Evaluation of *Aspergillus* as a host for the production of viral proteins using hepatitis B as a model

Annette Plüddemann

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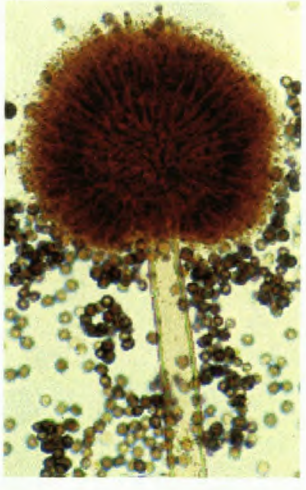
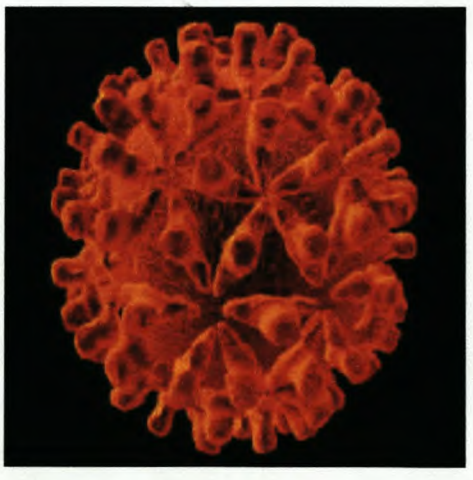
Supervisor: Prof. W. H. van Zyl





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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and has not previously in its entirety or in part submitted it at any university for a degree.

Annette Plüddemann

SUMMARY

Since the advent of recombinant DNA technology in the 1970s, various microbial hosts have been employed for the efficient high-level heterologous production of a variety of proteins, ranging from enzymes and reagents to therapeutics and vaccines. More recent microbial hosts to be employed for these purposes are filamentous fungi, and particularly the genus *Aspergillus*. Aspergilli have been associated with industrial processes for many years and are used in the production of antibiotics, enzymes, citric acid and Oriental foods and beverages, and thus strains such as *Aspergillus niger* and *Aspergillus oryzae* have been afforded GRAS (Generally Regarded As Safe) status. They also secrete copious amounts of homologous and heterologous proteins and are able to perform post-translational modifications effectively. Various proteins of pharmaceutical interest have been successfully expressed in *Aspergillus*, but the potential of these fungi to produce heterologous viral proteins has not been explored extensively.

In this study, we evaluated the potential of the filamentous fungi *A. niger* and *Aspergillus awamori* as alternative hosts for the heterologous production of hepatitis B viral proteins. Hepatitis B is a serious, potentially lethal liver disease that affects 2000 million people world-wide and has a high endemicity in Southern Africa. Currently there is no effective treatment for viral hepatitis and thus a mass vaccination strategy is the only solution to curb the spread of the disease. This kind of vaccination strategy requires a cheap, safe and effective vaccine and these objectives have been achieved in the development of recombinant subunit vaccines from yeasts such as *Saccharomyces cerevisiae, Hansenula polymorpha* and *Pichia pastoris* that are commercially available. The hepatitis B virus envelope consists of a membrane fraction and three proteins, namely the major (S) protein (encoded by the *S* gene), the middle (M) protein (encoded by the *preS2S* gene) and the large (L) protein (encoded by the *preS1preS2S* gene). When produced in the above-mentioned yeasts, the S protein was shown to spontaneously assemble into pseudoviral particles devoid of viral DNA, which were then purified and used as vaccine.

In the present study the *S* and *preS1preS2S* genes from a local isolate of hepatitis B subtype adw2 were placed under transcriptional control of the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter and the inducible *A. niger* glucoamylase (*glaA*) promoter. The respective viral genes were also fused to the region encoding the catalytic domain of the highly expressed and secreted *A. niger* glucoamylase, which served as a carrier moiety to possibly facilitate viral protein secretion. The various gene constructs were subsequently transformed to laboratory strains of *A. niger* and *A. awamori* and numerous transformants were obtained. One *A. niger* transformant carrying the *S* gene under control of the *gpdA* promoter contained approximately 7 integrated copies of the expression cassette and produced hepatitis B pseudoviral particles intracellularly at levels of 0.4 mg/l culture. These levels are approximately ten-fold higher than those initially obtained from the yeast *S.cerevisiae,* which showed yields of 0.01 to 0.025 mg/l. None of the other transformants could be shown to produce recombinant S or L protein and no secretion of viral protein could be demonstrated. This could be attributed to numerous factors, including vector copy number, site of integration or proteolytic activity. The most important insight emerging from this work regarding secretion of heterologous viral protein was that the addition of a carrier protein hampered, rather than enhanced secretion of the viral envelope protein, due to the inherent properties of viral protein assembly.

This work also serves as a "proof of principle", showing that *Aspergillus* is indeed a viable alternative host for the production of hepatitis B pseudoviral particles, and could be investigated further for its potential as host for the heterologous expression of other viral proteins.

OPSOMMING

Sedert die ontwikkeling van rekombinante DNA tegnologie in die sewentigerjare is verskeie mikroorganismes reeds gebruik vir die doeltreffende produksie van 'n verskeidenheid proteïne teen hoë vlakke; onder andere ensieme, reagense, terapeutiese middels en vaksiene. Onlangs is filamentagtige swamme, veral van die genus *Aspergillus*, ontwikkel vir heteroloë proteïenproduksie. Aspergilli word al vir baie jare in nywerheidsprosesse gebruik, onder andere in die vervaardiging van antibiotika, ensieme, sitroensuur en sekere Oosterse voedsel- en drankprodukte. As gevolg van hierdie jarelange gebruik van rasse soos *Aspergillus niger* en *Aspergillus oryzae,* word hulle algemeen aanvaar as veilig vir menslike gebruik. Hierdie swamme besit veral die vermoë om hoë vlakke van homoloë en heteroloë proteïene uit te skei en die na-translasie-modifisering van proteïene korrek uit te voer. Verskeie proteïene van farmaseutiese belang is al suksesvol in *Aspergillus* uitgedruk, maar die potensiaal van hierdie swamme om virale proteïene te vervaardig is nog nie deeglik ondersoek nie.

Hierdie studie ondersoek die geskiktheid van die filamentagtige swamme *A. niger* en *Aspergillus awamori* om as alternatiewe gashere vir die heteroloë produksie van hepatitis B proteïene te dien. Hepatitis B is 'n ernstige en selfs dodelike lewersiekte. Omtrent 2000 miljoen mense wêreld-wyd is met die virus geïnfekteer en dit is veral endemies in Suiderlike Afrika. Daar is tans geen doeltreffende behandeling vir virale hepatitis en dus is wêreld-wye inentingsprogramme die enigste oplossing om die verspreiding van die siekte te bekamp. Hierdie inentingsstrategie vereis die beskikbaarheid van 'n bekostigbare, veilige en doeltreffende vaksien. Die rekombinante subeenheid-vaksiene wat ontwikkel is deur van gashere soos *Saccharomyces cerevisiae, Hansenula polymorpha* en *Pichia pastoris* gebruik te maak, voldoen aan hierdie vereistes en is kommersieel beskikbaar. Die omhulsel van die hepatitis B virus bestaan uit 'n membraangedeelte en drie proteïene, naamlik die hoofproteïen (S) (gekodeer deur die *S*-geen), die middelproteïen (M) (gekodeer deur die *preS2S*-geen) en die grootproteïen (L) (gekodeer deur die *preS1preS2S*-geen).

Wanneer die S-proteïen in bo-genoemde giste uitgedruk word, vorm dit spontaan pseudovirale partikels wat nie virale DNA bevat nie. Hierdie partikels word dan gesuiwer en as vaksien gebruik.

In hierdie studie is die *S*- en *preS1preS2S*-gene, vanaf 'n plaaslike isolaat van hepatitis B subtipe adw2, onder transkripsionele beheer van die konstitutiewe *Aspergillus nidulans* gliseraldehied-3-fosfaat-dehidrogenasepromoter (*gpdA*) en die induseerbare *A. niger* glukoamilasepromoter (*glaA*) geplaas. Die onderskeie virale gene is ook aan die koderende gedeelte vir die katalitiese domein van *A. niger* glukoamilase gelas om fusieproteïene te vorm. Glukoamilase word teen hoë vlakke deur *Aspergillus* vervaardig en uitgeskei en kan dus moontlik dien as draerproteïen om sekresie van die proteïne te bevorder. Transformasie van die geenkonstrukte na laboratoriumrasse van *A. niger* en *A. awamori* het verskeie transformante gelewer. Een *A. niger* transformant bevattende die *S*-geen onder transkripsionele beheer van die *gpdA* promoter het minstens sewe kopieë van die uitdrukkingskaset in sy genoom geïntegreer en het hepatitis B pseudovirale partikels intrasellulêr teen vlakke van 0.4 mg/l swamkultuur vervaardig. Hierdie vlakke is omtrent tien-voudig hoër as die vlakke van 0.01 – 0.025 mg/l wat *S.cerevisiae* oorspronklik opgelewer het. Nie een van die ander transformante het rekombinante S of L proteïene vervaardig nie en sekresie van virale proteïen kon nie getoon word nie. Hierdie verskynsel mag te wyte wees aan verskeie faktore insluitende vektor-kopiegetal, setel van integrasie en proteolitiese aktiwiteit. Die belangrikste insig uit hierdie studie aangaande sekresie van heteroloë virale proteïene is dat die koppeling van die virale omhulsel-proteïen aan 'n draerproteïen sekresie benadeel het, eerder as om dit te bevorder. Hierdie verskynsel is te wyte aan die inherente geneigdheid van virale omhulsel-proteïene om 'n kompleks te vorm.

Die studie dien ook as "bewys van beginsel" dat *Aspergillus* wel 'n werkbare alternatiewe gasheer vir die produksie van hepatitis B pseudovirale partikels is, en dat dit verder ondersoek sou kon word as potensiële gasheer vir die heteroloë uitdrukking van ander virale proteïene.

"Molecular biology is the art of the inevitable. If you do it, it's inevitable you will find how it works – in the end."

Sydney Brenner

This thesis is dedicated to Gerhard and Ingrid Plüddemann

In loving memory of Harold and Lotte Plüddemann and Karl and Martha Kroll

BIOGRAPHICAL SKETCH

Annette Plüddemann was born in Geneva, Switzerland, on 21 July 1972. She matriculated from The Settlers High School in Bellville, South Africa, in 1990. Annette enrolled at the University of Stellenbosch in 1991 and obtained the degrees B.Sc. (Microbiology and Biochemistry), B.Sc.Hons. (Microbiology) and M.Sc. (Microbiology) *cum laude* from this university. As part of her studies towards the degree Ph.D. (Microbiology) she spent a period of 7 months in 1998 in the laboratory of Prof. C.A.M.J.J. van den Hondel at the Netherlands Organisation for Applied Scientific Research (TNO) Nutrition and Food Research Laboratories in Zeist, The Netherlands.

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My **grandfather**. I wish you were here to witness the completion of this project that you were so genuinely interested in. It means so very much to me that you wanted to understand every detail of this new technology.

The National Research Foundation (NRF) and the University of Stellenbosch for financial support.

PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the journal to which the manuscript was submitted.

Chapter 3 "Evaluation of *Aspergillus niger* as host for virus-like particle production using the hepatitis B surface antigen as a model" will be submitted to *Journal of Virology.*

The **South African Provisional Patent** (no. 2001/9777) that has been filed encompassing the work detailed in chapter 3 is provided as an **Appendix**.

CONTENTS

Chapter 1

Introduction

1. INTRODUCTION

Hepatitis B is a serious, potentially lethal liver disease caused by the hepatitis B virus (HBV) and there are an estimated 2000 million HBV carriers in the world today (Vryheid *et al.*, 2001). The disease is especially prevalent in underdeveloped areas of the world, such as Africa, where 60% to 99% of healthy adults show evidence of exposure to HBV (Moradpour and Wands, 1995; Ayoola, 1988). In South Africa, more than 70% of the population has been exposed to HBV, with an estimated 10% being carriers of the virus (Tsebe *et al.*, 2001). Hepatitis B surface antigen proteins have important functions in the viral life cycle, such as attachment and penetration, envelopment and virus maturation (Gerlich, 1991). These proteins also spontaneously assemble into pseudoviral particles devoid of viral DNA and these particles play a role in neutralising virus-eliminating immune reaction. Thus, these proteins have been targeted for heterologous expression to produce recombinant vaccines. HBsAg proteins are encoded by two adjacent regions of the HBV envelope gene, namely the *preS* and the *S* region (Tiollais *et al.,* 1985). The *S* gene encodes the major (S) protein (226 amino acids) which constitutes most of the HBV envelope. S protein monomers can also spontaneously assemble into pseudoviral particles of about 22 nm in size. The middle (M) protein is coded for by the *preS2-S* gene and contains an additional 55 amino acids at the N-terminus, while the large (L) protein is encoded by the *preS1-preS2-S* gene and is 389 amino acids in size. No effective treatment for this disease currently exists and an extensive programme of vaccination presents the only solution to curb the spread of the disease (Kassianides *et al.*, 1988; Dehoux *et al.*, 1986). In order to make a mass vaccination strategy feasible, large amounts of safe, affordable vaccine must be available. Recombinant DNA methods have provided an alternative to the blood-origin vaccine obtained from plasma of chronic carriers and to date, bacteria, yeasts and mammalian cells transformed with appropriate expression vectors are able to synthesise hepatitis B surface antigen (HBsAg) for immunisation purposes (Dehoux *et al.*, 1986; Valenzuela *et al.*, 1982; Michel *et al.*, 1984).

