activity stated by the manufacturer and the reactions were allowed to proceed for at least 2 hr. For the ligations, a molar ratio of vector to insert of 1:4 was usually used.

Escherichia coli strain DH5αF′ was used for transformation and propagation of plasmids. Transformation of *E. coli* was carried out either by the $CaCl_2$ procedure (Hanahan, 1983) or by electroporation.

Small scale preparations of plasmid DNA were performed by the

alkaline lysis method (Sambrook *et al.*, 1989). Large scale preparations of plasmid DNA were performed either using the CsCl density gradient centrifugation procedure (Sambrook *et al.*, 1989) or using the Qiagen Plasmid Purification Kit (Qiagen Inc.).

2.2.2 PCR amplification of DNA

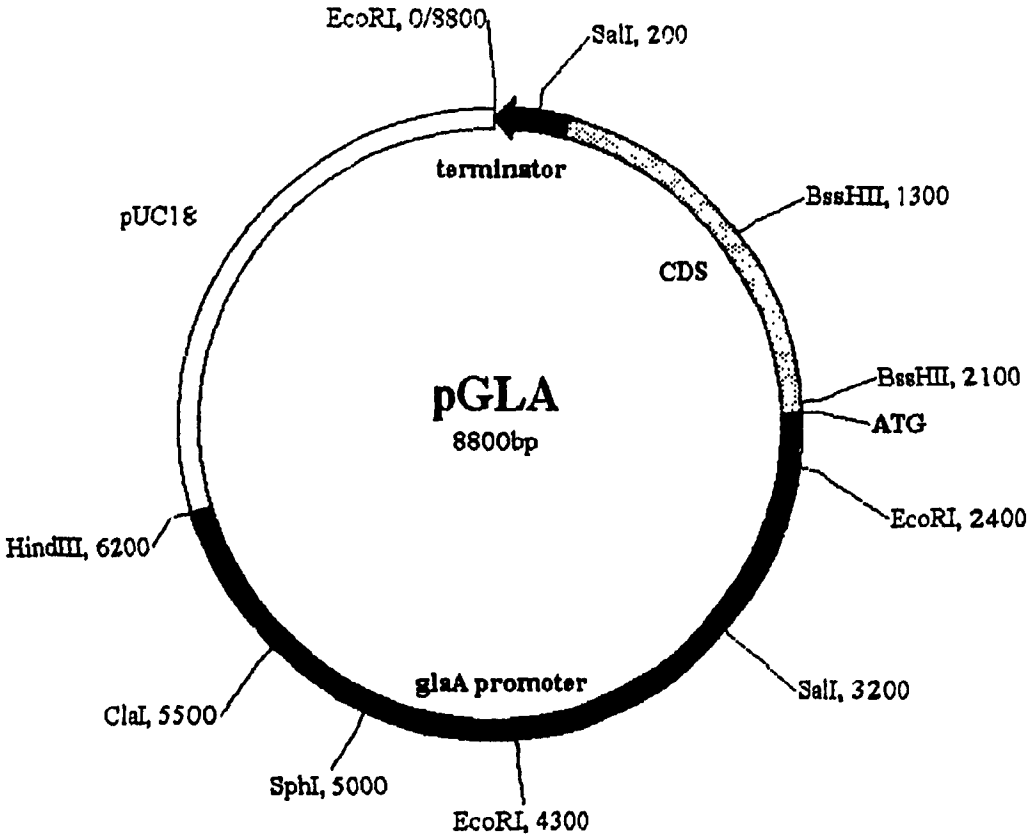
Polymerase chain reactions were done using the Expand long template PCR system (Boehringer Mannheim) following the manufacturer's instructions. The reaction was carried out in 50 µl final volume with 350 µM of each dNTP, 300 nM of each primer, 0.1 ng of plasmid pGLA (Figure 2; The plasmid pGLA carries a copy of the *glaA* gene.) as template, and 0.75 µl of the supplied enzyme mix (Taq and Pwo DNA polymerases). The initial denaturation was 95°C for 3 min, for each subsequent cycle it was 95°C for 30 sec. Annealing was done at 55°C for 30 sec and elongation at 68°C for 3 min. There were 30 cycles altogether. After the last cycle, there was a prolonged elongation at 68°C for 7 min. A Bio/Can Scientific Hybaid Thermal Reactor was used for the PCR reactions.

2.3 Assays for glucuronidase (GUS)

2.3.1 Filter GUS assay

Whatman # 1 filters were placed on top of CM agar and *A. niger* transformants were inoculated on the filters. After 1 day of incubation at 30°C, the filters were lifted from the plates and placed in liquid nitrogen. Then the filters were thawed at room

Figure 2. Plasmid map of pGLA. It carries a copy of the *glaA* gene. The positions of the *glaA* promoter, start codon (ATG), coding sequence (CDS) and the transcription terminator region are indicated. The two forms of glucoamylase proteins are generated as a result of an alternative RNA spicing event as described in **Section 1.1.2.**


EcoRI, 0/8800
SalI, 200
terminator
BssHII, 1300
CDS
pUC18
pGLA
8800bp
BssHII, 2100
ATG
EcoRI, 2400
HindIII, 6200
glaA promoter
SalI, 3200
ClaI, 5500
SphI, 5000
EcoRI, 4300

temperature and soaked in the assay solution [1 ml Z-buffer (Miller, 1972), 11.1 μl X-gluc (52 mg/ml in N,N - dimethylformamide)]. The X-gluc (Sigma) is a color substrate for β-glucuronidase. A blue color develops when X-gluc is broken down by glucuronidase. It could take from 2 hr to more than 24 hr for the blue color to appear.

2.3.2 Liquid GUS assay

Approximately 5 x 10^7 spores of *A. niger* transformants were inoculated in 50 ml of MM5 medium [minimal medium with 5% maltose as single carbon source], grown for 48 hr at 30°C, 300 rpm in 250 ml flasks. The mycelia were harvested by filtration, washed with 0.7% NaCl, squeezed, and powdered in liquid nitrogen. The powdered mycelia were resuspended in about 3 ml extraction buffer [50 mM $NaPO_4$, pH 7.0, 1 mM EDTA and 20 μM PMSF] by vortex mixing and kept on ice for 15 min to extract the proteins. After centrifugation at 12000 g for 15 min, a dilution of the supernatant was added to the GUS assay buffer [50 mM $NaPO_4$, pH 7.0, 5 mM DTT, 1 mM EDTA, 1 mM PNPG] and the reaction was allowed to proceed at 37°C. Then a 300 μl aliquot of the reaction mixture was added to 2.4 ml of 0.4 M Na_2CO_3 to stop the reaction, and the optical density at OD_{415} was measured to quantify the relative GUS activity. GUS activity of the growth media was assayed by adding the media directly to the GUS assay buffer. The protein concentration was determined by the Bradford method (Bio-rad Bradford assay protocol) using BSA as the protein standard. One unit of GUS activity is defined as one

nanomole of PNPG cleaved per minute per mg of protein.

2.3.3 *In situ* detection of glucuronidase using zymogram

An 8% SDS-polyacrylamide gel (Laemmli, 1970) was prepared and the crude extracts were mixed with cold loading buffer (composition varies as described in the **RESULTS**) and applied directly. Electrophoresis was done at 4°C, 200 volts for 45 min. Then the gel was washed once in cold Z-buffer (Miller, 1972) for 10 min, twice in Z-buffer for 15 min at room temperature. The substrate 4-methyl-umbelliferyl-ß-D-glucuronide (Boehringer Mannheim) was dissolved at 20 mg/ml in DMSO and diluted 1000-fold in Z-buffer containing 50 mM ß-mercaptoethanol. The gel was agitated for 10 min in the substrate solution at room temperature, rinsed with water and florescent enzyme bands were photographed under UV illumination through a pale yellow filter (Kodak Wratten 2E).

The gel was stained afterwards in Coomassie blue R-250 (0.1% in 50% methanol, 10% acetic acid) and destained in 10% methanol, 10% acetic acid to visualize the proteins.

2.4 DNA blot hybridization

2.4.1 Isolation of *A. niger* genomic DNA

Approximately 5×10^7 *A. niger* conidia were inoculated in 50 ml CM and grown in a 250 ml flask at 30°C, 300 rpm for 16 hr. The mycelia were harvested by filtration, washed with cold water and blot dried. The dried mycelia were weighed and ground to a fine

powder in liquid nitrogen. Added to each gram wet weight of the mycelia being processed were 5 ml of solution A1 [10 mM Tris·HCl, pH 8.0, 100 mM EDTA], 0.5 ml of solution A2 [20% Sarkosyl] and 0.5 ml of solution B [10 mg/ml RNase A in 50 mM NaOAc, pH 4.8]. The mixture was incubated at 65°C for 30 min, then proteinase K was added to a final concentration of 200 μg/ml and the mixture was incubated at 37°C for 3 hr. The mixture was centrifuged at 30,000 g for 30 min to remove the cell debris. The supernatant was extracted with phenol:chloroform several times until no protein was visible at the interface. The genomic DNA in the supernatant was precipitated with NH_4OAc and ethanol. The pellet was washed with 70% ethanol, air dried, and dissolved in a small volume of water. Optical density at OD_{260} and OD_{280} were measured to estimate the approximate concentration of the DNA.

2.4.2 Preparation of genomic DNA blots

Two micrograms of *A. niger* genomic DNA were digested with restriction enzymes and separated on a 0.7% agarose gel. The DNA was depurinated by soaking the gel in 0.25 N HCl for 15 - 30 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min, and neutralized in 1.5 M NaCl, 0.5 M Tris·Cl (pH 8.0) for 30 min. The DNA was blotted onto NYTRAN PLUS membranes (Schleischer & Schuell) as described in Sambrook *et al.* (1989) and fixed onto the membranes by UV irradiation for 30 sec using a UV Stratalinker 1000 (Stratagene).

2.4.3 Preparation of radioactive DNA probes

The probe was prepared by the random primer method (Bethesda Research Laboratories Random Primer Kit) using gel-purified GUS fragment (*Bss*HII fragment from pGGC16, Figure 3) as template and [α-^{32}P] dCTP (ICN Biomedicals Canada Ltd., 3000 Ci/mmole) as labelled substrate. The probe was purified by gel filtration through Sephadex G-50 and quantified using liquid scintillation counting. The probe was boiled for 5 min, then chilled on ice before adding to the hybridization bag.

2.4.4 Hybridization conditions

For prehybridizations, the membranes were kept in a Seal-a-meal bag with about 10 ml of hybridization solution [10% dextran sulfate, 50% deionized formamide, 1% SDS, 900 mM NaCl, 200 μg/ml denatured, sheared salmon sperm DNA] and incubated at 37°C for 1 hr. The denatured probe was added to the hybridization bag and mixed well. Hybridization was carried out at 37°C for 16 - 20 hr. After hybridization, the blot was washed once in 1 X SSC, 0.1% SDS at room temperature for 10 min, then 3 times in 0.1 X SSC, 0.1% SDS at 50°C for 15 min each.

After washing, the blot was wrapped in saran wrap and either exposed to X-ray film (Kodak) at -70°C with intensifying screens or exposed to the BI screen (BIORAD Molecular Imager system). The relative amount of signals was quantified using the software of the BIORAD imaging system.