Since the 1980's genetic engineering procedures have been developed for the filamentous fungi, in particular aspergilli (Kinghorn and Unkles, 1994). Subsequently various heterologous proteins of fungal and non-fungal origin have been expressed in these fungi (Gouka *et al.*, 1997; Kinghorn and Unkles, 1994; Verdoes *et al.*, 1995). The use of the yeast *Saccharomyces cerevisiae* as heterologous host for the production of pharmaceutical (viral) proteins has already highlighted limitations, such as low product and biomass yields, lack of very strong, tightly regulated promoters and hyperglycosylation of glycoproteins (Romanos *et al.,* 1992). This initiated a search for alternative heterologous hosts, and non-*Saccharomyces* yeasts (e.g. *Pichia pastoris, Hansenula polymorpha, Kluyveromyces lactis*) as well as filamentous *Aspergillus* species have gained increasing importance as hosts for the production of eukaryotic proteins in particular. Several characteristics of filamentous fungal expression hosts give them potential advantages over *S. cerevisiae*. These include the production of fungal proteins (homologous and heterologous) in copious amounts; their extensive use in the production of enzymes, citric acid and Oriental foods; efficient post-translational modification of proteins and effective secretion of heterologous proteins (Harvey and McNeil, 1994; Kinghorn and Unkles, 1994). Viral protein production in filamentous fungi has not been extensively evaluated to date, and the hepatitis B viral proteins provide the ideal basis for such an evaluation. Furthermore, new fungal systems offer alternatives for the commercial production of proteins already encumbered by process patent applications involving other systems.

1.1. Aims of the study

This study comprised an investigation into the potential of the filamentous fungus *Aspergillus* to produce hepatitis B virus envelope proteins and serve as a possible alternative vaccine production system. The specific aims of the study were:

1. Cloning of the hepatitis B *S* gene encoding the major viral surface antigen under control of the inducible *Aspergillus niger* glucoamylase (*glaA*)

promoter and the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter.

2. Cloning of the hepatitis B *preS1preS2S* gene, encoding the large viral envelope protein, under control of the inducible *A. niger* glucoamylase (*glaA*) promoter and the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter.

3. Construction of an in-frame fusion, separated by a dibasic proteolytic cleavage site, between the catalytic domain of the *A. niger* glucoamylase and the *S* and *preS1preS2S* genes, respectively, in order to enhance secretion of the viral proteins.

4. Transformation of the various expression vectors to laboratory strains of *A. niger* and *Aspergillus awamori* and evaluation of intracellular and extracellular recombinant viral protein production.

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Chapter 2

Literature Review

Filamentous Fungi: Vaccine factories of the future?

2. FILAMENTOUS FUNGI: VACCINE FACTORIES OF THE FUTURE?

2.1. Introduction

Fungi commonly receive negative attention for their harmful effects, such as causing disease and food spoilage, and they have proven themselves a force to be reckoned with for anyone who has ever tried to keep a bathroom mould-free! Despite all these negative aspects generally associated with fungi, they can rather be considered as "unsung heroes" with all the benefits that they have afforded humans. In the last century they have quietly been harnessed for the production of antibiotics (thereby removing the threat of many a bacterial infection), enzymes, fermented foods, vitamins, pharmaceuticals and organic acids, to name just a few (Alexopoulos and Mims, 1979). Filamentous fungi can now be considered to be "microbial factories", effectively and efficiently providing us with products we require every day. With the advent of gene technology, this capability has been further expanded and now fungi are employed to produce not only homologous, but heterologous proteins as well (Davies, 1994; Kinghorn and Unkles, 1994; Van den Hondel *et al.*, 1991). The technology of heterologous protein production by microorganisms was pioneered in the prokaryotic host *Escherichia coli* in the 1970s and subsequently expanded to eukaryotic yeasts, particularly *Saccharomyces cerevisiae* (Hadfield *et al.*, 1993). Today, filamentous fungi, in particular aspergilli, are emerging as another important eukaryotic host for the cost-effective production of functional proteins from a variety of sources (Davies, 1994; Van den Hondel *et al.*, 1991). With the increasing demand for vaccines as a means to control and eradicate disease and the inherent advantages of filamentous fungi as protein production systems, the evaluation of *Aspergillus* for the production of immunogenic viral proteins has emerged as a promising alternative to the current vaccine production technologies.

2.2. The fungus *Aspergillus*

The first person to study the aspergilli was a priest by the name of Micheli in 1729, who published Nova Plantarum Genera in which his microscopical research on fungi was included, distinguishing stalks and spore heads (Alexopoulos and Mims, 1979; Raper and Fennell, 1965). He noted that the spore chains or columns radiated from a central structure to produce a pattern that suggested the aspergillum (a device used to sprinkle holy water) and thus he applied the name *Aspergillus* to the moulds he observed. Species of *Aspergillus* belong to the first fungal organisms that were cultivated on artificial media and studied for their biochemical properties and they are one of the most common fungi found in man's environment (Samson, 1994). Since ancient times, *Aspergillus* species have been used in fermentation of food in Japan and other Asian countries and the early discovery of their ability to produce organic acids was made at the turn of the century. By 1928 more than 2000 papers had been published which in some way concerned aspergilli (Raper and Fennell, 1965).

2.2.1. Taxonomy

Various criteria are employed in the classification and identification of filamentous fungi. Morphological structures are still important for classification and *Aspergillus* species typically produce the aspergillum, a conidiophore with an aseptate stipe terminating in a vesicle, on which the conidiogenous cells (phialides and metulae) are borne (Samson, 1994). The phialides produce conidia in long chains with different pigmentation and ornamentation. Furthermore, colony colour and diameter, characteristics of the vegetative mycelium as well as pigments produced provide a basis for classification. More recently, several new approaches have also been employed in the identification and classification of aspergilli. These include profiles of secondary metabolites (Rahbaek *et al.*, 2000), steroid transformation (Mostafa, 1995), isozyme analysis (Kozlowski and Stepien, 1982; Loudon and Burnie, 1995) nuclear restriction fragment length polymorphisms (RFLPs) combined with DNA probes (James *et al.*, 2000), ribosomal banding patterns and mitochondrial RFLPs (Varga *et al.*, 1994). Polymerase chain reaction (PCR)-based methods that

have been employed include amplification of the mitochondrial cytochrome b gene (Yokoyama *et al.*, 2001), internal transcribed spacer (ITS) regions of the ribosomal DNA (Henry *et al.*, 2000; Kumeda and Asao, 1996; Zhao *et al.*, 2001) and the utilisation of sequence-specific DNA primers of RAPD (random amplified polymorphic DNA) fragments (Mondon *et al.*, 1997). The black aspergilli (*Aspergillus* section *Nigri,* which includes *Aspergillus niger* and *Aspergillus awamori*) have always been an important group in biotechnology and their taxonomy was based primarily on morphological criteria (Kusters-van Someren *et al.*, 1991). This hampered the taxonomy as morphology is determined by both genetic and environmental factors and morphological criteria are rather subjective. Furthermore, distinct morphological differences may be caused by simple mutations and are thus not always reliable for establishing genetic relationships. The integration of molecular, physiological and biochemical methods has recently facilitated the understanding of the classification of aspergilli (Samson and Pitt, 2000). Currently it is accepted that the genus *Aspergillus* belongs to the family Trichocomaceae and contains 182 species with eight holomorphic genera accepted as being associated with *Aspergillus* anamorphs, namely *Chaetosartorya, Emericella, Eurotium, Fennellia, Hemicarpenteles, Neosartorya, Petromyces* and *Sclerocleista* (Pitt *et al.*, 2000). *Aspergillus* and its teleomorphs have been divided into three subgenera and 15 sections (Peterson, 2000). The biotechnologically important *Aspergillus oryzae* belongs to the subgenus *Aspergillus,* section *Flavi,* whereas *A. niger* and *A. awamori* are grouped in the subgenus *Aspergillus* section *Nigri* (Peterson, 2000). *Aspergillus nidulans* is phylogenetically distinct from these strains and has been grouped in subgenus *Nidulantes,* section *Nidulantes.*

2.2.2. Physiology and Life cycle

Fungi of the genus *Aspergillus* occur in a wide variety of habitats, such as soil, stored food and feed products and decaying vegetation (Kozakiewicz and Smith, 1994). They grow readily at temperatures between 15 and 30°C with some tolerating temperatures as low as 3°C and as high as 65°C. *A. niger* has a temperature range of 9 to 60°C with a temperature optimum of 17 to 42°C. Aspergilli can also tolerate a wide range of pH, with *A. niger* able to grow over a

pH range of 1.5 to 9.8. In *A. nidulans* and *A. niger* eight linkage groups have been identified and seven of the eight have been correlated with a specific band in the electrophoretic karyotype (Debets *et al.*, 1993; Swart *et al.*, 1994). Some species of *Aspergillus* exhibit an ascosporic stage, but for most aspergilli the teleomorph is unknown (Raper and Fennell, 1965). For the anamorph *A. nidulans* the teleomorph has been identified and was named *Emericella nidulans* and a graphic representation of the life cycle of the holomorph is given in Figure 1 (Pitt *et al.*, 2000).

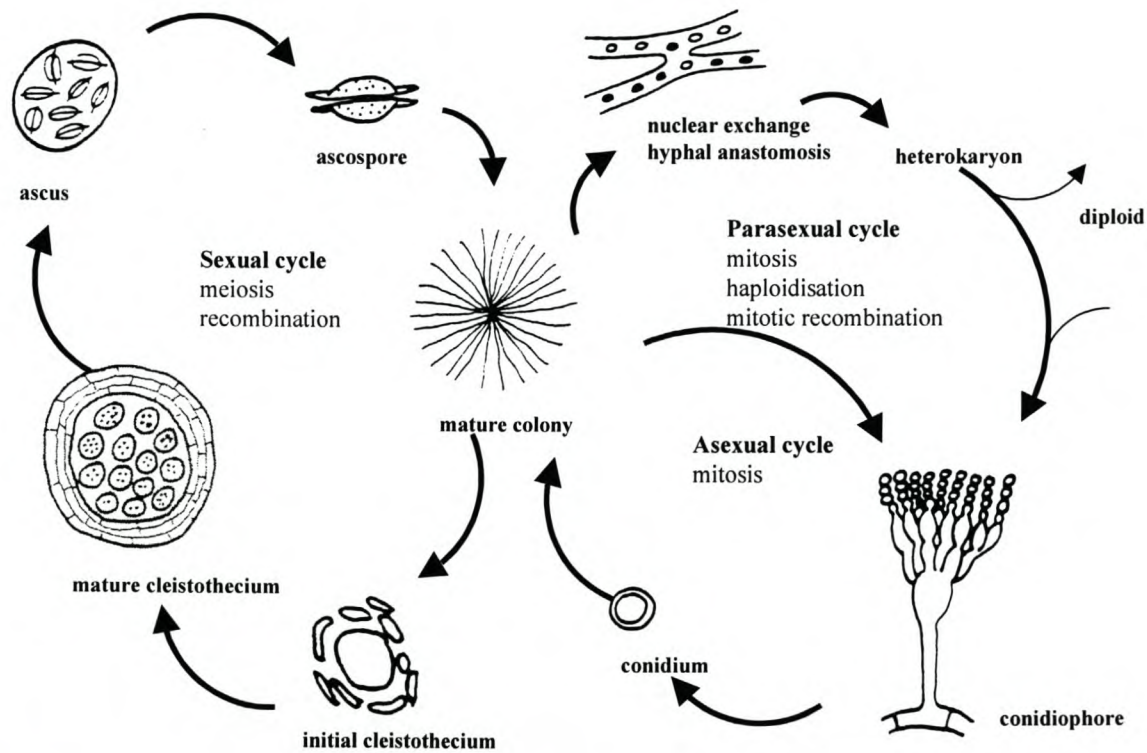

Figure 1. Life cycle of *A. nidulans* (Martinelli, 1994).

2.2.3. Industrial uses

For centuries fungi have been found to be both useful and harmful to humans (Alexopoulos and Mims, 1979). Fungi are the agents responsible for much of the disintegration of organic matter and as such they affect man directly by

destroying food, fabrics, leather and other consumer goods manufactured from raw materials subject to fungal attack. They cause the majority of known plant diseases and many diseases of animals and humans. Furthermore, they are the basis of a number of industrial processes involving fermentation, such as the fermentation of the cacao bean and the preparation of certain cheeses. They are employed in the commercial production of many organic acids, some drugs such as ergometrine and cortisone and some vitamin preparations, and are responsible for the manufacture of a number of antibiotics, particularly penicillin and griseofulvin.