2.5 RNA blot hybridization

2.5.1 Isolation of total cellular RNA from *A. niger*

The cultures for the RNA extraction were grown under the same conditions as those prepared for GUS activity assay (see **section 2.3.2**). After grinding the mycelia with liquid nitrogen, 10 ml of GSEM [50% guanidine thiocyanate, 0.5% sarkosyl, 25 mM EDTA, pH 7.0, 0.1% β-mercaptoethanol] was added per 1 g wet weight of the ground mycelia and the mixture was vortexed for 5 min. Two volumes of phenol:chloroform was added and the mixture was vortexed for 1 min then another 1 min with chilling on ice for 1 min in between. The mixture was centrifuged at 2000 rpm, 4°C for 5 min in a table-top centrifuge. The aqueous fraction was transferred to a fresh tube. The organic fraction was back extracted with phenol:chloroform and the aqueous fraction added to the original. Then 4 M NaOAc was added to the pooled aqueous fractions to a final concentration of 0.3 M. The mixture was subjected to multiple rounds of phenol:chloroform extractions until no debris was visible at the interface. Two volumes of 95% ethanol was added to the aqueous fraction to precipitate the RNA. The RNA was rinsed with 70% ethanol, and dissolved in RNase-free H_2O. Concentration of RNA was estimated by determining the optical density at OD_{260} and OD_{280}.

2.5.2 Hybridization conditions

Five micrograms of total RNA extracted from *A. niger* were denatured in sample buffer and run in a formaldehyde gel as described by Fourney *et al.* (1988). The gel was soaked in 0.05 M NaOH made in 1 X SSC for 15 min, then twice in 20 X SSC for 20 min

each. RNA was transferred to NYTRAN PLUS membranes (Schleischer & Schuell) and then linked to the membrane by UV irradiation. Prehybridization and hybridization conditions were the same as for the DNA blots except the hybridization solution used was 10% dextran sulfate, 50% deionized formamide, 1% SDS, 1 M NaCl, 100 µg/ml denatured, sheared salmon sperm DNA. Washing and signal quantification were the same as DNA blotting.

3. RESULTS

My objectives were to construct and characterize a series of *A. niger* expression vectors. To accomplish these goals it was necessary to begin by constructing a number of plasmid vectors. All the expression vectors constructed use the GUS gene as a reporter to monitor gene expression. In addition, they all utilize *glaA* sequences to direct reporter gene expression. Once constructed these expression vectors were used to study three aspects of gene expression. First I studied the role that the *glaA* coding region played in controlling gene expression. Next I studied the relationship between levels of *glaA* directed gene expression and its copy number. Finally, I studied how varying some growth condition parameters influenced the expression of my glucoamylase-GUS fusion genes. In the first section of the results I describe these plasmids and how they were constructed.

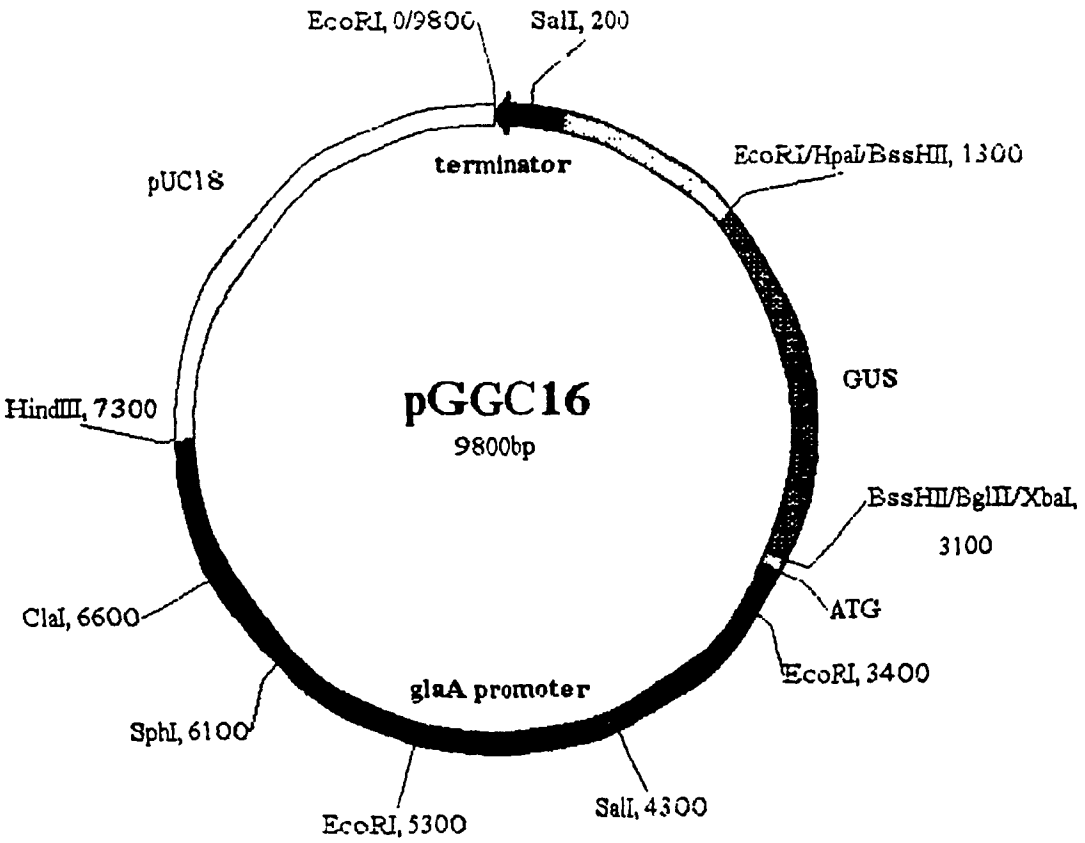
3.1 Expression of glucoamylase-GUS fusion genes

3.1.1 Plasmid construction

3.1.1.1 Construction of plasmids containing GUS fused to different regions of glucoamylase

The previously constructed plasmid pGGC16 (Figure 3, constructed by Dr. Yue Huang) carries an *E. coli* gene for glucuronidase fused to the *A. awamori* gene for glucoamylase. The

Figure 3. Restriction map of pGGC16 (constructed by Dr. Yue Huang). This plasmid carries a fusion of the first 24 aa of *A. awamori* glucoamylase with the *E. coli* GUS. To construct this fusion plasmid, a portion of the *glaA* coding sequence (Figure 2, the *Bss*HII fragment) was replaced with the PCR amplified GUS as indicated in the map.


EcoRI, 0/9800
SalI, 200
terminator
EcoRI/HpaI/BssHII, 1300
pUC18
GUS
pGGC16
9800bp
HindIII, 7300
BssHII/BglII/XbaI,
3100
ATG
ClaI, 6600
EcoRI, 3400
glaA promoter
SphI, 6100
EcoRI, 5300
SalI, 4300

coding region of GUS is fused in-frame to the DNA encoding the 24 N-terminal aa of glucoamylase. These 24 aa include the signal sequence for glucoamylase (Svensson *et al.*, 1983). In an attempt to reveal the correlation between the length of the glucoamylase protein fused before GUS and the expression of GUS, three longer fusion plasmids were constructed.

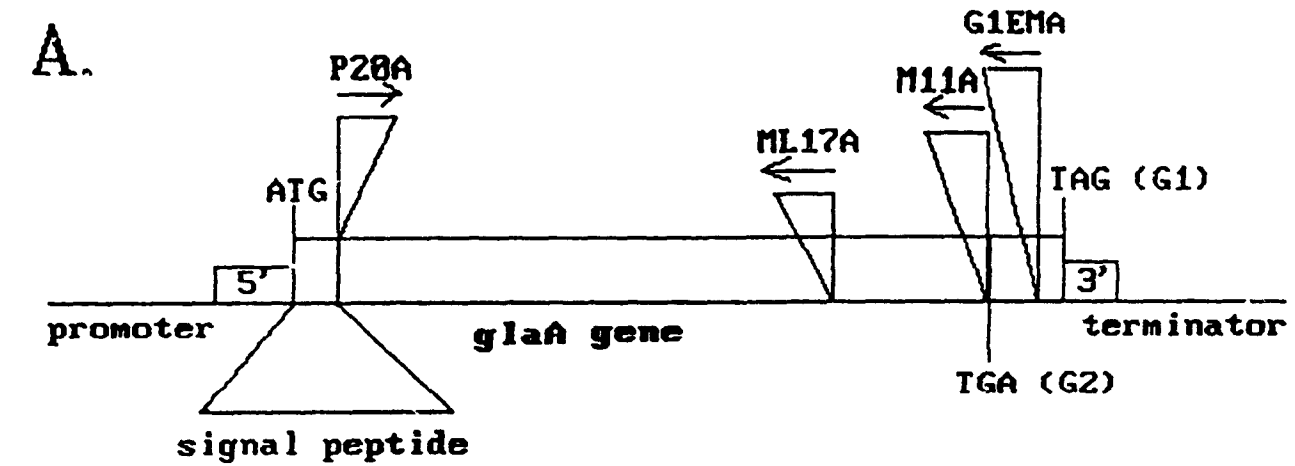
Three DNA fragments encoding different portions of the *glaA* coding sequence were amplified by PCR and placed in front of the GUS gene in pGGC16 generating 3 new fusion plasmids: pGL-GUS, pG2E-GUS and pG1E-GUS. The oligonucleotide primers and the strategy for PCR are illustrated in Figure 4. The PCR amplified DNA fragments were 1.7, 2.0, 2.1 kb in length for pGL-GUS, pG2E-GUS and pG1E-GUS respectively which resulted in the fusion of the *E. coli* GUS with the first 517 aa (end of the hinge region of glucoamylase), 533 aa (end of the G2 protein), 627 aa (end of the G1 protein) of the *A. awamori* glucoamylase in the final plasmids (Figure 5).

After the desired fragments were generated using PCR, the DNA fragments were digested with *Bcl*I and ligated with the *Bgl*II digested pGGC16, then transformed into *E. coli*. The desired plasmids pGL-GUS, pG2E-GUS and pG1E-GUS (Figure 5, 6) were identified by restriction digests of the plasmid DNA.

3.1.1.2 Construction of a plasmid containing *E. coli* GUS and *A. nidulans amdS*

The plasmid pGGC16 (Figure 3) lacks a selectable marker for *A. niger*. To construct an *A. niger* vector with a selectable marker, I

Figure 4. Diagram showing the strategy for the construction of longer fusion plasmids. **A.** The PCR amplification scheme. The start and stop codons of the 2 forms of glucoamylases (G1 and G2) are indicated. Positions of the sense and antisense primers are shown on the bar representing the *glaA* gene. Arrows indicate directions of extension. The primer sequences are shown, homologous sequences between the primers and the template are in bold face. Also shown in the primer sequences are the reading frames of the PCR products in the final fusions, indicating that they are in frame. *BclI* sites used for cloning are underlined. **B.** Strategy for the generation of different fusions. Fused DNA sequences in pGGC16 are represented in the diagram. The start and stop codons are indicated. Part of the signal peptide amino acid sequence is shown below the DNA sequence. The *BglII* site used for cloning is underlined. Also shown are the reading frame and the KEX2 site that was placed right in front of GUS in pGGC16. The PCR products were cut with *BclI* and inserted in the *BglII* site of pGGC16, generating 3 new fusion plasmids.



PCR products:

1.7 kb — GL-GUS

2.0 kb — G2E-GUS

2.1 kb — G1E-GUS

BclI

P20A primer sequence: 5' c tat gat cag acc ttg gat tca tgg tt 3'

ML17A primer sequence: 5' ta atg atc agt ctt gct ggt cga ggt 3'

M11A primer sequence: 5' ta atg atc aga cat acc aga gcg ggt 3'

G1EMA primer sequence: 5' at atg atc agc ctg agg aac ggt gta 3'

B.

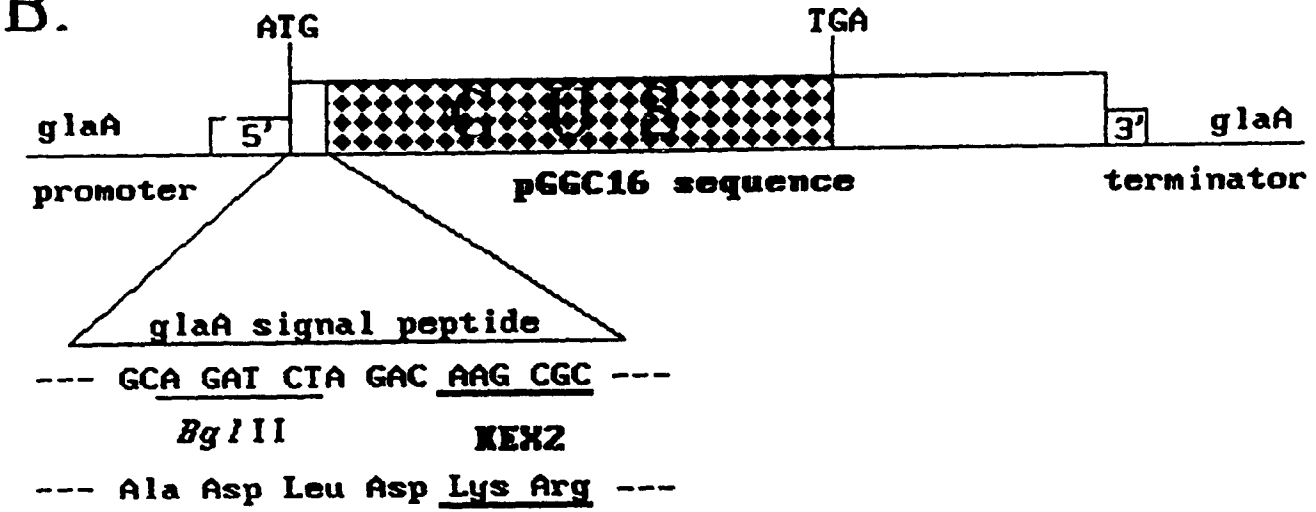
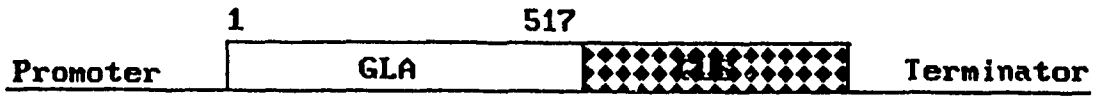
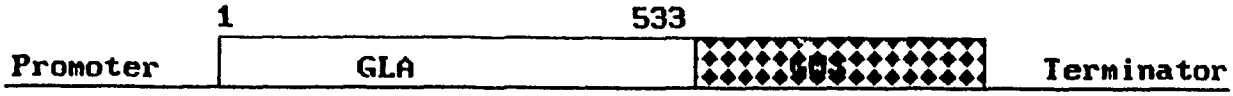

ATG
TGA
glaA
5'
3'
glaA
promoter
pGGC16 sequence
terminator
glaA signal peptide
--- GCA GAT CTA GAC AAG CGC ---
Bgl II
KEX2
--- Ala Asp Leu Asp Lys Arg ---

Figure 5. The different glucoamylase-GUS fusions used in this work. Numbers indicate the number of aa of the glucoamylase precursors (Figure 1) fused before GUS in each case.

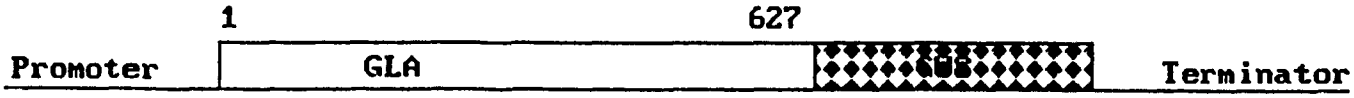
pGGC16



pGL-GUS



pG2E-GUS



pG1E-GUS

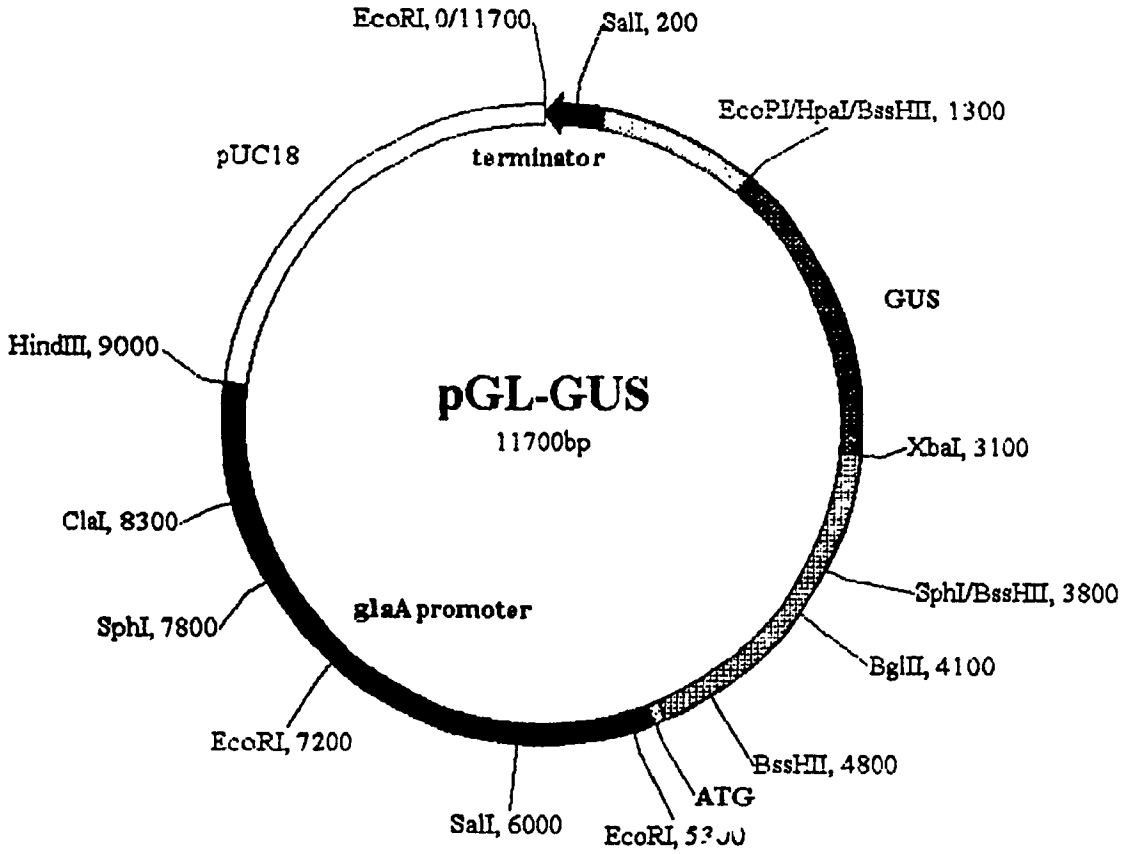


Figure 6. Plasmid map of pGL-GUS. The only difference among pGL-GUS, pG2E-GUS and pG1E-GUS is the length of the glucoamylase protein fused before GUS, as shown in Figure 5.

inserted the *A. nidulans* gene for acetamidase into pGGC16.

The plasmid pGGC16 was digested with *Cla*I, end-filled with Klenow and dNTP and then cut with *Sph*I. The digested DNA fragments were separated by agarose gel electrophoresis. The fragment containing the glucoamylase-GUS fusion was purified using the Geneclean II Kit (Bio 101 Inc.). The *amdS* gene was derived from the plasmid p3SR2 (Figure 7; Hynes *et al.*, 1983; a gift from Dr. E. Kafer, Institute of Molecular Biology and Biochemistry, Simon Fraser University, British Columbia). The plasmid p3SR2 was cut with *Eco*RI, end-filled, and then digested with *Sph*I and *Sca*I at the same time. The *amdS* containing fragment was purified by agarose gel electrophoresis. The purified fragments were ligated at 16°C overnight using T4 DNA ligase. The ligated DNA was transformed into *E. coli* strain DH5αF′ cells and transformants selected in the presence of 100 μg/ml ampicillin. The transformants were analyzed by restriction enzyme digests and the resulting construct was designated pGAMD (Figure 8).

3.1.2 **GUS activity in transformants**

3.1.2.1 GUS activity in different fusion construct transformants

The fusion plasmids pGGC16, pGL-GUS, pG2E-GUS and pG1E-GUS (Figure 5) were individually cotransformed with pPYRG into *A. niger* strain RS31. The filter assay was used to determine whether or not the *pyrG*$^+$ transformants obtained expressed the reporter gene (GUS). Out of a total of 153 cotransformants assayed, 51 turned blue in