2.2.3.1. Citric acid production

Citric acid is the most important organic acid produced by modern biotechnological means (Bodie *et al.*, 1994). It is commercially used as an acidulant in the food and pharmaceutical industries. In the food industry it is used in foods such as soft drinks, fruit juices, desserts, jams and jellies, candy and wine. In the pharmaceutical industry iron citrate is used as a source of iron and citric acid as a preservative for blood. Citric acid is also used in cosmetics and toiletries as a buffer, for pH adjustment and as an anti-oxidant. It is also used as a chelating and sequestering agent and citrate and citrate esters are used as plasticizers (Casida, 1968). At the beginning of the last century, citric acid could only be obtained commercially from citrus fruits (Johnson, 1954). Today, however, the product is obtained by fungal fermentation. The development of citric acid fermentation may be divided into three historical phases. The first phase started in 1893, with the discovery that certain *Penicillium* species produced citric acid. Various attempts were made to utilise this process commercially and many of these failed due to problems with contamination, selection of proper organisms, long fermentation time and high plant construction costs.

In 1917 during World War 1 it was discovered that *A. niger* is an excellent citric acid producer, marking the beginning of the second historical phase of citric acid production. During this phase, citric acid was produced by means of a surface culture method and since then a large amount of research has contributed to our knowledge of citric acid fermentation. The third phase was

inaugurated in 1938 when submerged culture methods supplanted the surface-culture methods. Advantages of submerged cultures include lower labour cost, higher yield, shorter time cycle, simpler operation and easier maintenance of asepsis. The main factors that are especially important for production in a submerged process include mycelium morphology, medium composition, pH, aeration and high quality materials used for fermenter construction (Bodie *et al.*, 1994). The most commonly used substrate for fermentation is beet molasses, a by-product of the sugar industry containing 10 to 20% sugar. Optimal yields of citric acid have been observed at sugar concentrations of 10% (w/v) (Xu *et al.*, 1989). High level accumulation of citric acid by *A. niger* is induced by a nutrient deficiency in the form of trace metals or phosphate. Fermentation is carried out at 28 to 30°C and the citric acid yield is in the order of 60 to 80 g of anhydrous citric acid per 100 g sugar (Kiel *et al.*, 1981). The inoculant consists of a high concentration of spores to produce an optimal form of pellet growth. Pellets are 0.1 to 0.5 mm in diameter, consisting of stubby mycelia with many swellings and with a granulated and vacuolated structure (Calam, 1986). Various strategies are continuously studied to improve the citric acid yield. One of these was a study on the effect of CO_2 on microbial growth (McIntyre and McNeil, 1997). Low levels of dissolved CO_2 were shown to stimulate growth, but at high levels of CO_2 there was a reduced growth rate, thus decreasing biomass concentration. The morphology of *A. niger* was also affected by higher CO_2 levels in that longer hyphae were present rather than the pellets with short stubby lateral hyphae, which are commonly accepted to be essential in achieving high citric acid yields.

2.2.3.2. Enzyme production

The major applications for industrial enzymes are in the manufacture of foods and beverages, waste-water treatment and the manufacturing of fine chemicals (Bodie *et al.*, 1994). The current world market for industrial enzymes is estimated at US$1.5 billion a year and a significant growth is predicted for the future (refer to Table 1) (Maister, 2001). Filamentous fungi are sources of about 40% of available enzymes (Archer and Peberdy, 1997). One of the industrially important enzymes produced by *Aspergillus* is glucoamylase (EC 23.2.1.3), also

referred to as amyloglucosidase (Bodie *et al.*, 1994). This is an exoamylase that catalyses the release of successive glucose units from the non-reducing ends of starch by hydrolysing α-1,4-D-glucosidic linkages. The glucose syrup and alcohol industries are the principal users of glucoamylase for saccharification of liquefied starch. Aspergilli such as *A. niger* produce two forms of glucoamylase, namely GA1 (Mr 85 300) and GA2 (Mr 77 600). Both forms are glycosylated and are capable of hydrolysing oligosaccharides, but GA1 hydrolyses starch nearly three times more rapidly than GA2. Both forms of *A. niger* glucoamylase are encoded by the single copy *glaA* gene and secreted proteases and/or glycosidases are involved in the generation of the different forms of GA in the culture broth.

Table 1. Global sales (US$ Mio.) of industrial enzymes from 1992 to 1998 and projected to 2009 (Godfrey, 2001).

YEAR	GLOBAL SALES (US $ MIO)	% CHANGE
1992	500	
1993	600	+20
1994	720	+20
1995	780	+8
1996	950	+22
1997	1200	+26
1998	1300	+8
2005 predicted	1700	+5 average
2009 predicted	2250	+8.1 average

Many other important enzymes are produced by aspergilli. α-Amylase is also used in commercial starch saccharification (Bodie *et al.*, 1994). Pectinolytic enzymes are used in the production of fruit juice and wine for increased juice extraction and juice clarification. Lactase is used as a digestive aid and proteases are used in the fermentation of wheat, soybeans and rice in the manufacture of Asian foods and beverages such as *shochu, miso* and *sake,* in beer brewing for haze clarification and in meat tenderisation. Glucose oxidase is used in the determination of glucose concentrations in body fluids and is also a component of other diagnostic enzyme kits. Other industrially important enzymes include catalase, cellulase, lipase, xylanase and phytase. It is clear

that enzymes from aspergilli are being used in a large variety of industries, making these fungi essential for our everyday lives.

Thus, with the established industrial importance and fermentation technology, filamentous fungi, especially *Aspergillus* species, were attractive hosts for the development of heterologous protein production systems.

2.3. Development of filamentous fungi as hosts for heterologous protein production.

To develop a heterologous protein expression system for a particular host a plasmid is required which contains all the information necessary for expression of the protein of interest and a transformation method for the host. The basic components of plasmids are (1) a gene that can be used for the selection of fungal transformants, (2) transcription elements for expression and, in the case of secreted proteins, secretion signals to direct protein production outside the cell and (3) bacterial plasmid sequences that can be used for selection and propagation of the plasmid in *E. coli*. Sometimes sequences for selection of plasmids in yeast or other alternative hosts are also included (Timberlake, 1991).

2.3.1. Selectable markers

Selective markers can be divided into at least three functional groups: genes that complement pre-existing mutations and lead to prototrophic growth (auxotrophic markers), genes that provide a new function and lead to drug resistance or growth on a previously non-utilisable nutrient source (drug resistance or added-function markers, also referred to as dominant selectable markers) and DNA fragments that give rise to selectable mutations when integrated into the genome of the recipient strain at specific locations (mutagenic markers). Each type of marker has specific uses, and in some cases it is advantageous to use combinations of markers.

2.3.1.1. Dominant selectable markers

Dominant selectable markers, also referred to as inhibitor resistance markers, can be used in transformation of both wild-type and mutant fungal strains and several of these markers are "broad-host range" markers which can be employed in different fungal species (Turner, 1994; Van den Hondel and Punt, 1991). Most of these markers are based on drug resistance, as reviewed by Van den Hondel and Punt (1991). Drug resistance markers that have been successfully employed in fungal transformation include genes conferring resistance to oligomycin, bleomycin, phleomycin, hygromycin and benomyl (Austin *et al.*, 1990; Dunne and Oakley, 1988; Kolar *et al.*, 1988; Punt *et al.*, 1987; Ward *et al.*, 1988). The *oliC3* gene of *A. niger* encoding an oligomycin-resistant allele of subunit 9 of the mitochondrial ATP synthetase complex confers resistance against oligomycin to *A. niger* recipient strains (Ward *et al.*, 1988). Similarly the *A. nidulans oliC31* allele confers resistance against the antibiotic to *A. nidulans*, however the *A. nidulans* gene is not able to act as a marker in *A. niger,* indicating the species specificity of this marker and thereby limiting its wide-spread use. Bleomycin and phleomycin are very closely related classes of metalloglycopeptide antibiotics produced by *Streptomyces verticillus* (Austin *et al.*, 1990). They are cytotoxic because they cause single-stranded and double-stranded breaks in DNA. The bleomycin and phleomycin resistance-encoding gene *ble* obtained from the bacterial transposon Tn5 has been used successfully in *Neurospora crassa* as well as *A. nidulans*, *Penicillium chrysogenum, Schizophyllum commune* and *Trametes versicolor* when placed under control of a fungal promoter and terminator (Austin *et al.*, 1990; Bartholomew *et al.*, 2001; Kolar *et al.*, 1988). Hygromycin resistance is conferred by the *E. coli* hygromycin B phosphotransferase gene (*hph*) (Punt *et al.*, 1987). This marker was also placed under control of a fungal promoter and terminator and used effectively in transformation of fungi such as *A. nidulans, A. niger* and the nematode-trapping fungus *Arthrobotrys oligospora* (Punt *et al.*, 1987; Tunlid *et al.*, 1999). In using antibiotic resistance markers, however, the sensitivity of the host strain to the antibiotic is a critical factor as some fungi have low sensitivity to some of the antibiotics. For example, at least 1 g/l hygromycin B is required to select for *A. nidulans* hygromycin B resistant

transformants (Punt *et al.*, 1987), and *P. chrysogenum* grows at concentrations of more than 1 g/l hygromycin B (Kolar *et al.*, 1988). Recently, a homologous drug-resistant marker gene has been developed for *Pleurotus ostreatus* (Irie *et al.*, 2001b). The marker gene Cbx^R encodes a mutant iron-sulphur protein subunit of succinate dehydrogenase and confers dominant resistance to a systemic fungicide, carboxin.

One of the most commonly used dominant markers is the *amdS* gene from *A. nidulans* that encodes the enzyme acetamidase (E.C.3.5.1.4) which hydrolyses acetamide to acetate and ammonium, permitting utilisation of acetamide as sole carbon and/or nitrogen source (Hynes, 1972; Hynes, 1973). It has been widely used as a dominant marker since fungi such as *A. nidulans* are insensitive to many antibiotics (Wernars *et al.*, 1985). Caesium chloride is added to the medium for reduction of background growth (Huge-Jensen *et al.*, 1989; Kelly and Hynes, 1985). In *A. nidulans* one copy of the *amdS* gene is sufficient for growth on acetamide, however growth on acrylamide is only possible if several copies of the gene are present (Van den Hondel *et al.*, 1992). The 1.5 kb *amdS* gene containing three small introns is located on chromosome III of *A. nidulans.* Acetamidase production is subject to multiple controls including nitrogen and carbon catabolite repression and induction by ω-amino acids, acetate and benzoate (Hynes, 1978). Therefore, production of the enzyme is regulated by several *trans*-acting regulatory genes affecting specific control mechanisms, as well as *cis*-acting regulatory sites closely linked to the structural genes (Corrick *et al.*, 1987; Hynes, 1979; Hynes, 1980; Hynes *et al.*, 1983). *Trans*-acting regulatory genes involved in *amdS* induction by small molecular weight effectors include *amdR* (ω-amino acids), *facB* (acetate) and *amdA* (acetate) (Apirion, 1965; Arst, 1976; Hynes and Davis, 1986). The *areA* gene is also a major activator involved in nitrogen metabolite repression of *amdS,* where the availability of easily metabolisable nitrogen sources such as ammonium or glutamine represses *amdS* expression (Hynes, 1972; Hynes, 1973). Since wild-type strains of *A. nidulans* do not grow strongly on acetamide medium it is possible to detect over-producing strains directly by their increased growth, and so *amdS* was one of the first genes used as selective marker for *A. nidulans* transformations (Hynes and Davis, 1986; Tilburn *et al.*, 1983). This

marker has since been successfully used to transform various fungi such as *A. niger* (Kelly and Hynes, 1985), *Trichoderma reesei* (Pentillä *et al.*, 1987), *P. chrysogenum* (Beri and Turner, 1987; Kolar *et al.*, 1988) and the plant pathogen *Cochliobolus heterostrophus* (Hynes, 1986). The homologous *amdS* gene from *A. oryzae* has also been isolated (Gomi *et al.*, 1991). Transformation occurs by homologous or non-homologous integration of vector DNA into the genome and frequently multiple copies of the vector are present in tandem repeats (Beri and Turner, 1987; Kelly and Hynes, 1985; Pentillä *et al.*, 1987; Wernars *et al.*, 1985). It is not known whether multi-copy transformants are generated by repeated integration events of plasmid monomers or by the formation of concatamers followed by a single integration event (Hynes, 1986). However, very high copy numbers of *amdS* have been shown to cause titration of *trans*-acting regulatory proteins, especially the *amdR* regulatory gene product (Arst, 1976; Arst *et al.*, 1978; Kelly and Hynes, 1987).

2.3.1.2. Auxotroph complementation

Transformation of nutritional mutants with cloned, wild-type genes requires the availability of an appropriate, cloned, prototrophic gene and stable, matching mutant (Turner and Ballance, 1985). In the initial transformation of *N. crassa*, the *N. crassa qa-2*$^{+}$ gene encoding catabolic dehydroquinase (3-dehydroquinate hydrolyase, EC 4.2.1.10) required for the catabolism of quinic acid, was used to obtain transformation of *N. crassa qa-2 arom-9* mutants (Case *et al.*, 1979). The *qa-2* gene was first obtained by complementation of an *E. coli aro* mutation and subsequently other fungal genes have been isolated in a similar manner and have been employed as nutritional markers in fungal transformation (Hynes, 1986). Some of the markers used (e.g. *pyrG, niaD* and *trpC*) have proven to be very useful, since they are functional in several species (Van den Hondel and Punt, 1991) (refer to Table 2).