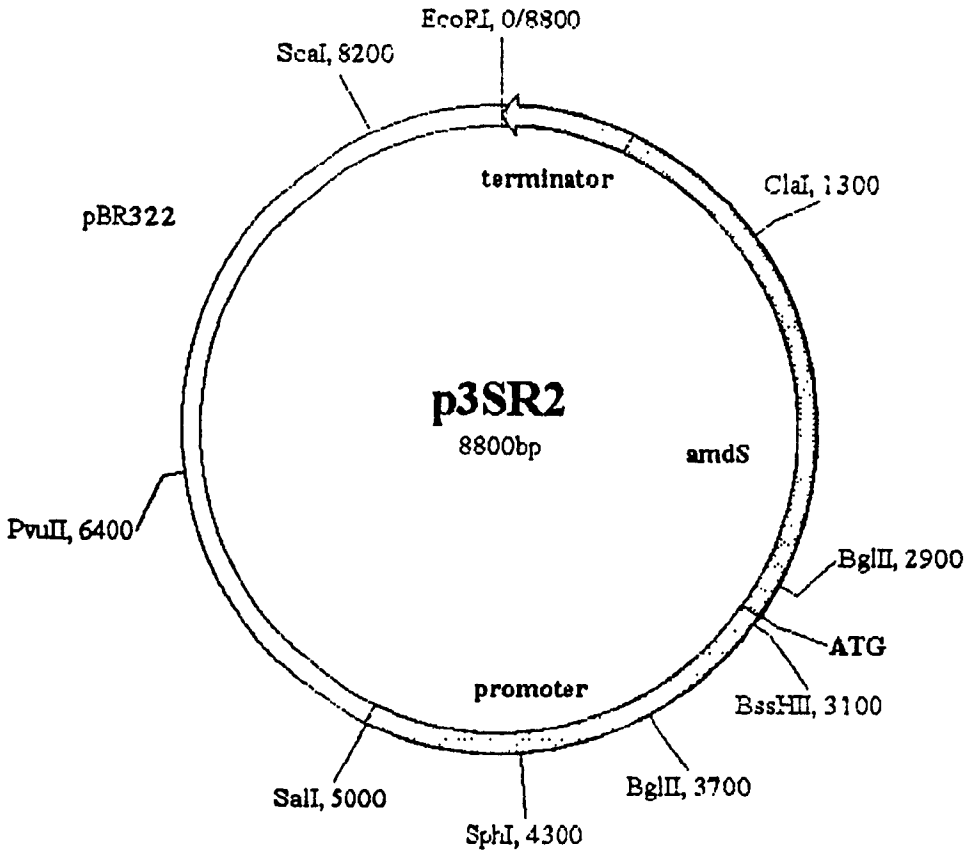
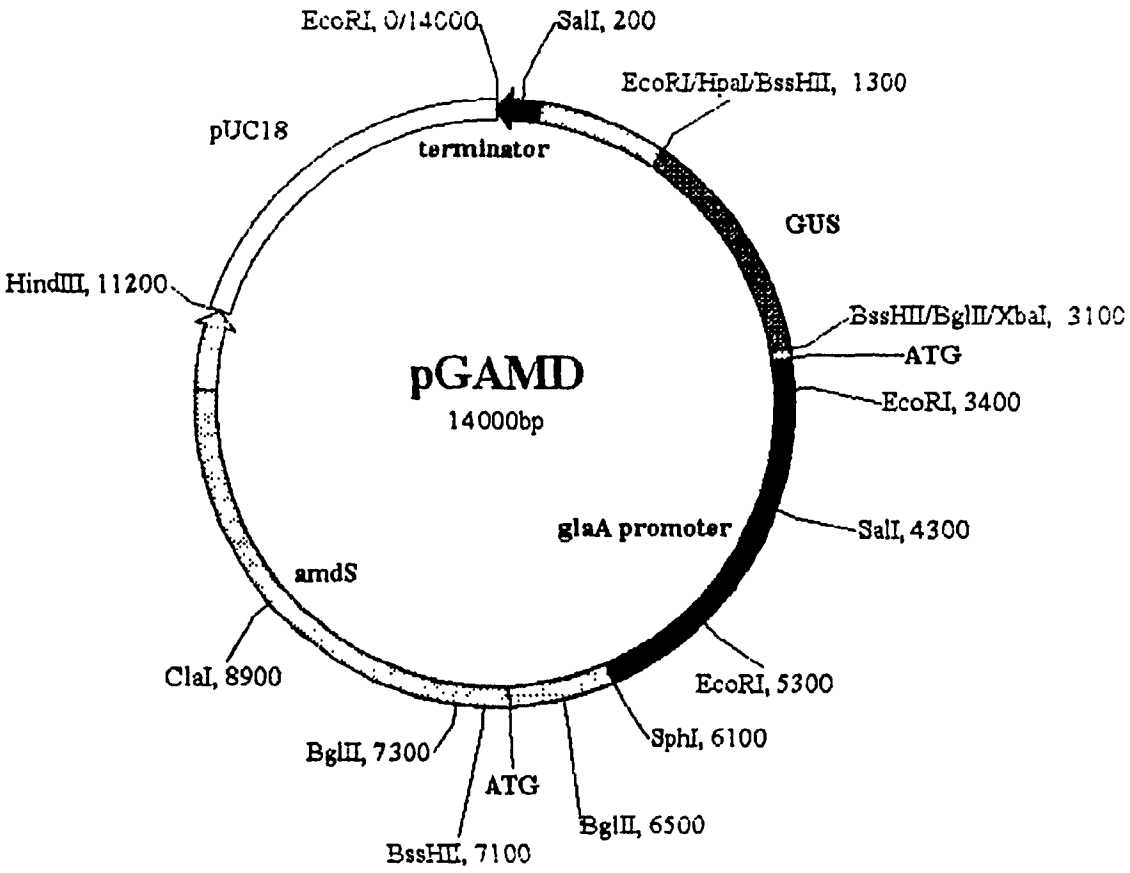


Figure 7. Restriction map of p3SR2 (Hynes *et al.*, 1983). This plasmid carries the *A. nidulans amdS* gene.

Figure 8. Restriction map of pGAMD. It has a selectable marker (*amdS*, from p3SR2, Figure 7) in addition to the fusion reporter gene (GUS). The advantage of using this plasmid is that all transformants must have the reporter gene.


EcoRI, 0/14000
SalI, 200
EcoRI/HpaI/BssHII, 1300
pUC18
terminator
GUS
HindIII, 11200
BssHII/BglII/XbaI, 3100
ATG
pGAMD
14000bp
EcoRI, 3400
glaA promoter
SalI, 4300
amdS
ClaI, 8900
EcoRI, 5300
SphI, 6100
BglII, 7300
ATG
BglII, 6500
BssHII, 7100

the filter assay. Three transformants that were a deep blue color on the indicator plates for each fusion construct were chosen for further analysis. First GUS activity was quantitatively determined using the liquid GUS assay (**section 2.3.2**). The results showed clear differences in GUS activity between individual transformants (Table I). First large differences were seen between transformants obtained using each of the fusion plasmids. Second, GUS expression levels varied significantly depending upon the fusion plasmid. The overall trend for the results obtained was that the pGGC16 transformants expressed the most activity, then pG1E-GUS transformants, the pGL-GUS and pG2E-GUS transformants expressed the least activity. It must be noted that some individual transformants did not conform to the overall trend.

3.1.2.2 GUS activity in acetamide transformants

The *amdS* gene of *A. nidulans* can be expressed in *A. niger* (Kelly and Hynes, 1985). When expressed in *A. niger*, the *amdS* gene enables some transformants to grow using acetamide or acrylamide as the carbon and/or nitrogen source(s). An interesting feature of this *amdS* gene is that it is very poorly expressed in *A. niger*, therefore, to grow efficiently on acetamide, *A. niger* transformants must harbour many copies of the *amdS* gene. We attempted to take advantage of this to isolate *A. niger* transformants with many copies of the glucoamylase-GUS fusion gene.

Aspergillus niger strain RS31 was transformed with pGAMD (Figure 8) and transformants selected on their ability to grow

Table I. GUS activity in different fusion construct transformants

Fusion plasmid	Number of aa fused before GUS	Strain[a]	GUS expression[b]
pGGC16	24	A2-3	1233
		A2-16	1694
		A1-4	262
pGL-GUS	517	B2-3	41
		B1-13	33
		B2-2	72
pG2E-GUS	533	C3-1	10
		C2-17	76
		C1-17	34
pG1E-GUS	627	D4-3	119
		D4-6	92
		D3-9	7
-	-	E	0.62

[a]Strain E (RS31 transformed with only pPYRG) was used as the negative control.

[b]MM5 medium (minimal medium with 5% maltose as single carbon source) was used to grow the transformants. GUS expression is indicated in nanomoles of PNPG cleaved/min/mg protein. Values shown are the mean value of at least 2 independent experiments each done in triplicate. Between experiments typical differences are within 20%.

Only background GUS activity could be detected in the medium (data not shown).

Table II. GUS activity in acetamide transformants

Strain[a]	GUS expression[b]
Ac1	190
Ac2	645
Ac3	1692
Ac4	184
Ac5	893
Ac6	503
Ac7	1714
Ac9	762
RS31	0.78

[a]The strains assayed were all RS31 (*pyrG*$^-$) pGAMD (Figure 8) transformants. The untransformed strain RS31 was included as a negative control.

[b]The culture medium used to grow the transformants was the MM5 medium supplemented with 10 mM uracil and 5 mM uridine. GUS expression is indicated in nanomoles of PNPG cleaved/min/mg protein. Values shown are the mean value of at least 2 independent experiments each done in triplicate. Between experiments considerable differences in expression level may be observed.

Only background GUS activity could be detected in the culture medium used to induce GUS expression (data not shown).

efficiently using acetamide as the single nitrogen source. GUS assays were performed on these transformants. The results in Table II show that considerable variations exist in the expression of GUS between the different transformants tested. The average level of expression of these acetamide transformants (823 units) is lower than that of the pGGC16 *pyrG*$^{+}$ transformants (1063 units, Table I). The pGGC16 *pyrG*$^{+}$ transformants assayed (Table I) were selected using the filter assay out of 14 blue transformants (40 transformants were screened in all to find these 14), whereas the acetamide transformants assayed (Table II) were taken at random. This may explain the differences in the average level of expression between these transformants.

3.1.3 **Detection of glucuronidase using zymogram**

GUS activity can be detected after size fractionation by SDS-PAGE (Gallagher, 1992). In this work, a zymogram was employed to try to size the GUS fusions while maintaining the GUS catalytic activity. The protocol used was as described by Dingerman *et al.* (1989).

Initially, SDS sample buffer without ß-mercaptoethanol (SDS SB) [62.5 mM Tris·Cl, pH 6.8, 2.3% SDS, 10% glycerol] was used for loading the crude extracts containing the fusion proteins, but only the positive control (ß-glucuronidase from *Helix pomatia*, SIGMA) had GUS activity in the gel (results not shown). It therefore appeared that the SDS SB buffer destroyed the glucuronidase activity of the fusion proteins. In order to determine what

component of the SDS SB was responsible for the inactivation, extracts prepared from strains B1-13 or A2-3 were incubated with SDS SB and SB. It was found that the positive control and the extracted fusion proteins reacted differently to the SDS SB (Table III) and the inhibitory effect of the SDS SB on the fusions is mainly due to the SDS (Table IV).

The results obtained also indicated that sensitivity to high concentrations of SDS is a characteristic of the fusion protein itself (Table V) and is not associated with some other component in the crude extract, because the wild type GUS did not exhibit similar characteristic in the presence of a crude extract prepared from transformant E (the negative control).

A zymogram experiment (Figure 9) supports the results of the GUS assay shown in Table V. That is, wild type GUS mixed with extracts from the negative control (transformant E) and in 2.3% SDS (Figure 9, lane 4), produced a bright florescent band after fractionation by gel electrophoresis. In contrast the A2-3 extract did not retain activity even when it was in Z buffer (Figure 9, lane 7).

In an attempt to make this system work, I increased the amount of GUS activity loaded onto the gel. As shown in Figure 10, even when A2-3 extract corresponding to about 20X more GUS activity than the positive control was loaded in lanes 2, 4 and 6 respectively, no activity was detected.

Table III. SDS sample buffer (SDS SB) inhibits GUS activity

Sample tested	Volume of extract used (μl)	GUS activity (OD_{405})	
		with SDS SB	without SDS SB
Positive control	2	0.012	0.020
	10	0.061	0.114
	100	0.557	0.941
B1-13 extract	2	0	0.005
	10	0	0.019
	100	0.003	0.185
E extract (negative control)	2	0	0
	10	0	0
	100	0.001	0.015

The volume of different of samples (2, 10, 100 μl) was adjusted to 100 μl with 1X M9 salts. The mixture was either mixed with 100 μl of 1X M9 salts or SDS SB (without β-mercaptoethanol), then assayed for GUS activity.