Table 2. Auxotrophic markers used for homologous and/or heterologous transformation of filamentous fungi.

Marker	Species from which marker is derived	Encoded protein	First transformed species	Reference
acuA$^+$	*Ustilago maydis*	acetyl-coA synthase	*U. maydis*	Hargreaves and Turner, 1989
acuD$^+$	*Aspergillus nidulans*	isocitrate lyase	*A. nidulans*	Ballance and Turner, 1986
ade-2$^+$	*Schizophyllum commune*	unknown	*Phanerochaete chrysosporium*	Alic *et al.*, 1989
am$^+$	*Neurospora crassa*	glutamate dehydrogenase	*N. crassa*	Kinsey and Rambosek, 1984
amdS$^+$	*A. nidulans*	acetamidase	*A. nidulans*	Tilburn *et al.*, 1983
			Aspergillus niger	Kelly and Hynes, 1985
			Penicillium chrysogenum	Beri and Turner, 1987
argB$^+$	*A. nidulans*	L-ornithine-carbamoyl-transferase	*A. nidulans*	John and Peberdy, 1984
			A. niger	Buxton *et al.*, 1985
			Aspergillus oryzae	Gomi *et al.*, 1987
			Trichoderma reesei	Pentillä *et al.*, 1987
facA	*P. chrysogenum*	acetyl-coenzyme A synthetase	*P. chrysogenum*	Gouka *et al.*, 1993
inl$^+$	*N. crassa*	unknown	*N. crassa*	Akins and Lambowitz, 1985
leu$^+$	*Mucor circinelloides*	unknown	*M. circinelloides*	Van Heeswijck and Roncero, 1984
met$^+$	*A. oryzae*	unknown	*A. oryzae*	Iimura *et al.*, 1987
met-2$^+$	*Ascobolus immersus*	homoserine-O-trans acetylase	*A. immersus*	Goyon and Faugeron, 1989
niaD$^+$	*A. nidulans*	nitrate reductase	*A. nidulans*	Malardier *et al.*, 1989
			Fusarium oxysporum	Malardier *et al.*, 1989
			P. chrysogenum	Whitehead *et al.*, 1989
niaD$^+$	*A. niger*	nitrate reductase	*A. niger*	Campbell *et al.*, 1989; Unkles *et al.*, 1989a
			A. nidulans	Campbell *et al.*, 1989
			A. oryzae	Campbell *et al.*, 1989
			P. chrysogenum	Campbell *et al.*, 1989; Whitehead *et al.*, 1989

Table 2. Continued.

Marker	Species from which marker is derived	Encoded protein	First transformed species	Reference
niaD$^+$	*A. oryzae*	nitrate reductase	*A. oryzae* *A. nidulans* *A. niger* *P. chrysogenum*	Unkles *et al.*, 1989b
niaD$^+$	*P. chrysogenum*	nitrate reductase	*P. chrysogenum*	Gouka *et al.*, 1991
nic-1$^+$	*N. crassa*	unknown	*N. crassa*	Akins and Lambowitz, 1985
Nit1	*Fusarium oxysporum*	nitrate reductase	*F. oxysporum*	Garcia-Pedrajas and Roncero, 1996
pkiA$^+$	*A. nidulans*	pyruvate kinase	*A. nidulans*	De Graaff *et al.*, 1988
prn$^+$	*A. nidulans*	proline catabolism	*A. nidulans*	Durrens *et al.*, 1986
pyr-3$^+$	*U. maydis*	dihydroorotase	*U. maydis*	Banks and Taylor, 1988
pyr-4$^+$	*N. crassa*	orotidine-5'-phosphate decarboxylase	*A. nidulans* *Aspergillus flavus*	Ballance *et al.*, 1983 Woloshuk *et al.*, 1989
pyr-6$^+$	*U. maydis*	orotidine-5'-phosphate decarboxylase	*U. maydis*	Kronstad *et al.*, 1989
pyrG$^+$	*Aspergillus awamori*	orotidine-5'-phosphate decarboxylase	*A. awamori*	Gouka *et al.*, 1995
pyrG$^+$	*A. nidulans*	orotidine-5'-phosphate decarboxylase	*A. nidulans*	Oakley *et al.*, 1987a
pyrG/A$^+$	*A. niger*	orotidine-5'-phosphate decarboxylase	*A. niger* *A. nidulans* *A. oryzae*	Goosen *et al.*, 1987; Van Hartingsveldt *et al.*, 1987 Goosen *et al.*, 1987 Mattern *et al.*, 1987
pyrG$^+$	*A. oryzae*	orotidine-5'-phosphate decarboxylase	*A. oryzae* *A. niger*	De Ruiter-Jacobs *et al.*, 1989
pyrG$^+$	*Aspergillus parasiticus*	orotidine-5'-phosphate decarboxylase	*A. parasiticus*	Skory *et al.*, 1990
pyrG$^+$	*M. circinelloides*	orotidine-5'-phosphate decarboxylase	*M. circinelloides*	Benito *et al.*, 1992
pyrG$^+$	*Claviceps purpurea*	orotidine-5'-phosphate decarboxylase	*C. purpurea* *A. niger*	Smit and Tudzynski, 1992
pyrG$^+$	*Solorina crocea*	orotidine-5'-phosphate decarboxylase	*A. nidulans*	Sinnemann *et al.*, 2000
pyroA$^+$	*A. nidulans*	unknown	*A. nidulans*	Van den Hondel and Punt, 1991
qa-2$^+$	*N. crassa*	catabolic dehydroquinase	*N. crassa*	Case *et al.*, 1979
qUTE$^+$	*A. nidulans*	catabolic dehydroquinase	*A. nidulans*	Da Silva *et al.*, 1986
riboB$^+$	*A. nidulans*	unknown	*A. nidulans*	Oakley *et al.*, 1987b

Table 2. Continued.

Marker	Species from which marker is derived	Encoded protein	First transformed species	Reference
sC1$^+$	*A. nidulans*	ATP sulphurylase	*A. nidulans*	Buxton *et al.*, 1989
sA$^+$	*A. nidulans*	phosphoadenosyl phosphosulphate reductase	*A. nidulans*	Turner, 1994
trp-1$^+$	*Cochliobolus heterostrophus*	trifunctional enzyme of tryptophan biosynthesis*	*A. nidulans*	Turgeon *et al.*, 1986
trp-1$^+$	*Coprinus cinereus*	trifunctional enzyme of tryptophan biosynthesis*	*C. cinereus*	Binninger *et al.*, 1987
trp-1$^+$	*S. commune*	trifunctional enzyme of tryptophan biosynthesis*	*S. commune* *C. cinereus*	Munoz-Rivas *et al.*, 1986 Casselton and De la Fuente Herce, 1989
trp-1$^+$	*N. crassa*	trifunctional enzyme of tryptophan biosynthesis*	*N. crassa*	Kim and Marzluf, 1988
trp-3$^+$	*N. crassa*	tryptophan synthetase	*N. crassa*	Vollmer and Yanofsky, 1986
trpC$^+$	*A. nidulans*	trifunctional enzyme of tryptophan biosynthesis*	*A. nidulans* *A. niger*	Yelton *et al.*, 1984 Goosen *et al.*, 1989
trpC$^+$	*A. parasiticus*	trifunctional enzyme of tryptophan biosynthesis*	*A. nidulans*	Horng *et al.*, 1989
trpC$^+$	*P. chrysosporium*	trifunctional enzyme of tryptophan biosynthesis*	*C. cinereus*	Casselton and De la Fuente Herce, 1989
trpC$^+$	*P. chrysogenum*	trifunctional enzyme of tryptophan biosynthesis*	*P. chrysogenum* *A. nidulans*	Picknett *et al.*, 1987; Sanchez *et al.*, 1987 Picknett *et al.*, 1989
ura3$^+$	*T. reesei*	orotidine-5'-phosphate decarboxylase	*T. reesei*	Bergès and Barreau, 1991
ura5$^+$	*T. reesei*	orotate phosphoribosyl transferase	*T. reesei*	Bergès and Barreau, 1991
ura-5$^+$	*Podospora anserina*	orotidylic acid pyrophosphorylase	*P. anserina*	Begueret *et al.*, 1984

*encodes for glutamine amidotransferase, indoleglycerolphosphate synthetase and phosphoribosylanthranilate isomerase

The most common selection systems based on complementation include use of the cloned *trpC*, *argB, niaD* and *pyrG* genes, as these were the earliest markers where mutant recipient strains were already available (John and Peberdy, 1984; Johnstone, 1985; Turner, 1994; Unkles *et al.*, 1989b). The *trpC* gene

complements mutants lacking phosphoribosylanthanilate isomerase activity and was isolated from *A. nidulans* by complementation of an *E. coli trpC* mutant (Yelton *et al.*, 1983). The *A. nidulans argB* gene encodes ornithine carbamoyl transferase (E.C.2.1.3.3.), an enzyme involved in arginine biosynthesis, and was cloned by complementation of the yeast *arg3* mutation (Berse *et al.*, 1983). This gene has been used to transform *argB*$^-$ mutants of *A. nidulans, A. niger, A. oryzae* and *T. reesei* (Buxton *et al.*, 1985; Gomi *et al.*, 1987; John and Peberdy, 1984; Pentillä *et al.*, 1987). In the nitrate assimilation pathway, the *niaD* gene codes for the nitrate reductase apoprotein (EC 1.6.6.8). This gene has been cloned from fungi including *A. nidulans, A. niger* and *A. oryzae* and has been employed in the transformation of various fungal species (Malardier *et al.*, 1989; Unkles *et al.*, 1989b; Unkles *et al.*, 1989a). The *pyrG* gene codes for the enzyme orotidine-5'-phosphate decarboxylase (EC4.1.1.23) which catalyses the final enzymatic step in the *de novo* biosynthesis of uridine monophosphate (UMP) (Benito *et al.*, 1992). Therefore, *pyrG*$^-$ mutants are auxotrophic for uracil or uridine (Goosen *et al.*, 1987). The equivalent *N. crassa pyr-4* gene was first used to successfully transform a *pyrG*$^-$ *A. nidulans* strain, demonstrating that a gene from one euascomycete can function in another (Ballance *et al.*, 1983). Subsequently, the *pyrG* genes of fungi such as *A. niger, A. nidulans, A. oryzae, A. awamori, Claviceps purpurea, Mucor circinelloides* and *Solorina crocea* have been isolated and employed in the development of transformation systems (Benito *et al.*, 1992; De Ruiter-Jacobs *et al.*, 1989; Goosen *et al.*, 1987; Gouka *et al.*, 1995; Mattern *et al.*, 1987; Oakley *et al.*, 1987a; Sinnemann *et al.*, 2000; Smit and Tudzynski, 1992; Van Hartingsveldt *et al.*, 1987;). The popularity of these genes as selective markers is due to (1) their early cloning in the development of fungal transformation systems, (2) the ready availability of the corresponding mutant strains, and (3) the simplicity and dependability of selection schemes for transformants (Timberlake, 1991). Markers such as *pyrG* and *niaD* are also attractive markers for developing a gene transfer system in poorly characterised fungal species, since the required mutants can be isolated by positive selection (Van den Hondel and Punt, 1991). In the case of *pyrG* they can be isolated by resistance to 5-fluoro-orotic acid (Goosen *et al.*, 1987; Mattern *et al.*, 1987; Smit and Tudzynski, 1992; Van

Hartingsveldt *et al.*, 1987) and in the case of *niaD*, by resistance against chlorate (Gouka *et al.*, 1991; Unkles *et al.*, 1989a).

2.3.1.3. Mutagenic Markers

In yeast and some filamentous fungi, plasmids carrying DNA fragments from the recipient species often integrate into the chromosome by homologous recombination (Timberlake, 1991). This integration often leads to loss of gene function and can sometimes be used for selection of the integration event and also transformation. For example, integration of an internal fragment of the *A. nidulans pyrG* gene will lead to loss of orotidine-5'-phosphate decarboxylase, which can be selected for by resistance to 5-fluorootic acid, as described above.