Table IV. SDS inhibits GUS activity of the fusion proteins

	GUS activity (OD_{415})
1X M9	0.349
10% Glycerol	0.345
2.3% SDS	0
62.5 mM Tris·Cl, pH 6.8	0.276
SDS SB	0.007

GUS assays were done on 20 μl of A2-3 extract in either 1X M9 salts, 10% glycerol, 2.3% SDS, 62.5 mM Tris·Cl (pH 6.8) or SDS SB (without β-mercaptoethanol) as indicated in the table. The final volume of the mixtures was 200 μl for each. Results presented are the average of 2 independent experiments. Differences between experiments are within 10%.

Table V. High SDS concentration eliminates GUS activity of the fusion proteins

	1 μl +'ve[a] control		10 μl A2-3 extract	10 μl -'ve[b] extract (from E)
	+ 9 μl extraction buffer	+ 9 μl -'ve extract (from E)		
Z Buffer	0.365	0.418	0.581	0
0.1% SDS	0.212	0.257	0.492	-
2.3% SDS	0.187	0.200	0	-

GUS assays were done on the extract mixes shown on the top of the table in either Z buffer, 0.1% SDS, or 2.3% SDS as indicated. Values presented (OD_{415}) are the average of 3 independent experiments. Differences between experiments are within 10%.

[a]Positive

[b]Negative

Figure 9. Zymogram showing the unusual characteristic of the fusion protein. Lane 1, 1 μl of wild type GUS + 9 μl of extraction buffer in 2.3% SDS; Lanes 2-5, 1 μl of wild type GUS + 9 μl of E extract (the negative control) in Z buffer, 0.1% SDS, 2.3% SDS, 2.3% SDS + 20 μM PMSF respectively; Lane 6, 10 μl of E extract in Z buffer; Lanes 7-10, 10 μl of A2-3 extract in Z buffer, 0.1% SDS, 2.3% SDS, 2.3% SDS + 20 μM PMSF respectively. All samples contained 10% glycerol and were prepared on ice. There was a florescent band in every lane loaded with the wild type GUS, but not in any of the lanes loaded with A2-3 extract or extract from E.

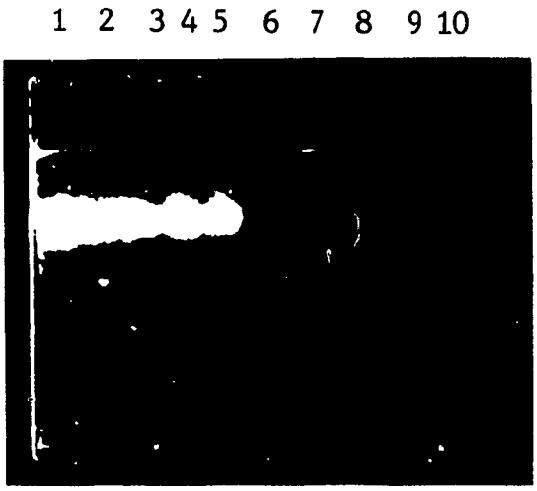

1 2 3 4 5 6 7 8 9 10

Figure 10. GUS fusion can not maintain its catalytic activity in the zymogram system. Lane 1, 1 μl of wild type GUS + 19 μl of extraction buffer in 2% SDS; Lanes 2, 4, 6: 20 μl of A2-3 extract in extraction buffer, 0.1% SDS, or 2% SDS respectively; Lanes 3, 5, 7: 20 μl of D4-3 extract in extraction buffer, 0.1% SDS or 2% SDS respectively; Lane 10, 20 μl of E extract in extraction buffer. All samples were prepared on ice and contained 10% glycerol.

1 2 3 4 5 6 7 8 9 10

3.2 Determination of glucoamylase-GUS copy number and relative mRNA concentration in transformants

3.2.1 Estimation of glucoamylase-GUS gene copy number in transformants

In order to quantify the copy number of the GUS fusion genes in the transformants as well as to have an idea about how they were integrated into the genome, DNA blot hybridizations were performed.

Genomic DNA was isolated from *A. niger pyrG*$^{+}$ transformants (section 3.1.2.1), digested with *Eco*RI or *Bgl*II, fractionated in a 0.7% agarose gel and blotted onto NYTRAN PLUS membranes. The blots were probed with the 1.8 kb GUS fragment isolated from *Bss*HII digested pGGC16 (Figure 3).

*Eco*RI digests were done to quantify the copy number of the GUS fusion genes in the transformants. GUS is not homologous to *A. niger* genomic DNA sequences, if integration of the fusion plasmids into the *A. niger* genome happens via homologous recombination (Fowler *et al.*, 1990), then recombination between the fusion plasmids and *A. niger* genomic DNA should not happen within the GUS gene. Since there is no *Eco*RI site in the coding region of the fusion genes (Figure 2, 6), digesting genomic DNA from the transformants with *Eco*RI would result in the generation of size-specific DNA fragments containing the GUS gene for the fusion plasmids (2.1, 4.0, 4.3, 4.4 kb for pGGC16, pGL-GUS, pG2E-GUS, pG1E-GUS respectively).

The size of major fragments observed for pGGC16 transformants

Figure 11. DNA blot used to determine GUS copy number in transformants. Two micrograms of genomic DNA were digested with *Eco*RI for each lane. The blot was probed with the 1.8 kb GUS fragment. Lanes 1-13 had genomic DNA from the following transformants: A2-3, A2-16, A1-4, B2-3, B1-13, B2-2, C3-1, C2-17, C1-17, D4-3, D4-6, D3-9, E (described in Table I, section 3.1.2.1). Loaded in lanes 14-16 were genomic DNA from E containing different amounts of pGGC16 DNA corresponding to 1, 5, 25 copies of the GUS gene. These were used as standards. Copy number of the GUS fusion genes in a transformant was determined by comparing the intensity of its signal on the DNA blot with the standards. The estimated copy number of the GUS gene for each transformant is shown in Table VI.

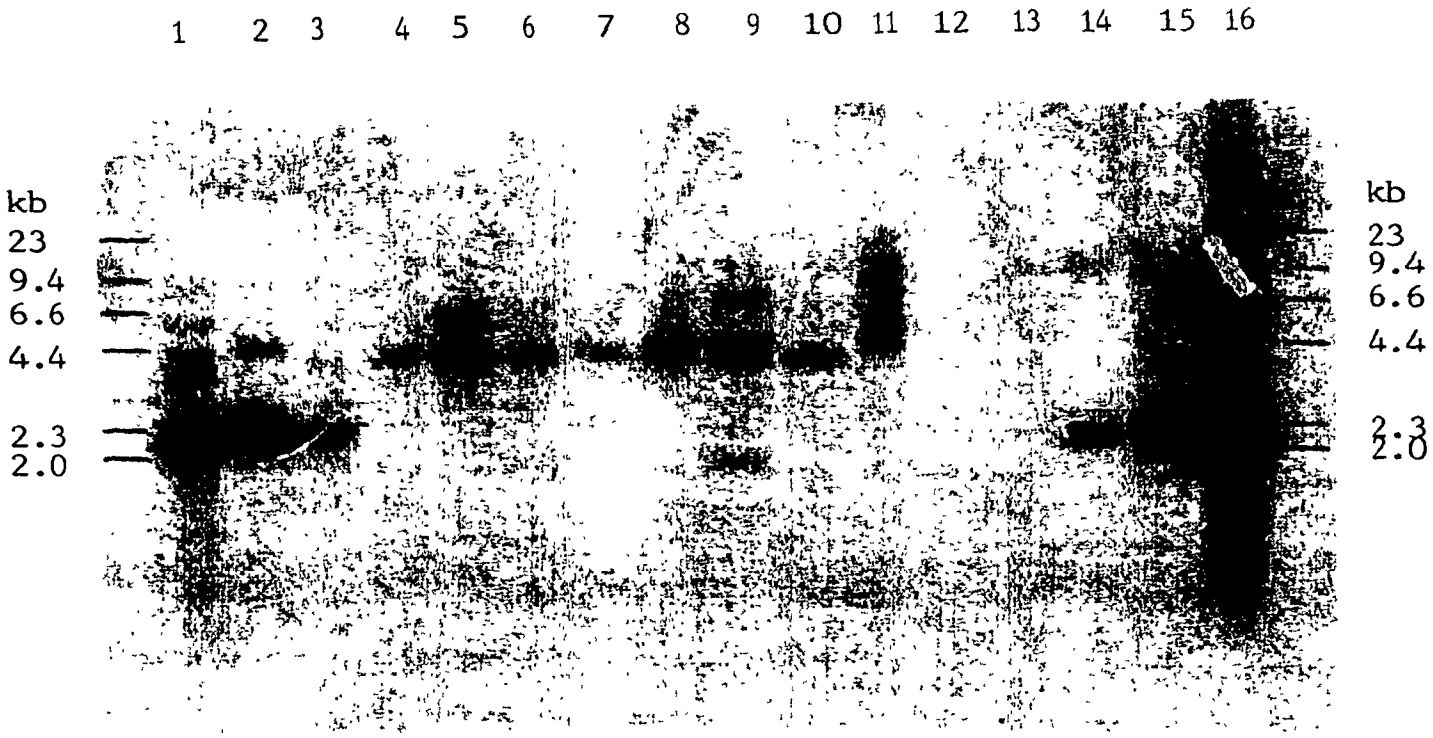

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
kb
23
9.4
6.6
4.4
2.3
2.0
kb
23
9.4
6.6
4.4
2.3
2.0

Table VI. Observed correlation between the glucoamylase-GUS fusion gene copy number, relative mRNA concentration and its expression

Strain	copy number[a]	mRNA concentration[b]	GUS activity[c]
A2-3	14	1.9	1233
A2-16	9	2.0	1694
A1-4	3	1.3	262
B2-3	1	2.5	41
B1-13	6	6.2	33
B2-2	1	8.7	72
C3-1	1	1.0	10
C2-17	4	5.6	76
C1-17	4	9.9	34
D4-3	1	6.9	119
D4-6	3	2.3	92
D3-9[d]	0	0	7
E	0	0	0.62

[a]Genomic DNA extracted from all the *pyrG*$^+$ transformants listed in the table were subjected to DNA blot analysis using the 1.8 kb GUS fragment as a probe. Transformant E was used as the negative control. Genomic DNA from transformant E was mixed with pGGC16 DNA corresponding to 1, 5, 25 copies of the GUS gene and used as a standard for the determination of copy numbers.

[b]Total cellular RNA was extracted from the *pyrG*$^+$ transformants and probed with the 1.8 kb GUS fragment for the RNA blot. Relative signal intensities determined using the software of the BIORAD imaging system, were used to compare mRNA levels.

[c]The *pyrG*$^+$ transformants were grown in MM5 medium (minimal medium with 5% maltose) at 30°C for 2 days for the GUS assay. The extracts prepared from the mycelia were assayed for GUS activity. GUS expression is indicated in nanomoles of PNPG cleaved/min/mg protein.