2.3.2. Promoters

For efficient heterologous expression, the gene of interest must be flanked by transcription control sequences that are functional in the host organism (Van den Hondel *et al.*, 1991). *S. cerevisiae* promoters have generally not been used successfully to drive gene expression in filamentous fungi, which implies that transcription control sequences probably differ fundamentally from the corresponding sequences of *S. cerevisiae* (Hynes and Andrianopoulos, 1989; Van den Hondel *et al.*, 1991). In filamentous fungi consensus TATA-boxes have been found in very few fungal promoters. Some genes possess TATA-like sequences at the expected position, while others simply have an AT-rich region 30-100 bases upstream of the transcription start point. Sequences related to the CAAT consensus have also been described. For a review see Ballance, 1986; Ballance, 1991 and Jeenes *et al.*, 1991. In some cases, remarkably short (± 50 bp) upstream sequences can initiate transcription precisely in filamentous fungi, which may explain why transcriptional signals appear to transfer so readily between filamentous fungi (Hamer and Timberlake, 1987; Punt *et al.*, 1990; Van den Hondel *et al.*, 1991). Various promoters from filamentous fungi have been isolated which include constitutive and regulated promoters (refer to Table 3) (Van den Hondel *et al.*, 1991). One of the strongest inducible promoters available to date is the alcohol dehydrogenase I (*alcA*) promoter, which shows low expression on glucose, but is strongly induced by ethanol or

threonine under glucose depleted conditions (Devchand *et al.*, 1989; Gwynne *et al.*, 1989; Hintz and Lagosky, 1993). Other regulatable strong promoters include the glucoamylase (*glaA*) promoter of *A. niger, A. oryzae* and *A. awamori;* the Taka-amylase A gene promoter (*amyB*) of *A. oryzae* and the *A. awamori* 1,4-β-endoxylanaseA (*exlA*) promoter (Fowler *et al.*, 1990; Gouka *et al.*, 1996a; Hata *et al.*, 1992; Kanemori *et al.*, 1999; Nunberg *et al.*, 1984). The *glaA* and *amyB* promoters are all inducible by starch or maltose and repressed by xylose or glycerol, while the *exlA* promoter is strongly inducible by D-xylose.

Table 3. Fungal promoters used for heterologous protein production.

Organism	Promoter	Encoded protein	Type[a]	Heterologous Hosts	Reference
Aspergillus awamori	*amyA*	α-amylase	I	*A. awamori*	Korman *et al.*, 1990
	amyB	α-amylase	I	*A. awamori*	Korman *et al.*, 1990
	exlA	1,4-β-endoxylanase A	I	*A. awamori*	Gouka *et al.*, 1997a; Van Gemeren *et al.*, 1996;
	gdhA	glutamate dehydrogenase	C	*A. awamori*	Moralejo *et al.*, 1999
	glaA	glucoamylase	I	*A. awamori*	Dunn-Coleman *et al.*, 1991
				Aspergillus nidulans	Dunn-Coleman *et al.*, 1991
				Aspergillus niger	Archer *et al.*, 1990a
A. nidulans	*alcA*	alcohol dehydrogenase	I	*A. nidulans*	Gwynne *et al.*, 1987; Ward *et al.*, 1992b
	alcC	alcohol dehydrogenase	I	*A. nidulans*	Upshall *et al.*, 1987
	amdS	acetamidase	I	*A. nidulans*	Turnbull *et al.*, 1989; Turnbull *et al.*, 1990
				A. niger	Turnbull *et al.*, 1990
	gpdA	glyceraldehyde-3-phosphate dehydrogenase	C	*A. awamori*	Moralejo *et al.*, 1999
				Aspergillus ficuum	Mullaney *et al.*, 1988
				Aspergillus giganteus	Wnendt *et al.*, 1990
				A. nidulans	Punt *et al.*, 1991b; Roberts *et al.*, 1989
				A. niger	Archer *et al.*, 1990a ; Punt *et al.*, 1991b; Roberts *et al.*, 1989
				Aspergillus oryzae	De Ruiter-Jacobs *et al.*, 1989
				Cladosporium fulvum	Oliver *et al.*, 1987; Roberts *et al.*, 1989
				Claviceps purpurea	Van den Hondel *et al.*, 1991
				Cryphonectria parasitica	Churchill *et al.*, 1990

Table 3. Continued.

Organism	Promoter	Encoded protein	Type[a]	Heterologous Hosts	Reference
A. nidulans	*gpdA*	glyceraldehyde-3-phosphate dehydrogenase	C	*Curvularia lunata*	Osiewacz and Weber, 1989
				Fusarium culmorum	Van den Hondel *et al.*, 1991
				Laccaria laccata	Barrett *et al.*, 1990
				Leptosphaeria maculans	Farman and Oliver, 1988
				Penicillium chrysogenum	Kolar *et al.*, 1988
				Penicillium roqueforti	Van den Hondel *et al.*, 1991
				Pseudocercosporella herpotrichoides	Blakemore *et al.*, 1989
				Schizophyllum commune	Mooibroek *et al.*, 1990
				Septoria nodorum	Cooley *et al.*, 1988
				Tolypocladium geodes	Calmels *et al.*, 1991
				Trichoderma hamatum	Van den Hondel *et al.*, 1991
				Trichoderma harzianum	Herrera-Estrella *et al.*, 1990
				Trichoderma viride	Herrera-Estrella *et al.*, 1990
				Trichosporon cutaneum	Glumoff *et al.*, 1989
	oliC	ATPase subunit 9	C	*A. nidulans*	Ward, 1991
	pgk	3-phosphoglycerate kinase	C	*A. nidulans*	Streatfield *et al.*, 1992
	tpiA	triosephosphate isomerase	C	*A. nidulans*	Upshall *et al.*, 1987
	trpC	tryptophan biosynthesis enzyme	C	*A. nidulans*	Hamer and Timberlake, 1987; Kos *et al.*, 1988
				C. parasitica	Churchill *et al.*, 1990
A. niger	*adhA*	alcohol dehydrogenase I	C	*A. nidulans*	Upshall *et al.*, 1987
	aphA	acid phophatase	I	*A. niger*	MacRae *et al.*, 1993
				A. nidulans	MacRae *et al.*, 1993
	glaA	glucoamylase	I	*A. niger*	Fowler *et al.*, 1990; Jeenes *et al.*, 1993
				Ustilago maydis	Smith *et al.*, 1990
				A. nidulans	Cullen *et al.*, 1987; Gwynne *et al.*, 1987
				A. awamori	Faus *et al.*, 1998
	god	glucose oxidase	I	*A. nidulans*	Whittington *et al.*, 1990
	gpdA	glyceraldehyde-3-phosphate dehydrogenase	C	*A. niger*	Van den Hondel and Punt, 1991
				A. nidulans	Van den Hondel and Punt, 1991
	oliC	ATPase subunit 9	C	*A. niger*	Ward *et al.*, 1988

Table 3. Continued.

Organism	Promoter	Encoded protein	Type[a]	Heterologous Hosts	Reference
A. niger	*pe*	pectin esterase	I	*A. niger*	Khanh *et al.*, 1991
	pelA	pectin lyase	I	*A. niger*	Gysler *et al.*, 1990
	pg	polygalacturonase	I	*A. awamori* *A. niger*	Ruttkowski *et al.*, 1991
	pkiA	pyruvate kinase	C	*A. nidulans* *A. niger*	Bartling *et al.*, 1996 Bartling *et al.*, 1996
	pkiA	pyruvate kinase	C	*A. awamori*	Bartling *et al.*, 1996
A. oryzae	*agdA*	α-glucosidase	I	*A. oryzae*	Minetoki *et al.*, 1995
	amyA	α-amylase	I	*A. oryzae* *A. niger*	Christensen *et al.*, 1988; Huge-Jensen *et al.*, 1989 Jeenes *et al.*, 1991
	amyB	Taka-amylase A	I	*A. nidulans* *A. oryzae*	Kanemori *et al.*, 1999 Takashima *et al.*, 1998
	glaA	glucoamylase	I	*A. oryzae*	Hata *et al.*, 1992; Tsuchiya *et al.*, 1993
Cephalo-sporium acremonium	*isp*	isopenicillin N synthetase	C	*C. parasitica*	Churchill *et al.*, 1990
Neurospora crassa	*β-tubulin*	β-tubulin	C	*C. parasitica*	Churchill *et al.*, 1990
	am	glutamate dehydrogenase	C	*N. crassa*	Kinnaird and Fincham, 1983
P. chrysogenum	*oliC*	ATPase subunit 9	C	*P. chrysogenum*	Bull *et al.*, 1988
	pcbC	isopenicillin N synthetase	I[b]	*P. chrysogenum* *A. awamori*	Cantwell *et al.*, 1990 Moralejo *et al.*, 1999
Pleurotus ostreatus	*sdi1*	component of the mitochondrial respiratory chain	C	*P. ostreatus*	Irie *et al.*, 2001a
Penicillium simplicissimum	*vaoA*	vanillyl-alcohol oxidase	I	*A. niger*	Benen *et al.*, 1998
Rhizomucor miehei	*mprA*	aspartyl protease	I	*Mucor circinelloides*	Dickinson *et al.*, 1987
Acremonium chrysogenum	*AlpA*	alkaline protease	C	*A. chrysogenum*	Isogai *et al.*, 1991
	cesB	B2 esterase	C	*A. awamori*	Moralejo *et al.*, 1999
S. commune	*Gpd*	glyceraldehyde-3-phosphate dehydrogenase	C	*Trametes versicolor*	Bartholomew *et al.*, 2001
Trichoderma reesei	*cbh1*	cellobiohydrolase	I	*T. reesei* *A. nidulans*	Harkki *et al.*, 1989; Nyyssönen *et al.*, 1993; Uusitalo *et al.*, 1991 Berka and Barnett, 1989
	eg1	endoglucanase	I	*A. nidulans*	Berka and Barnett, 1989
U. maydis	*hsp*	heat shock promoter	I	*C. parasitica*	Churchill *et al.*, 1990
Saccharomyces cerevisiae	*gpd*	glyceraldehyde-3-phosphate-dehydrogenase	C	*A. oryzae*	Hahm and Batt, 1990

[a]C = constitutive, I = inducible; [b]secondary metabolism

The *A. niger glaA* promoter has been studied using green fluorescent protein (GFP) as a reporter, as well as with the aid of deletion analyses (Fowler *et al.*, 1990; Santerre Henriksen *et al.*, 1999). These studies revealed that the promoter is induced to high levels when the fungus is grown on starch or maltose, to intermediate levels when grown on glucose, but that it is repressed in the presence of xylose (Santerre Henriksen *et al.*, 1999). The promoter region is 1966 bp and two regions essential for transcription have been identified. Region I (between position –318 and –562) is required for high-level expression and region II (which lies within the first 214 bp upstream of the translation initiation site) directs low-level expression and is the minimum region that can act as a promoter (Fowler *et al.*, 1990). Putative binding sites for the protein that mediates carbon catabolite repression (CREA) have also been identified (Archer and Peberdy, 1997; Kelly, 1994). The *A. niger glaA* promoter can function in other ascomycetes (such as *A. nidulans*), as well as in the basidiomycete *Ustilago maydis* (Smith *et al.*, 1990).

The most commonly used constitutive promoter is the *gpdA* promoter from *A. nidulans* (Punt *et al.*, 1988). Glyceraldehyde-3-phosphate-dehydrogenase (GPD) is a key enzyme in glycolysis and gluconeogenesis and in *A. nidulans* up to 5% of the soluble cellular proteins consists of GPD (Punt *et al.*, 1990). This indicates that the expression signals of the *gpdA* gene are very strong, making this an ideal promoter for heterologous expression. Promoter studies of the 1.3 kb region upstream of the ATG using a *lacZ* fusion strategy revealed two elements (at position –650 and –250) which act as transcription activation elements (Punt *et al.*, 1990; Punt *et al.*, 1995). A third element (a C + T- rich region directly upstream from the transcription start point) was shown to be involved in correct initiation of transcription.

Vectors have also been developed carrying various reporter genes to facilitate the analysis of promoter fragments as well as the identification of novel genomic fragments with strong promoter activity, which could have applications in the high-level expression of heterologous proteins (Ozeki *et al.*, 1996; Punt *et al.*, 1991a; Roberts *et al.*, 1989; Van Gorcom *et al.*, 1985; Van Gorcom *et al.*, 1986; Van Gorcom and Van den Hondel, 1988).

2.3.3. Secretion signals

A minimum requirement for secretion of proteins is that a sequence encoding a secretory signal peptide is included in the expression cassette (Van den Hondel *et al.*, 1991). This signal peptide is necessary for the translocation of a polypeptide across the membrane of the endoplasmic reticulum, the first step in the protein secretion pathway. Studies have shown that signal peptides do not contain certain recognition amino acid sequences that are necessary for secretion, rather the physical characteristics of the leader peptide are important (Jarai, 1997). Although a signal sequence from a protein in one species usually functions to direct secretion of that protein in an organism of a different species, secretion efficiency is often lower (Moir and Mao, 1990). Therefore host secretion signals are mostly employed for secretion of heterologous proteins in filamentous fungi. One of the most commonly used secretion signals is the *A. niger* glucoamylase secretion signal. Glucoamylase is secreted at high levels by various *Aspergillus* species (Van den Hondel *et al.*, 1992). Other secretion signals that have been employed include the α-amylase signal peptide from *A. oryzae* (Hellmuth *et al.*, 1995), and the signal sequences from the *A. niger* acid phosphatase (MacRae *et al.*, 1993), polygalacturonase II and pectin lyase (Bartling *et al.*, 1996). In some cases secretion vectors have also been constructed containing entire genes or N-terminal sequences of efficiently secreted homologous enzymes to aid the secretion of heterologous proteins by fusion to these sequences (Gouka *et al.*, 1997b; Jeenes *et al.*, 1993; Spencer *et al.*, 1998). This aspect is discussed in detail elsewhere (section 4.1.5.). Briefly, for this gene fusion strategy mainly the *A. niger* or *A. awamori* glucoamylase genes have been used (Gouka *et al.*, 1997b). Other genes that have also been employed for gene fusion strategies to improve secretion include the *T. reesei* cellobiohydrolase I, *A. awamori* α-amylase and the *A. oryzae* Taka-amylase genes (Gouka *et al.*, 1997b; Harkki *et al.*, 1989; Korman *et al.*, 1990). Since both *A. nidulans* and *A. niger* have been shown to exhibit an endoproteolytic activity equivalent to that of the yeast KEX2 protease, a yeast KEX2-like cleavage site is generally introduced between the homologous secretion sequence and the heterologous gene to facilitate the cleavage of the heterologous protein from the secretory sequences

(Broekhuijsen *et al.*, 1993; Contreras *et al.*, 1991; Jarai, 1997). There have also been cases where the signal peptide of a vertebrate gene itself has been used successfully and cleaved correctly from the mature protein, for example human tissue plasminogen activator and hen egg white lysozyme (Archer *et al.*, 1990a; Upshall *et al.*, 1987). This indicates that the signal cleavage enzymes and recognition signals of vertebrates, filamentous fungi and insects can be compatible, in contrast to the general failure of mammalian signal peptides in yeast (Davies, 1994).