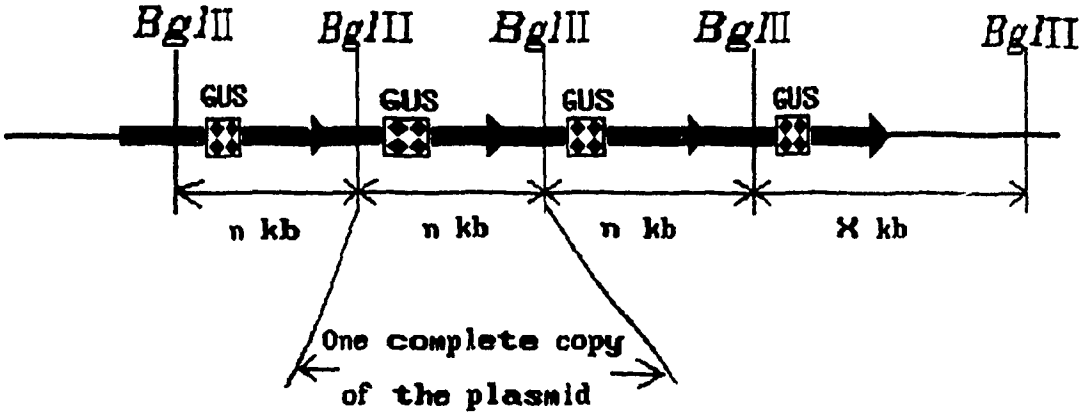
[d]Although there was no integration of the fusion gene for strain D3-9 (Figure 11), a low level GUS activity was detected, which could be the result of unspecific hydrolysis of the colorimetric substrate (PNPG) used in the GUS assays by some other cellular components. This is corroborated by the RNA blot hybridization analysis (Figure 13), which did not detect GUS mRNA in this transformant.

(Figure 11, lanes 1-3) were about 2.1 kb as expected. The major fragments observed for the longer fusion transformants (Figure 11, lanes 4-11) were close to their expected sizes. No band was observed in lane 12 for transformant D3-9, which indicated that there was no fusion plasmid integrated into the genome of this transformant. As expected, no band was observed for the negative control E (Figure 11, lane 13). The presence of additional although less intense bands in most lanes could be the result of rearrangements of some copies of the fusion plasmids in the genome of the transformants. The copy number for each transformant, estimated as described in the legend of Figure 11, is shown in Table VI.

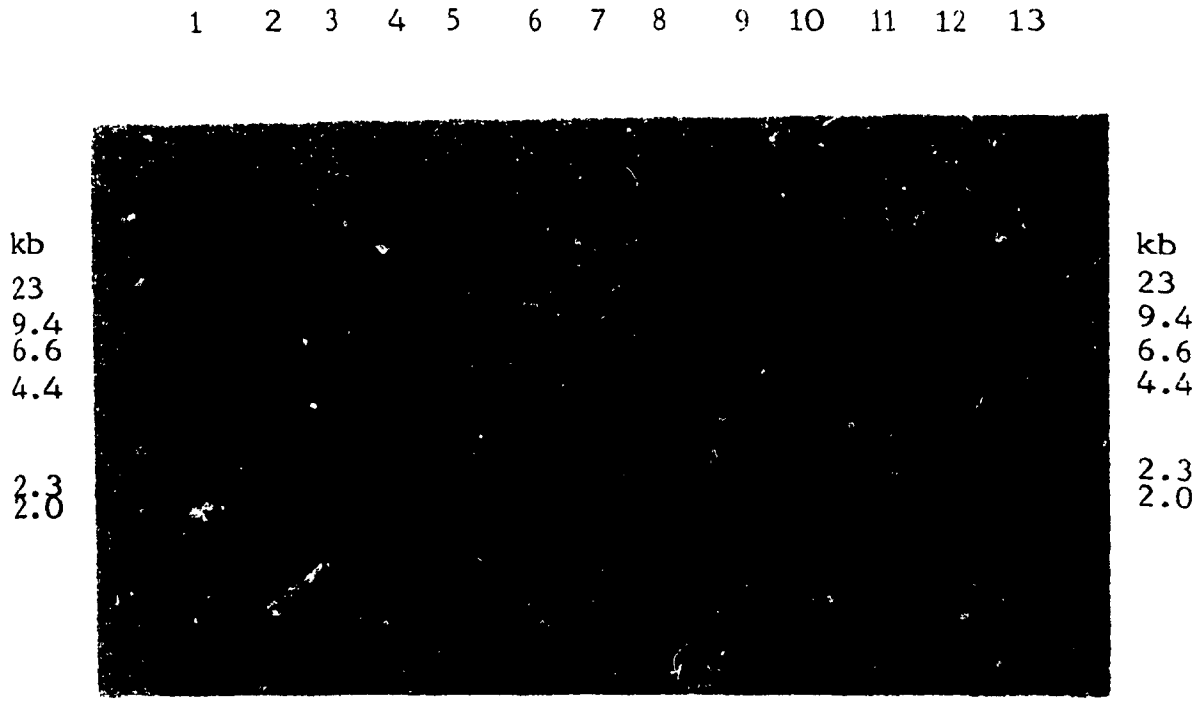
*Bgl*II digests of the genomic DNA were done to see if all the GUS fusion gene copies in a transformant were integrated at a single location in the *A. niger* genome. There is only one *Bgl*II site in each of the fusion plasmids. Since it is outside of GUS, any GUS gene copies present in a transformant should not be cleaved by *Bgl*II digestion. If all the fusion genes were integrated tandemly at a single site in a transformant, probing the *Bgl*II digested genomic DNA with GUS would reveal 2 bands, one of which would correspond to the size of the plasmids, as shown in Figure 12 A. There were 3 bands in a single lane for some transformants (Figure 12 B, lanes 1, 3, 5). In lanes 2, 4, 6, 7 and 11 (Figure 12 B), there were bands close to the size of the plasmids, which indicated that in these cases, the fusion plasmids may have integrated into the *A. niger* genome tandemly at a single site.

Figure 12. **A.** If a *pyrG*$^+$ transformant has many copies of GUS integrated tandemly at a single site as shown in the figure, probing *Bgl*II digested genomic DNA with GUS would show 2 bands, since digesting the genomic DNA with *Bgl*II would only generate 2 kinds of fragments (n kb and X kb respectively) containing the GUS gene. The size of the most intense band on the DNA blot (n kb) would correspond to the size of the fusion plasmids which is 9.8, 11.7, 12, 12.1 kb for pGGC16, pGL-GUS, pG2E-GUS, pG1E-GUS respectively. **B.** DNA blot used to determine how the GUS genes were integrated into the *A. niger* genome. Two micrograms of genomic DNA were digested with *Bgl*II for each lane. The 1.8 kb GUS fragment was used as a probe. The order of loading for lanes 1-13 was same as in Figure 11: A2-3, A2-16, A1-4, B2-3, B1-13, B2-2, C3-1, C2-17, C1-17, D4-3, D4-6, D3-9, E.

A


BglII
BglII
BglII
BglII
BglII
GUS
GUS
GUS
GUS
n kb
n kb
n kb
X kb
One complete copy
of the plasmid

B


1 2 3 4 5 6 7 8 9 10 11 12 13
kb
23
9.4
6.6
4.4
2.3
2.0
kb
23
9.4
6.6
4.4
2.3
2.0

3.2.2 Accumulation of glucoamylase-GUS mRNA in transformants

To reveal the relationship between the GUS copy number, mRNA expression and GUS activity in the transformants, RNA blot hybridizations were also performed.

Total cellular RNA from the *pyrG*$^+$ transformants (section 3.1.2.1) was size fractionated in a denaturing 1% agarose-formaldehyde gel. RNA was transferred to NYTRAN PLUS membrane and probed with the 1.8 kb GUS fragment. The amount of mRNA hybridized to the GUS probe varied for different transformants (Figure 13). Estimated relative amount of signals for each transformant is shown in Table VI. No band was observed for both the negative control E (Figure 13, lane 13) and transformant D3-9 (Figure 13, lane 12). There were two bands in a single lane for some transformants (Figure 13, lanes 6, 9, 10), which may be generated by alternative transcript splicing. The sizes of the transcripts seen for these transformants are all in the expected range, which is about 3 kb for the shortest fusion and around 5 kb for all the longer fusions.

3.3 The effect of growth conditions on the expression of glucoamylase-GUS

3.3.1 Induction media, temperature and culture volume

Growth conditions dramatically affect glucoamylase expression (Nunberg *et al.*, 1984). The growth medium could be complete medium or minimal medium, the incubation temperature could range from 25°C to 37°C (Jeenes *et al.*, 1993; Fowler *et al.*, 1990), the culture

Figure 13. RNA blot hybridization to quantify the expression of GUS mRNA in the cells of the transformants. Total cellular RNA (5 μg each lane) was probed with the 1.8 kb GUS fragment. Transformants were arranged in the same order as for the DNA blots in Figures 11 and 12 from lane 1 to lane 13. Lane 13 had the negative control transformant E. Estimated relative amount of signals is shown in Table VI.


1 2 3 4 5 6 7 8 9 10 11 12 13
kb
3.5
1.8
kb
3.5
1.8

Table VII. Effective growth conditions for the expression of glucoamylase-GUS

Growth condition	Strain								
	RS34#2[a]	RS34#6[a]	A2-16	A1-4	A2-3	B2-3	C3-1	D4-3	E
CMM5 medium, 37°C, 50 ml culture	1.4	7.3	-	-	175	5.21	1.04	2.68	0.37
CMM5 medium, 30°C, 50 ml culture	27.4	66.2	204	104	-	-	-	-	-
CMM5 medium, 30°C, 5 ml culture	48.7	-	132	87	-	-	-	-	-
MM5 medium, 30°C, 50 ml culture	-	-	1694	262	1233	41	10	119	0.62

Aspergillus niger pyrG$^+$ transformants were assayed for GUS activity after growing for 2 days under conditions indicated. MM5 medium: minimal medium with 5% maltose as single carbon source; CMM5 medium: complete medium containing 5% maltose as carbon source. Values presented are the average or assays done in triplicate on at least one culture. GUS activity is indicated in nanomoles of PNPG cleaved/min/mg of protein.

[a]*Aspergillus niger* strain RS34 (*cspA*1; (I) *fwn*A1; (III) *pyr*G5; (VII) *nic*B5) was cotransformed with plasmids pG1E-GUS and pPYRG using the *pyr* selection. The transformants obtained were selected for GUS expression using the filter GUS assay. RS34#2 and RS34#6 are 2 such transformants that were very blue on the filter, they were from S. Sillaots.

volume may also influence the glucoamylase-GUS gene expression as *A. niger* mycelia would form clumps during growth unless they are grown in a certain volume in an appropriate flask. To find out how growth conditions affect expression of the glucoamylase-GUS fusion gene, cultures of *A. niger* transformants were grown in MM5 and CMM5 media. Culture volume and incubation temperature were also varied (Table VII).

As shown in Table VII, both RS34#2 (a transformant of *A. niger* strain RS34 harbouring both pPYRG and pG1E-GUS) and RS34#6 (another transformant of RS34 like RS34#2) showed higher GUS activity when grown at 30°C compared to 37°C. At 30°C, for strains A2-16 and A1-4, higher GUS activity was obtained with the 50 ml rather than the 5 ml culture. However, for RS34#2, a higher value was obtained with the 5 ml culture. Since the growth of the mycelia in 50 ml cultures was much more uniform and comparable, values obtained with the 50 ml cultures were expected to be more reproducible. The results for A2-16 and A1-4 show that GUS was expressed at much higher levels using MM5 medium (minimal medium with 5% maltose) than CMM5 medium (complete medium with 5% maltose) (Table VII).