2.3.4. Terminators

The mechanism of transcription termination and polyadenylation in filamentous fungi is unknown (Van den Hondel *et al.*, 1991). Filamentous fungal mRNAs tend to have heterogeneous 3' ends with several apparent polyadenylation sites, sometimes resulting in mRNA transcripts of varying lengths (Kleene *et al.*, 1987; Mullaney *et al.*, 1985). The higher eukaryotic polyadenylation signal AATAAA is seen intact or degenerate preceding polyadenylation sites in some fungal genes, but is more often absent (Clements and Roberts, 1986; Hata *et al.*, 1991; Mullaney *et al.*, 1985). Transcription termination and polyadenylation is however important to achieve optimal levels of mRNA and therefore all expression cassettes contain termination regions, often originating from the same gene as the promoter region (Davies, 1991).

2.3.5. DNA Transformation Methods

DNA transformation of a filamentous fungus was first achieved with *N. crassa* in 1979 (Case *et al.*, 1979). The method was adapted from the yeast spheroplast transformation method of Hinnen and co-workers and involved the glusulase-mediated spheroplasting of germinated conidia in the presence of sorbitol as osmotic stabiliser. DNA uptake was mediated by polyethylene glycol (PEG) 4000 and $CaCl_2$. The mechanism by which cellular membranes become competent for DNA uptake is not known, but PEG induces protoplast fusion and calcium chloride precipitated DNA may be internalised along with membrane during this process (Johnstone, 1985; Timberlake and Marshall, 1989).

Since this first transformation experiment, the protocol has been adapted to transform various filamentous fungi including *A. nidulans* (Ballance *et al.*, 1983; Tilburn *et al.*, 1983; Yelton *et al.*, 1984), *A. niger* (Buxton *et al.*, 1985; Kelly and Hynes, 1985), *T. reesei* (Pentillä *et al.*, 1987) and *P. chrysogenum* (Beri and Turner, 1987). For an overview of filamentous fungi which have been transformed see Ballance, 1991. All these procedures involve removal of the cell wall of germinated spores or hyphae with lytic enzymes (most commonly Novozym 234, an enzyme preparation from *Trichoderma harzianum* primarily consisting of glucanases and chitinase), in the presence of osmotic stabilisers, followed by co-precipitation of DNA with Ca^{2+} and polyethylene glycol. In some protocols $MgSO_4$ has been employed in the preparation of protoplasts for transformation in order to facilitate separation of protoplasts from mycelial debris (Tilburn *et al.*, 1983). In this medium, protoplasts vacuolate and become less dense than the solution, allowing their purification by centrifugation: mycelial debris forms a pellet at the bottom of the centrifuge tube while the protoplasts float (Johnstone, 1985). Various combinations of lytic enzymes (Novozym 234, helicase, β-glucuronidase, driselase) and osmotic stabilisers (sorbitol, sucrose, KCl, NaCl) have been used by different workers, and for each laboratory and organism it is necessary to establish appropriate protocols (Ballance *et al.*, 1983; Jeenes *et al.*, 1991; Tilburn *et al.*, 1983; Yelton *et al.*, 1984). There does not seem to be a great deal of difference in transformation frequency between these methods, all achieving about 10–100 transformants per microgram DNA. In some protocols the nuclease inhibitor aurintricarboxylic acid is added to the DNA prior to transformation as it was found to increase transformation frequencies by approximately 2 to 5 fold (Ward *et al.*, 1988). Electroporation of protoplasts has also been employed as an alternative to PEG-mediated transformation (Ward *et al.*, 1989).

Transformation methods that do not require protoplasting have subsequently been developed. A lithium acetate-mediated method has been developed for *N. crassa* where germinated conidia are treated with lithium acetate and then incubated with DNA (Dhawale *et al.*, 1984). This is a relatively rapid, simple and reliable method and transformation frequencies are comparable to those obtained with protoplasting methods. Other transformation methods include

biolistic nuclear transformation (Armaleo *et al.*, 1990) and *Agrobacterium tumefaciens*-mediated transformation (De Groot *et al.*, 1998). Using *Agrobacterium,* both hyphal tissue and conidia can be transformed and when this technique was employed for transformation of *A. awamori* conidia, transformation frequencies improved up to 600-fold as compared with the conventional protoplasting technique (De Groot *et al.*, 1998). Other fungi that have been successfully transformed using *Agrobacterium* include *A. niger, Fusarium venetatum, T. reesei, Colletotrichum gloeosporioides, N. crassa* and the mushroom *Agaricus bisporus,* indicating that this technique can be very useful, especially for species that are recalcitrant to transformation by other means.

Various factors that may influence DNA transformation frequency include carrier DNA, incubation period, incubation temperature, PEG molecular weight, PEG concentration, addition of cations and purity and concentration of transforming DNA (Wernars *et al.*, 1985). The most important of these is the purity of the vector DNA. In transformation of *A. nidulans* a non-linear relationship was observed between the number of transformants and the amount of vector used. A minimum of 2 µg vector was required for transformation and the number of transformants increased exponentially with an increasing amount of DNA up to 10 µg. At higher concentrations the relationship became linear and above 50 µg transformation frequency dropped, possibly due to impurities in the DNA preparations. Cotransformation is also an efficient process in filamentous fungi and the cotransformation frequency of unlinked sequences can equal that of covalently linked genes (Wernars *et al.*, 1987). Cotransformation can be used for introduction of genes for which direct selection is not possible.

2.3.6. Transformation Events

Transformed plasmids may be maintained extrachromosomally or be integrated into the genome of the recipient strain at homologous or heterologous sites and the copy number may vary from one to many copies (Timberlake, 1991). Abortive transformants can occur and in filamentous fungi sometimes up to 90% of the initially observed colonies remain very small and are incapable of further

growth upon subculture (Turner and Ballance, 1985). This may result from transient expression without stabilised integration.

2.3.6.1. Integration

In most filamentous fungi, stable transformants result from various types of recombinational interaction between transforming DNA and the chromosome of the recipient strain (Turner, 1994). Circular vector DNA can become integrated into the genome, but in some species linearisation of vector DNA prior to transformation increases transformation frequency and the proportion of homologous recombinations (Berka *et al.*, 1990; Frederick *et al.*, 1989; Miller *et al.*, 1985; Paietta and Marzluf, 1985). Mechanisms of fungal recombination and integration are reviewed by Orr-Weaver and Szostak (1985). In general three types of integration events can occur: Type I, integration of the vector by homologous recombination; type II, ectopic integration of the vector by non-homologous recombination; and type III, gene replacement (Case *et al.*, 1979; Van den Hondel and Punt, 1991). For most homologous selectable markers, homologous interactions (type I and III integrations) predominantly occur, resulting in gene conversion or integration of the whole vector sequence (Ballance and Turner, 1985; Campbell *et al.*, 1989; Dunne and Oakley, 1988; Yelton *et al.*, 1984). However, in some cases type II transformants are preferentially found, e.g. in *A nidulans* with the *trpC* gene (Ballance and Turner, 1985; Yelton *et al.*, 1984) and with the *amdS* gene (Wernars *et al.*, 1985). The most striking feature of transformation of *Aspergillus* is that integration shows no dependence on extensive homology between the transforming DNA and the host chromosomal sequences (Ballance and Turner, 1985). Plasmids can integrate into the genome by single crossover events (Tilburn *et al.*, 1983; Yelton *et al.*, 1984). Alternatively, plasmid co-integrants can form by homologous recombination of a number of plasmid molecules prior to chromosomal recombination, resulting in integration of multiple plasmid copies arranged in head to tail arrays (Wernars *et al.*, 1985). Integration of a single plasmid molecule can also be followed by integration of additional plasmids to form tandemly repeated arrays, and different integration events result in different levels of expression (Tilburn *et al.*, 1983). The ratio of homologous to

heterologous (ectopic) integration varies according to the plasmid used and the species and genetic strain being transformed (Timberlake, 1991; Wernars *et al.*, 1985). For example, *A. nidulans* shows about 70% homologous integration (Tilburn *et al.*, 1983; Yelton *et al.*, 1984). Heterologous, seemingly random integration is presumably favoured with plasmids containing low amounts of DNA homology to the target genome. However, even with plasmids having no known homology with the target genome, small stretches of target-plasmid DNA identity could provide nucleation sites to initiate homologous recombination reactions. If these sites are small (e.g. 6-bp sequence), integration would be expected to occur at many genomic locations, thus approximating random integration. Targeting to a homologous sequence can also be improved by cutting and linearising the vector within the homologous sequence or by using a plasmid containing an internal restriction fragment from the gene to which integration is to be targeted (Streatfield *et al.*, 1992; Timberlake, 1991). However, even with this strategy, integration does not occur solely at the targeted site. The type of integration is dependent on the strain used for transformation (Wernars *et al.*, 1985). For example, in one *A. nidulans* strain transformed with a vector containing the *amdS* gene, most of the transformants showed integration of one copy of the vector at the partially deleted *amdS* gene, whereas in another strain tandem integration of multiple copies at non-homologous sites occurred.

Integrated plasmids of homothallic fungi such as *A. nidulans* and *A. niger* are generally mitotically stable in that they are stably maintained during vegetative growth and through the asexual reproductive cycle (even after 100 days of growth on non-selective medium), but show meiotic instability through the sexual cycle (Mohr and Esser, 1990; Tilburn *et al.*, 1983; Wernars *et al.*, 1985). Tandemly repeated sequences are frequently lost after sexual reproduction cycles in *A. nidulans* (Kelly and Hynes, 1987; Tilburn *et al.*, 1983; Yelton *et al.*, 1983). However, most economically important filamentous fungi lack a sexual phase, thus with many species the behaviour of inserted DNA sequences in meiosis is irrelevant (Timberlake and Marshall, 1989). In some cases it has proved possible to rescue the transforming DNA integrated into the chromosome by making use of *cos* sites present in the original vector utilising a

technique called cosmid rescue, where the DNA is packaged into viral particles (Yelton *et al.*, 1985). Integrated vectors can also be recovered by digestion with restriction enzymes, followed by circularisation with T4 DNA ligase and subsequent transformation of *E. coli* (Ballance and Turner, 1985; Yelton *et al.*, 1984). It is often also possible to recover plasmids by transforming *E. coli* with untreated transformant DNA, even when the plasmid has been shown to integrate into a chromosome (Ballance and Turner, 1985). There seems to be a limited degree of "pop-out", or reversal of the integration event, leading to a very low amount of free molecules, which are however not able to replicate autonomously (Ballance, 1991).

2.3.6.2. Autonomous replication

In single-celled yeasts, selection can be stringent enough to ensure that only plasmid-bearing cells continue to divide, but in coenocytic filamentous fungi, the flow of nutrients along the hyphae will allow division of plasmidless nuclei, resulting in a heterokaryon (Buxton and Radford, 1984). However, replicative plasmids are more suited for gene expression studies. They have the advantage over integrative systems in that transformation rates are high, the context of the expressed gene is free from effects of different chromosomal integration sites, the risk of rearrangement accompanying integration is eliminated and copy number is dependent only on the selective system (Aleksenko *et al.*, 1996b).

To develop autonomously replicating vectors there are five potential sources of replication origins: (1) native nuclear plasmids, (2) DNA viruses, (3) mitochondrial DNA, (4) mitochondrial plasmids and (5) chromosomal DNA (Gems *et al.*, 1991; Johnstone, 1985). Plasmids such as the freely replicating yeast episomal plasmid shuttle vectors based on the naturally occurring 2-µm plasmid of *S. cerevisiae* have not been found in the filamentous fungi and the 2-µm replication origin does not function in these fungi (Gems *et al.*, 1991). A native plasmid-like DNA inducing senescence has been identified in the ascomycete *Podospora anserina* which could be developed as a vector for gene transfer, but no such DNA molecules have thus far been identified in other ascomycetes (Tudzynski *et al.*, 1980). Also, no native DNA viruses have been

found in *Aspergillus* species, which might supply "replicons" suitable for autonomous replication (Johnstone, 1985). Mitochondrial DNA sequences from *Aspergillus amstelodami* and *A. nidulans* that have replicative activity in *S. cerevisiae* were isolated and incorporated into integrative transformation vectors (Beri *et al.*, 1988). Although the *A. nidulans* sequence enhanced transformation frequency, neither of the ARS-containing plasmids were mitotically stable in *A. nidulans* and no freely replicating plasmids were detected. Three mitochondrial plasmid DNAs have been isolated from *N. crassa* and when ligated to *E. coli* vectors one of these was found to replicate autonomously in both *N. crassa* and *E. coli* (Stohl and Lambowitz, 1983). Similarly hybrid bacterial-mitochondrial plasmids exhibiting autonomous replication have been constructed for the fungus *P. anserina* (Stahl *et al.*, 1982). These are, however, exceptions. It has not proved possible to develop such vectors for other ascomycetous fungal species, possibly due to differences in the organisation of origins of replication between species (Jeenes *et al.*, 1991).

The best-known chromosomal elements that support replication of extrachromosomal plasmids in yeasts are the autonomous replicating sequences (ARSs) which are chromosomal origins of bidirectional replication (Aleksenko and Clutterbuck, 1997). Among filamentous fungi, an *ARS* has only been successfully isolated from zygomycetous fungi like *M. circinelloides* and from the basidiomycete *U. maydis*, however the autonomous replicating plasmids carrying these sequences were found to be mitotically unstable in both these fungi (Tsukuda *et al.*, 1988; Van Heeswijk, 1986). More than 100 independent clones that had replicative activity in yeast were isolated from an *A. nidulans* genomic library, however only one (designated *ANS1* for *Aspergillus nidulans s*equence 1) enhanced *Aspergillus* transformation frequency 50 to 100 fold (Ballance and Turner, 1985). *ANS1*-bearing vectors replicate while retaining high recombinogenic potential and undergo reversible integration into the chromosome, but do not promote autonomous replication (Aleksenko and Clutterbuck, 1997; Ballance and Turner, 1985). When a genomic library of *A nidulans* was constructed in *A nidulans*, a 6.1 kb fragment designated *AMA1* (autonomously maintained in *Aspergillus*) was isolated (Gems *et al.*, 1991). Vectors carrying this fragment exhibit 2000-fold enhanced transformation

frequency of *A. nidulans*, are maintained in free form at 10 to 30 copies per cell, are rarely transferred between nuclei and are not integrated into the fungal chromosome. The *AMA1* sequence consists of an inverted repeat of a sequence that has other isolated copies throughout the genome (Aleksenko and Clutterbuck, 1996). These sequences (mobile *Aspergillus* transformation enhancers or MATEs) share a high degree of sequence similarity and exhibit some features characteristic of mobile elements. The nucleotide sequence does not encode any extended polypeptides but contains ARS-consensus matches and a multiply repeated "Spe" motif, which may be described as a symmetrically duplicated topoisomerase I recognition site. The two long perfect inverted repeats (MATE elements) are necessary for efficient plasmid replication (Aleksenko *et al.*, 1996a). *In vivo* rearrangements of plasmids containing the *AMA1* replication-promotion element have been reported and *AMA1* plasmids were also shown to recombine efficiently with co-transforming DNAs to form autonomously replicating co-integrates (Gems *et al.*, 1995; Gems and Clutterbuck, 1993). Concatenation occurs by end-to-end ligation of linear molecules, together with homologous or non-homologous recombination of linear and/or circular molecules (Aleksenko, 1994). This recombination of the autonomously replicating plasmids during co-transformation is a phenomenon generated entirely by the conditions of transformation, i.e. protoplasting, DNA uptake and regeneration (Gems *et al.*, 1995). After this period a plasmid is structurally stable in vegetative mycelium for many generations. However, during sexual reproduction, autonomous plasmids exhibit increased recombination, which results in both plasmid concatenation and integration into the chromosome. The *AMA1* sequence has been shown to function as an origin of replication not only in *A. nidulans,* but also in *A. niger, A. oryzae, P. chrysogenum* and *Penicillium canescens* (Aleksenko *et al.*, 1995; Fierro *et al.*, 1996; Gems *et al.*, 1991). Other chromosomal elements that can promote autonomous replication are telomeric sequences (Javerzat *et al.*, 1993). Linear plasmids containing two inverted stretches of human telomeric repeats transformed *A nidulans* at frequencies characteristic of autonomously replicating vectors, however replication of telomeric plasmids is never as efficient as that of circular *AMA1* plasmids (Aleksenko and Ivanova, 1998). Fungal sequences containing telomeric elements that promote autonomous

replication of linear plasmids have also been isolated (Javerzat *et al.*, 1993; Poplawski *et al.*, 1997; Powell and Kistler, 1990).

Although a variety of tools (transformation procedures, dominant and auxotrophic markers, transcriptional elements) have been developed for the genetic engineering of filamentous fungi, particularly aspergilli, the availability and application is still somewhat limited when compared to the tools available for yeasts such as *S. cerevisiae*, *P. pastoris* and *H. polymorpha*. This may be directly attributed to the greater degree of complexity of the multicellular filamentous fungi when compared to the unicellular yeasts.

2.4. Heterologous protein production in *Aspergillus*

There has been considerable commercial interest in exploiting filamentous fungi, especially aspergilli, as hosts for heterologous protein production (Kinghorn and Unkles, 1994). Aspergilli have the ability to secrete copious amounts of their own enzymes, which can be as much as 25 g/l of glucoamylase by *A. niger.* Through their history of industrial application in the production of antibiotics, enzymes, citric acid and oriental alcoholic beverages, fermentation and cultivation procedures, conditions and equipment has been established. *A. oryzae* has been used for more than 2000 years in the production of *koji*, a complex enzyme preparation for the production of soy sauce, *miso* and *sake* (Barbesgaard *et al.*, 1992). It has also been employed in Europe since the beginning of the 20th century to produce enzymes for baking and brewing. Cultivation took place in open trays, releasing massive amounts of conidia into the environment, but without adverse effect to the population. No proven carcinogenic metabolites have been identified in *A. oryzae* and aflatoxins are not formed although it belongs to the section *Flavi* (Barbesgaard *et al.*, 1992; Peterson, 2000). This group of mycotoxins is produced exclusively by other members of the section *Flavi*, notably *A. flavus, Aspergillus nominus* and *Aspergillus parasiticus. A. niger* is known to occur naturally in foods and is commonly present in products such as rice, seeds, nuts, olives and dried fruits (Coenen *et al.*, 1998). As mentioned previously it also has a long history (since 1919) in the industrial production of enzymes and citric acid. In the US, enzyme

preparations from *A. niger* have had a GRAS (Generally Regarded As Safe) status since 1973 and there is no evidence that industrial strains used are able to produce toxins. Detailed safety evaluations and toxicity tests of various homologous and heterologous enzyme preparations from *A. niger* and *A. oryzae* have clearly indicated that there are no reasons for safety concerns in the human consumption of these products (Barbesgaard *et al.*, 1992; Coenen *et al.*, 1998; Greenough *et al.*, 1996; Lane *et al.*, 1997). Therefore, the fungi *A. niger* and *A. oryzae* also have been granted GRAS status by the United States Food and Drug Administration, making them acceptable hosts for the production of recombinant proteins of pharmaceutical interest.

2.4.1. Factors affecting heterologous protein expression

When employing *Aspergillus* as a host for the production of heterologous proteins, various factors influence the level of protein yield as well as the integrity and functionality of the protein:

2.4.1.1. Site of integration

Transformation of filamentous fungi with recombinant DNA frequently results in non-specific integration of the molecule into the chromosome, as already described. A common feature of this process is the duplication of the integrated DNA, producing multiple copies of the target gene (Verdoes *et al.*, 1993). Although it can be generally said that increasing the copy number will result in improved yields, there appears to be no strict correlation between the two (e.g. Christensen *et al.*, 1988; Fowler *et al.*, 1990; Finkelstein *et al.*, 1989; Punt *et al.*, 1991b; Verdoes *et al.*, 1994b). This was also found to be the case in the expression of hepatitis B viral proteins in *Aspergillus* (Paper 1 and 2, this thesis). The most likely explanation is that the DNA is integrating into regions of the chromosome which are transcribed with different efficiencies (Verdoes *et al.*, 1994b). Nucleosome positioning can either restrict or provide access of transcription factors to their recognition sequences in promoters and is therefore a powerful element of control in gene expression (Archer and Peberdy, 1997). This problem can be overcome by using site-directed integration vectors,

resulting in gene replacement or integration into a non-essential gene which is known to be highly expressed (Gouka *et al.*, 1995; Van Gemeren *et al.*, 1996).

2.4.1.2. Transcriptional control

Several strong promoters from highly expressed genes are currently being exploited for the expression of recombinant genes, as has been discussed in section 2.2. Strategies for optimising the production of proteins have to ensure that transcription levels do not limit protein yields. The most straightforward approach to transcriptional enhancement has been to alter gene dosage, for example, glyceraldehyde-3-phosphate dehydrogenase overproducing strains were constructed containing 9 copies of the *gpdA* gene (Hanegraaf *et al.*, 1991) and glucoamylase overproducing strains of *A. niger* were constructed carrying around 20 copies of the *glaA* gene (Verdoes *et al.*, 1993; Verdoes *et al.*, 1994b). However, introduction of more copies led to reductions in glucoamylase levels due to gene rearrangements (Verdoes *et al.*, 1994a). One important factor affecting heterologous protein expression in this regard is regulatory factor limitation (Verdoes *et al.*, 1995). Limitation at the level of the amount of regulatory factors available has been observed for the overexpression of several fungal genes, including *glaA* (Verdoes *et al.*, 1994a), genes involved in lactam and ω amino acid catabolism (Kelly and Hynes, 1987), and genes involved in alcohol metabolism (Davies, 1991). Specific trans-acting regulatory proteins regulate expression of these genes. The regulatory proteins are limiting when multiple copies of these gene promoters are employed to direct expression of heterologous genes in transformants carrying multiple copies of the expression vector. If the regulatory factors have been identified, these limitations can be overcome by increasing the expression level of the gene encoding the limiting regulatory factor through the introduction of additional copies of this gene (Davies, 1991; Kelly and Hynes, 1987).

In general, with a few exceptions, the transcription of recombinant genes is not limiting in filamentous fungi and the majority of eukaryotic mRNAs are relatively stable in the cytoplasm (Jeenes *et al.*, 1991). Five structural features of mRNA have been shown to strongly influence this stability: (i) the 5' 7-methylguanosine

triphosphate cap, and (ii) the 3'-poly A tail, both of which have a protective function on mRNA stability; (iii) the mRNA length, which sometimes negatively regulates mRNA stability; (iv) posttranscriptional base modifications, such as methylation of adenine residues or the conversion of adenines to inosines; and (v) mRNA-stabilising or destabilising sequences (Gouka *et al.*, 1996b).

2.4.1.3. Intron splicing

The majority of filamentous fungal genes sequenced contain introns, ranging in size from 48 bp to 398 bp (Ballance, 1991; Clements and Roberts, 1986; Gomi *et al.*, 1991). In higher eukaryotes, the majority of genes contain introns, which can be several kilobases in size, whereas in yeasts introns are very much the exception and tend to be relatively short (a few hundred bases or less) (Ballance, 1986). Filamentous fungal genes appear to be somewhere in between in this respect. Sequences at the 5' and 3' splice site are highly conserved and are similar to those observed in yeast and in higher eukaryotes, though both yeast and filamentous fungi lack the pyrimidine-rich stretch which precedes the 3' splice site in higher eukaryotes. There are also sufficient differences between the conserved sequences of introns in yeasts and filamentous fungi. Therefore, some yeasts fail to splice fungal introns. However, filamentous Ascomycete genes are to a large extent interchangeable, indicating that the intron splice signals are conserved. For example, the *A. nidulans amdS* gene contains three introns, but is functional in various heterologous hosts, such as *A. oryzae, A. niger, P. chrysogenum* and *T. reesei* (Beri and Turner, 1987; Gomi *et al.*, 1991; Kelly and Hynes, 1985; Pentillä *et al.*, 1987). Therefore, fungal genes can be expressed in a heterologous host without the prior removal of introns. However, filamentous fungi have not been shown to remove introns from mammalian genes.