3.3.2 **Detecting GUS activity in media**

Previously significant GUS activity was not detected in the growth media. There are several possible reasons for this result. i) GUS is inhibited by certain divalent cations (Cu^{2+}, Zn^{2+}, *etc.*, Gallagher, 1992) which are present in the growth media (**section 2.1.1**), ii) secreted proteases destroy any GUS that is in the

media, iii) the activity is inactivated by oxidation, iv) GUS is not secreted. To investigate these possibilities, the experiments outlined in the following sections were performed.

3.3.2.1 Growth media pH and GUS expression

It is known that *A. niger* secrets a complex mixture of proteases (Mattern *et al.*, 1992; Thompson, 1991). This means that the glucoamylase-GUS fusions could be secreted into the media but are destroyed by proteolytic degradation. As the extracellular *A. niger* proteases are less active at high pH, we tested whether using a high pH medium for the growth of our GUS expressing strains would enable us to detect activity in the medium. Strains were grown in CM to generate lots of mycelia. After harvesting and washing with 0.7% NaCl different amounts of the blot-dried mycelia (about 1:3:9 ratio) were transferred to 50 ml of BMM5 medium (MM5 medium buffered with 100 mM $NaPO_4$, pH 7.0) and grown overnight. The next day mycelia were processed as before and GUS assays were done on both the medium and the cells. One positive control was included. This was produced by incubating 1/10 of the squeezed mycelia of transformant A2-16 in MM5 medium instead of BMM5 medium. The pH of these media were checked using a pH meter both before and after overnight growth.

Most cultures remained above pH 6.5 throughout the induction. There were no clear differences in GUS activity in the media between the fusion transformants and the negative control E (Table VIII). The GUS activity in the cellular extracts prepared from the

Table VIII. GUS activity of *pyrG*$^+$ transformants in a high pH medium

Strain[a]	Mycelia amount ratio	Induction media	pH of media		GUS activity[b]	
			before induction	after induction	in cell extract	in media
A2-16	1	MM5	6.5	5.83	294	-
A2-16	1	BMM5	7.0	6.83	283	22.6
	3	BMM5	7.0	6.70	295	32.2
	9	BMM5	7.0	6.57	116	36.5
B2-2	1	BMM5	7.0	6.93	-	12.4
	3	BMM5	7.0	6.86	349	3.14
	9	BMM5	7.0	6.62	163	3.08
C2-17	1	BMM5	7.0	6.85	140	21
	3	BMM5	7.0	6.60	116	24.8
	9	BMM5	7.0	6.22	110	15.7
D4-3	1	BMM5	7.0	6.88	229	28.6
	3	BMM5	7.0	6.62	263	32.5
	9	BMM5	7.0	6.15	300	11.1
E	1	BMM5	7.0	6.91	-	24.8
	3	BMM5	7.0	6.71	0.93	12.1
	9	BMM5	7.0	6.41	1.21	6.9

[a]The strongest GUS producing transformant of each fusion (see Table I) were used for this experiment. Strain A2-16 was grown in MM5 medium also as a positive control. Strain E was used as a negative control.

[b]One unit of GUS activity is one nanomole of PNPG cleaved/min/mg protein.

different cultures were all significantly above background. However, the expression levels were very different from previous results (Table I). For transformant A2-16, there was a 5-fold decrease in the amount of activity expressed. For all other longer fusion transformants, the GUS activity increased 2 to 4 fold. These differences may be the result of the different growth conditions (*i.e.*, pH of the media, *etc.*).

3.3.2.2 Other tests

To investigate the possibility of divalent cation inhibition, the following experiment was done. Eighty microliters of the cell free extracts of transformants A2-3, C3-1, D4-3 were mixed with equal volumes (80 μl each) of extraction buffer [50 mM $NaPO_4$, pH 7.0, 1 mM EDTA, 20 μM PMSF], its corresponding CMM5 growth medium [complete medium with 5% maltose], or sterile CMM5 medium. Added to the GUS assay buffer were 100 μl of each of the mixtures and the reactions were carried out at 37°C. The reactions were stopped after a light yellow color appeared by mixing 300 μl of the reaction mixture with 2.4 ml of 0.4 M Na_2CO_3. We thought if the proteases in the media were the problem, then by changing the order of addition, *i.e.*, by adding the GUS assay buffer to the extract first (elevating the pH), then adding the growth media, there should be a significant increase in GUS activity. Two such assays were done also in parallel with the above experiment using extracts of A2-3, C3-1.

The results in Table IX show that divalent cations are not the

problem, as GUS activity did not necessarily get higher when extraction buffer (which did not have divalent cations in it) was used instead of the growth medium or the sterile medium. By switching the order for adding the GUS assay buffer and the growth medium (the 2nd and 4th row), no differences were observed for A2-3 and C3-1. This suggested that proteases in the growth media were not a major problem. To investigate whether longer incubation times might affect activity, the following experiment was done. Extracts of B2-3 were mixed with an equal volume of its CMM5 growth medium (100 μl for each) and incubated at 37°C for 0, 10, 20, 40, or 80 min. Then the mixtures were assayed for GUS activity as above. It was found that there was no significant decrease of GUS activity even after 80 min of incubation (Figure 14).

Ammonium ions in the growth media are known to repress protease secretion (Cohen, 1977). Cultures were grown with $(NH_4)_2SO_4$ (300 mM) added to the growth medium. No significant increase in the amount of GUS activity present in the medium was observed (data not shown).

Table IX. GUS assay in media

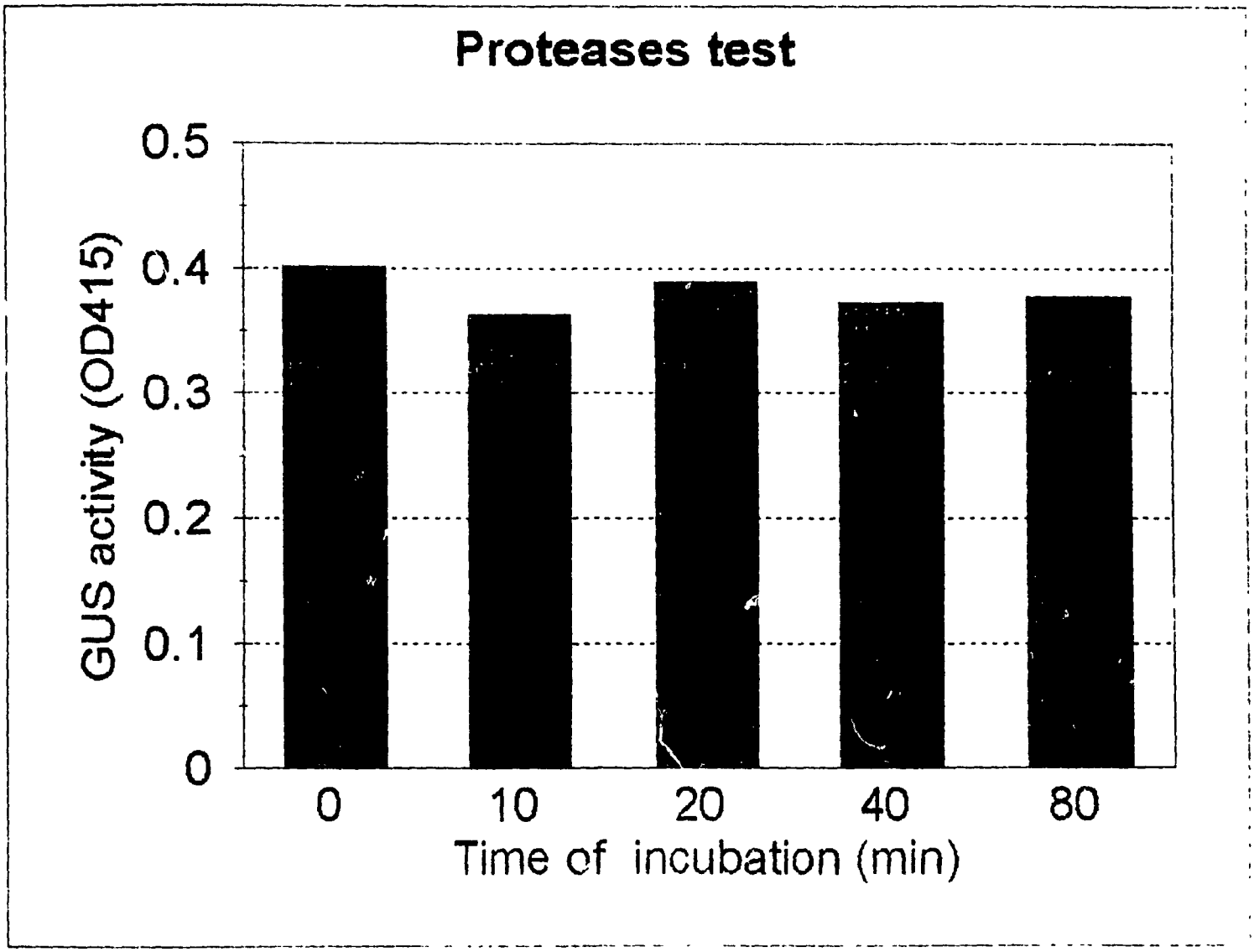
	A2-3	C3-1	D4-3
Extract + Extraction Buffer + GAB[a]	0.686	0.116	0.305
Extract + Growth Medium[b] + GAB	0.657	0.123	0.305
Extract + Sterile Medium + GAB	0.664	0.121	-
Extract + GAB + Growth Medium	0.733	0.129	-

All values (OD_{415}) shown are the average of assays done in triplicate. Differences between measurements are within 10%.

[a]GUS assay buffer

[b]The growth media used here were from cultures in which the *A. niger* transformants had been grown for 2 days.

Figure 14. Proteases test. Extracts were mixed with equal volumes of the growth medium and incubated at 37°C for different length of time then assayed for GUS activity.


Proteases test
GUS activity (OD415)
0.5
0.4
0.3
0.2
0.1
0
0
10
20
40
80
Time of incubation (min)

4. DISCUSSION

4.1 Expression of glucoamylase-GUS fusion genes

4.1.1 Role of the glucoamylase coding region in fusion gene expression

4.1.1.1 Extracellular production of the fusion proteins

One aspect of the present work was to characterize the secretion of a heterologous protein under the control of the *glaA* promoter and targeting signal. For this purpose, I fused different portions of the *glaA* gene to the *E. coli* GUS gene. We hoped that fusing GUS to part of glucoamylase a secreted protein would encourage the passage of the fusion protein through the secretory pathway. A KEX2 site was also inserted in each of these fusions between the glucoamylase and GUS portions so that GUS could be processed by the Kex2p protease.

We found that there was only background GUS activity in the growth medium of the *A. niger* transformants expressing these fusions (Table I). There could be a variety of reasons for this result. Growth in high pH media or in the presence of $(NH_4)_2SO_4$, conditions which repress protease activity or secretion, did not increase the amount of GUS in the media. These results suggested that the extracellular proteases were not preventing the appearance of activity in the media.

To test for the presence of extracellular proteases that

degraded GUS, cellular extracts prepared from transformant B2-3 were incubated in the presence of equal volumes of its growth medium at 37°C for different lengths of time. No obvious decrease in GUS activity occured even after 80 min of incubation (Figure 14). It thus appeared that the GUS activity expressed by these transformants was not very sensitive to the proteases in the media. From the above experiments, it appeared that proteolytic degradation was not responsible for the lack of GUS activity in the media.

We also assayed the GUS activity of mixtures containing GUS in cellular extracts (from transformants A2-3, C3-1 and D4-3) and either the extraction buffer (without divalent cations) or growth medium (with divalent cations). The results did not support the hypothesis that the presence of the divalent cations in the media inhibited GUS activity (Table IX). Therefore my results suggest that GUS is not inactivated or destroyed by something in the media.

It is possible that the glucoamylase-GUS fusion proteins were not present in the media because the *glaA* sequence used had acquired a mutation which prevented secretion. DNA sequence analysis of the *glaA* portion of our fusion genes would test this possibility. If a mutation were present, using the wild type *glaA* gene to generate a new set of fusions may result in glucuronidase secretion.

It is also possible that the *E. coli* GUS gene is unsuitable for studies of protein secretion. For example, it may be inactivated during passage through the secretory pathway. It is

noteworthy that there have been no reports describing the use of GUS as a reporter for protein secretion studies in *A. niger*, although it has been used as a reporter for intracellular expression (Roberts *et al.*, 1989; Verdoes *et al.*, 1994a). To test this possibility, another reporter gene could be used. Hen egg-white lysozyme, which is suitable for secretion studies (Archer *et al.*, 1990; Jeenes *et al.*, 1993), is one possibility.

4.1.1.2 Intracellular production of the fusion proteins

4.1.1.2.1 Effect of copy number on glucuronidase expression

For independent transformants harbouring the same fusion plasmid (Table I), large differences in glucuronidase expression were observed. These differences are not proportional to the gene copy number (Table VI). A similar variation between copy number and mRNA concentration was also seen (Table VI). In contrast there is a strong correspondence between glucuronidase activity and mRNA concentration for transformants harbouring the same fusion plasmid (Table VI). However, the corresponding increases are not proportional. For example, transformant D4-3 has 3 times more GUS mRNA than D4-6, but there is only a 1.3 fold increase in glucuronidase activity. This as well as the exceptions (B2-3 vs. B1-13; C2-17 vs. C1-17) could be the result of errors introduced by loading or RNA transfer when doing the RNA blot hybridizations.

4.1.1.2.2 Effect of fusion gene size on expression

Levels of GUS activity expressed by transformants harbouring the shortest fusion (only the signal peptide of glucoamylase fused) were much higher than that obtained with the longer fusions (Table I). The lowest levels of GUS expression were obtained when GUS was fused at the end of the G2 protein. The longest GUS fusion gene and the fusion with GUS after the linker region gave intermediate levels of expression. Levels of GUS mRNA expressed by the transformants do not seem to vary much with the length of the fusion gene (Table VI). Since the longer fusion protein is not very sensitive to the secreted proteases (Figure 14), it is unlikely that the lower GUS expression obtained with the longer fusions would be the result of proteolytic degradation. The GUS activities expressed from the longer fusions may represent only a small portion of the synthesized proteins. Perhaps most of the fusion protein is exported, but inactivation occurs during its transit through the secretory pathway. Also, as the fusions with glucoamylase get longer, the corresponding mRNA can be processed differently and may be translated with different efficiency.

GUS expression from my shortest fusion gene is comparable to those reported in the literature for fusion of GUS to the glyceraldehyde-3-phosphate dehydrogenase promoter and the glucoamylase promoter by Roberts *et al.* and Verdoes *et al.* (Table X).

4.1.2 Advantages of using the *amdS* gene as a selectable marker

The *amdS* gene provides positive selection. Further, since this

Table X. Comparison of GUS activity in *Aspergillus niger* strains

Strain[a]	Promoter[b]	Fusion	GUS activity[c]
tr10	Pgpd	-	5698
tr22	Pgpd	-	1535
tr24	Pgpd	-	1953
GUS64	PglaA	-	1360
A2-3	PglaA	24 aa	1233
A2-16	PglaA	*glaA*	1694
A1-4	PglaA	signal peptide	262

Values shown in this table are taken from either Roberts *et al.* (1989), Verdoes *et al.* (1994a) or the present work. Reporter genes used in all 3 cases were the *E. coli* GUS.

[a]Strains tr10, tr22, tr24 (Roberts *et al.*, 1989) and GUS64 (Verdoes *et al.*, 1994a) were the *pyrG*$^+$ transformants of strain AB4.1 (*pyrG*$^-$; Van Hartingsveldt *et al.*, 1987). Strains A2-3, A2-16 and A1-4 used in this work are the *pyrG*$^+$ transformants of RS31 (*pyrG*$^-$; Goosen *et al.*, 1987).

[b]The promoters used were either that of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter (Pgpd) or the *A. niger* glucoamylase promoter (PglaA). The GUS fusions used by Roberts *et al.* (1989) and Verdoes *et al.* (1994a) were designed to produce GUS intracellularly and do not contain any secretion signal sequence. The shortest fusion used in this work has the 24 aa signal sequence of the *gla*A gene.

[c]All assay conditions were the same as described for ß-gal assays (Van Gorcom *et al.*, 1985). One unit of GUS activity were all defined as one nanomole of PNPG cleaved/min/mg protein. It is known that the Pgpd promoter is not regulated by carbon source (Verdoes *et al.*, 1994a). Cultures of GUS64 and A2-3, A2-16, A1-4 were all grown using 5% maltose as single carbon source.

gene is poorly expressed in *A. niger*, selection for integrating plasmids harbouring the *amdS* gene by growth on acetamide selects for transformants harbouring many integrated copies of the plasmid. The 3 pGGC16 *pyrG*$^+$ transformants assayed were chosen as the highest GUS expressing transformants from a total of 40 transformants (Table I). Therefore, their fusion gene copy number is expected to be higher than average. The average level of GUS activity expressed by the acetamide transformants (Table II) is about the same as that for the 3 pGGC16 *pyrG*$^+$ transformants that expressed the most activity. The number of integrated fusion genes was not determined for the acetamide transformants; however, if we assume they have the same average number of integrated genes as the 3 pGGC16 *pyrG*$^+$ transformants assayed then *amdS* selection isolates transformants harbouring about 8 copies of the introduced genes.

amdS selection has been combined with a vector containing 4 copies of the *glaA* gene (Verdoes *et al.*, 1993). This vector was used to transform *A. niger* strains using the *amdS* selection. With this strategy strains were isolated which carried up to 200 copies of the *glaA* gene. This strategy is certainly worth trying in the search for transformants expressing the GUS reporter at high levels.

4.2 Plasmid integration events

One purpose of the DNA blot analysis in this work was to see how the fusion genes had integrated into the *A. niger* genome. The DNA blot prepared from *Eco*RI digested DNA showed 2 or more *Eco*RI

bands for about half of the transformants (Figure 11). If homologous recombination is the only mechanism by which the plasmids get integrated into the *A. niger* genome, assuming there were no rearrangements or deletions, there should be only one band of a specific size for each transformant. It thus appears that in these transformants not all the plasmids integrated into the homologous site (*i.e.*, the *gla*A locus). The multiple bands may reflect the rearrangements of the fusion plasmids in the genome of the transformants. As well, the DNA blot of *Bgl*II digested DNA (Figure 12) revealed that the multiple copies in a particular transformant did not necessarily integrate in a tandem fashion at a single site.

The results obtained here support previous results showing that the integration of plasmid DNA into the *A. niger* genome can happen at both homologous and nonhomologous sites (Fowler *et al.*, 1990; Verdoes *et al.*, 1993; 1994a; 1994b). This is also observed for both *A. nidulans* (Ballance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Wernars *et al.*, 1985) and *A. oryzae* (Hata *et al.*, 1992). It has been shown that when *A. niger* strains were transformed for the first time, virtually all integrations were at nonhomologous sites (>88%; Verdoes *et al.*, 1994c). Linearization of the transforming plasmid DNA does not significantly increase the frequency of homologous recombination (Finkelstein *et al.*, 1989). The initial homologous recombination in a strain has been used to do gene disruptions (Fowler *et al.*, 1990) or construct reporter strains harbouring gene fusions at a specific locus (Verdoes *et*

al., 1994a). In subsequent retransformation of the already-transformed strains, the frequency of homologous recombination apparently increases. Among the 33 retransformants examined by Fowler *et al.* (1990), 7 resulted from homologous recombination. In cases where the integration events were characterized, the integrated copies often formed tandem repeats (Kelly and Hynes, 1985; Fowler *et al.*, 1990; Verdoes *et al.*, 1993; 1994a; 1994b). The mechanism for the integration of the transforming DNA into the *A. niger* genome is still unknown, although this does not require large regions of strong homology, small regions of partial homology may be required (Kelly and Hynes, 1985).

4.3 Glucoamylase-GUS transcription

Transcription of the integrated fusion genes was characterized by RNA blot analysis. As all of our fusions have 1 alternatively spliced intron 3′ to the GUS sequences (Boel et al., 1984b), they could all be processed into different forms. On the RNA blot, only 1 mRNA species was observed for the shortest glucoamylase-GUS fusion (Figure 13, lanes 1-3). Alternative RNA splicing could explain the 2 mRNA species seen in some of the transformants obtained with the longer fusions. For example, in lane 6 the expected size range for the mRNAs is from 4.4 (all introns removed) to 4.8 (no introns removed) kb. The estimated sizes of the observed bands are 4.6 and 4.9 kb respectively.

Expression of the wild type *glaA* gene results in 2 forms of glucoamylases at close to equal molar amounts (Boel *et al.*, 1984b).

mRNA encoding G2 was shown by sequence analysis of cDNA clones of the *gla*A gene (Boel *et al.*, 1984b). However, only one glucoamylase mRNA band (that of G1's) could be seen on RNA blots probed with the *gla*A gene (Nunberg *et al.*, 1984; Verdoes *et al.*, 1993). It is possible, since there is only a difference of 169 bp in length between these 2 forms of mRNAs, that the resolution obtained using agarose gel electrophoresis to size fractionate mRNA may not have distinguished between these 2 species which are expected to be 2.15 kb and 1.98 kb.

4.4 Effect of growth conditions on the expression of glucoamylase-GUS

It has been shown that maltose strongly induces *gla*A expression (Nunberg *et al.*, 1984; Fowler *et al.*, 1990). I found that maltose did not induce *gla*A expression as strongly in complete medium (which contained in addition 0.2% peptone, 0.1% yeast extract, 0.1% casamino acids, 0.1% (v/v) vitamin solution), as it did in minimal medium (Table VII, see the 2nd and 4th row of data for strains A2-16, A1-4). The presence of additional nutrients did stimulate the growth of *A. niger* transformants in this case, but significantly lowered *gla*A expression. The lower expression could be due to a repressor that responds to the additional components in the complete medium. It is doubtful that this repressor is the same as the one hypothesized to respond to xylose (Verdoes *et al.*, 1994a), since repression by xylose could be overcome by maltose (Fowler *et al.*, 1990).

We also investigated the effect of temperature on the expression of glucoamylase-GUS. Substantially higher expression was observed when transformants RS34#2 and RS34#6 were grown at 30°C compared to 37°C (Table VII). The exact cause for this difference remains unknown.

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