2.4.1.4. Codon bias

All organisms tend to favour a subset of codons and this bias is generally most marked in highly expressed genes (Ballance, 1991). Thus different genes in the same genome have related codon preference rules and it seems clear that certain codons are optimal for the translation system in a particular organism

(Sharp and Cowe, 1991). These "optimal" codons are those that are recognised most efficiently by the most abundantly available tRNA species and, just as the optimal codons vary among species, so do the anticodon sequences and relative abundance of different tRNAs. The codons are translated at different rates and the rate of polypeptide chain elongation will influence the efficiency with which ribosomes are utilised, affecting the growth rate of the cell and thus providing the basis for a selective advantage of optimal codons in highly expressed genes. This implies that genes which are strongly expressed are more biased towards optimal codons than genes expressed at a lower level and this leads to the conclusion that, in addition to the regulatory flanking elements of a structural gene, the coding sequence itself contains crucial information with regard to expression levels of a gene (Bennetzen and Hall, 1981; Hoekema *et al.*, 1987). In filamentous fungi the degree of bias varies, being particularly marked in *N. crassa* and less so in *A. nidulans* (Ballance, 1986). The preferred codons are similar in all ascomycete filamentous fungi for which genes have been sequenced. There is a preference for codons ending in pyrimidines, or where this is not possible, codons ending in G. In both genera there is a tendency to avoid usage of codons ending in A. This is also seen in *S. cerevisiae* (Bennetzen and Hall, 1981), though there are several cases where the codons preferred by *S. cerevisiae*, e.g. GAA for Glu and AGA for Arg, are different from those preferred by filamentous fungi, where GAG is preferred for Glu and GGU or CGC for Arg (Ballance, 1986). In highly expressed or constitutive genes in *Aspergillus* there is generally a preference for G or C in the third codon position (Clements and Roberts, 1986; Gomi *et al.*, 1991; Lloyd and Sharp, 1991). Only 10 of the codons identified as being optimal for *A. nidulans* are among the 22 optimal codons in *S. cerevisiae* (Lloyd and Sharp, 1991; Sharp and Cowe, 1991). Interestingly, the codon dialects in these two species are at least as divergent, if not more divergent, than those of *E. coli* and *S. cerevisiae* (Lloyd and Sharp, 1991). In mammalian genes, the percentage of A-ending codons is low, with G- and C-ending codons prevailing, corresponding more closely to the codon strategy of *Aspergillus*, than that of yeast and bacteria (De Boer and Kastelein, 1986). However, mammalian viruses (SV40, adenovirus, hepatitis) differ again from their hosts with A-ending codons being exceptionally frequent. Codon usage in *A. nidulans* is summarised in Lloyd and

Sharp, 1991. Codon usage may influence the translation of heterologous genes, although this has not been investigated in any detail in filamentous fungi. Codon preference was examined in the expression of *Cyamopsis tetragonoloba* α-galactosidase in *A. awamori* (Gouka *et al.*, 1996b). Full-length mRNA was not detected from the wild-type gene, but was detected when the codon preference was altered to that of *S. cerevisiae,* suggesting that mRNA stability had been affected due to the presence of rarely used codons, possibly resulting in temporary translation arrest and subsequent degradation of the transcript. Therefore, the codon context of a heterologous gene may influence the yield of a protein where mRNA is produced at high levels (Romanos *et al.*, 1992). This may be more likely to occur in minimal growth medium when the cell produces a wide variety of biosynthetic enzymes, encoded by genes containing rare codons. Highly efficient translation of cloned mammalian or other heterologous genes may thus require careful comparison of codon usage of the gene in relationship to the codon preference of the host cell. Also, an understanding of the role of the coding sequence in controlling gene expression is required to achieve an expression level of heterologous genes in *Aspergillus* that is equivalent to that of homologous genes (Gomi *et al.*, 1991). Therefore the construction of a synthetic gene with a codon usage optimised for *Aspergillus* species might improve the translation efficiency in some proteins, resulting in increased protein levels.

2.4.1.5. Secretion

Many proteins of biotechnological importance produced in filamentous fungi are secreted because it makes the product easier and cheaper to purify (Archer and Peberdy, 1997). Continuous fermentation can then be employed, because cell lysis to recover an intracellular product is unnecessary. Continuous fermentation reduces product loss which can occur due to inefficient lysis or internal proteolysis during intracellular product recovery, and facilitates product purification (Jeenes *et al.*, 1991; Smart, 1991). Also, possible toxic effects of intracellular product build-up are reduced and, by passing through the secretory pathway, processing events such as glycosylation and proteolytic maturation can occur to yield correctly folded, fully active molecules. However, there are

some disadvantages to secreting heterologous proteins into the growth medium. Several fungal strains secrete a wide array of proteases that can degrade the product (Van den Hombergh *et al.*, 1997c). Furthermore, many fungi also produce large amounts of organic acid, resulting in a low pH (pH 1.5-2.0 in some cases), which can alter the structure of certain proteins or lead to increased sensitivity to acid proteases active in the culture broth (Jeenes *et al.*, 1991). Although homologous proteins such as secreted filamentous fungal gene products (both homologous and heterologous) can often attain levels measured in g/l, secreted proteins derived from non-fungal sources tend to give yields measured in mg/l (Jeenes *et al.*, 1991). Thus, for these proteins the secretory pathway may present a bottleneck. Intracellular compartmentalisation of recombinant proteins appears to be less of a problem in filamentous fungi than in *S. cerevisiae* or *E. coli,* indicating that many of the problems of expression are due to the secretion pathway or the extracellular environment.

To identify possible bottlenecks in the secretory pathway, the processes involved in secretion need to be elucidated. The protein secretion pathway processes in filamentous fungi do not essentially differ from those in yeast and mammalian cells (Moir and Mao, 1990) and are reviewed by Gouka *et al.* (1997b). Essentially, co-translational entry of a protein into the lumen of the endoplasmic reticulum (ER) with concomitant processing of the N-terminal signal peptide is followed by translocation through the ER lumen to membrane-bound vesicles and targeting to the Golgi compartment. In the Golgi compartment processes such as glycosylation and processing occur and finally the protein is transported to the cell wall, again in vesicles, and is secreted into the extracellular medium through the fusion of the vesicles with the hyphal membrane. The precise location of protein secretion is under debate, but occurs primarily at the hyphal tips (Gordon *et al.*, 2000a; Gordon *et al.*, 2000b). The wall at the hyphal tips is newly synthesised and may be more porous than elsewhere, thereby facilitating secretion.

Studies on protein secretion in fungi have shown that in most cases the presence of a fungal (or non-fungal) sequence encoding a secretion signal is sufficient to promote secretion of a protein into the culture fluid (Brandhorst and

Kenealy, 1995; Punt *et al.*, 1991b; Punt *et al.*, 1994; Van den Hondel *et al.*, 1992). However, in some cases the heterologous gene is fused to the 3' end of a highly expressed and efficiently secreted fungal gene, such as glucoamylase, as described by Gouka *et al.* (1997a). Fusion can be effected to the complete fungal gene or to a truncated gene (Tsuchiya *et al.*, 1994). For these gene fusion strategies, the *A. niger* or *A. awamori* glucoamylase (*glaA*) genes have been mainly employed, resulting in improved levels of secreted bovine prochymosin (Ward *et al.*, 1990), porcine pancreatic phospholipase A2 (Roberts *et al.*, 1992), human interleukin-6 (Contreras *et al.*, 1991), hen egg-white lysoszyme (Jeenes *et al.*, 1993) and human lactoferrin (Ward *et al.*, 1992a). The production increase varies from 5 to 1000-fold, depending on the protein, resulting in levels varying from 5 mg/l to 250 mg/l. A similar gene fusion strategy was therefore also employed to possibly direct and enhance secretion of the hepatitis B virus major (S) and large (L) proteins by *A. niger* (Paper 2, this thesis). However, in this case the strategy was unsuccessful, probably due to the underlying mechanisms of viral protein assembly (Huovila *et al.*, 1992; Prange and Streeck, 1995; Paper 2, this thesis).

Apart from *glaA* gene fusions, fusion constructs utilising the *A. awamori* α-amylase (Korman *et al.*, 1990) and the *A. oryzae* Taka-amylase/glucoamylase (Tsuchiya *et al.*, 1994) have also been successful in *Aspergillus.* The success of this strategy can be attributed to the structure of these carrier enzymes. The glucoamylases can be divided into three domains: an N-terminal catalytic domain, a C-terminal starch-binding domain and a flexible *O*-glycosylated linker region that separates the two domains (Archer, 1994). The C-terminal starch-binding domain can be efficiently replaced by the heterologous protein (Broekhuijsen *et al.*, 1993; Jeenes *et al.*, 1993; Ward *et al.*, 1995), although fusions to full-length glucoamylase have also been successful (Contreras *et al.*, 1991; Roberts *et al.*, 1992; Ward *et al.*, 1990). The positive effect of the fusion is probably caused by the fact that the linker region permits the catalytic domain and the rest of the fusion protein to fold independently. In general, the N-terminal fungal protein is believed to serve as a carrier, improving the translocation of the protein into the ER, to aid folding and to protect the heterologous protein from degradation. The fusion protein is then cleaved

further along the secretory pathway, resulting in the secretion of the separate proteins. Cleavage occurs either by autocatalytic processing by an unknown fungal protease (Baron and Tiraby, 1992; Nyyssönen *et al.*, 1993; Roberts *et al.*, 1992; Tsuchiya *et al.*, 1994; Ward *et al.*, 1990) or by a KEX2-like protease, for which a dibasic amino acid recognition site had been introduced specifically into the fusion protein (Broekhuijsen *et al.*, 1993; Calmels *et al.*, 1991; Contreras *et al.*, 1991; Mikosch *et al.*, 1996). The latter has proven an effective method of obtaining correctly processed mature heterologous proteins because an effective kexin-like maturase has been identified in *A. niger* (Jalving *et al.*, 2000). However, the sequences upstream and downstream of the KEX2 site affect the fidelity of the cleavage, with more faithful processing occurring when the downstream sequence is less prone to helix formation (Spencer *et al.*, 1998).

2.4.1.6. Chaperones

In the secretory pathway, many proteins (collectively known as chaperones) are involved in the transport of secretory proteins by membrane translocation and vesicular transport from the endoplasmic reticulum (ER) via the Golgi apparatus to the cell surface. Only a few genes encoding such proteins involved in the secretory pathway of aspergilli have been isolated to date (Punt *et al.*, 1998). These proteins are all resident in the ER lumen and are encoded by the *srpA* (Thompson *et al.*, 1995), *pdiA* (Ngiam *et al.*, 1997), *tigA* (Jeenes *et al.*, 1997), *prpA* (Wang and Ward, 2000), *sarA* (Punt *et al.*, 1994) and *bipA* (Van Gemeren *et al.*, 1997) genes. The *pdiA, tigA* (Jeenes *et al.*, 1997) and *prpA* (Wang and Ward, 2000) genes all encode protein disulphide isomerases which possess disulphide-bond exchange activities and, together with other chaperone proteins, are required for proper folding of soluble ER proteins (Jeenes *et al.*, 1997; Wang and Ward, 2000).

BiP (encoded by the *bipA* gene) is a chaperone protein first identified in the ER of mammalian cells, which belongs to the HSP70 class of stress-inducible proteins and is a homologue of the *S. cerevisiae* Kar2p (Normington *et al.*, 1989). In mammalian cells it was shown to bind transiently to nascent proteins, but permanently to misfolded proteins whose transport from the ER is blocked.

Therefore BiP has been afforded a dual role in preparing normal proteins for export from the ER by presenting them to PDI and other catalysts of polypeptide folding, and acting as quality control monitor which binds and retains unassembled or misfolded proteins in the ER where they are degraded (Kim *et al.*, 1992; Knittler *et al.*, 1995). In normal cells, the concentration of chaperones is likely to be sufficient for proper folding and assembly of secretory proteins. However, in expression systems where there is a greater flux of proteins being translocated into the ER, the folding, assembly and secretion machinery may become saturated, leading to improperly folded structures or protein aggregates which are not secreted (Ngiam *et al.*, 2000). Both PDI and BiP transcription levels increase in response to stress (Van Gemeren *et al.*, 1997; Wang and Ward, 2000). Higher levels of *prpA* transcript levels were found in *A. awamori* producing bovine prochymosin (Wang and Ward, 2000) and *bipA* expression was induced in recombinant *A. niger* strains overexpressing glucoamylase and human interleukin-6 (IL-6), respectively, and in recombinant *A. awamori* strains overexpressing single-chain antibody fragments (scFv4715) (Punt *et al.*, 1998). To overcome the secretion bottleneck, overexpression of BiP and/or PDI could increase the yield of secreted heterologous protein and this has proven successful in *S. cerevisiae* expressing bovine prochymosin (Gouka *et al.*, 1997b; Harmsen *et al.*, 1996). However, overexpression of BiP did not improve yields of IL-6 or scFv4715 from *Aspergillus*, nor did the overexpression of PDIA improve yields of hen egg white lysozyme in *A. niger* (Ngiam *et al.*, 2000; Punt *et al.*, 1998). In fact, in some cases the overexpression of chaperones negatively affects heterologous protein expression. This phenomenon was observed when BiP was overexpressed in an *H. polymorpha* strain expressing *A. niger* glucose oxidase (Van der Heide *et al.*, 2002). BiP overexpression also significantly reduced recombinant manganese peroxidase production by *A. niger* (Conesa *et al.*, 2002). With increased understanding of the transcriptional regulation of the chaperone proteins and their role in the secretory pathway, they will however play a key part in improving heterologous protein production by filamentous fungi.

2.4.1.7. Glycosylation

Glycosylation is one of several important events associated with the post-translational modification of proteins and plays an important role in protein folding, stability and function (Sorensen *et al.*, 1996; Wang *et al.*, 1996). It is perceived that this process is a bottleneck at several levels, which affects biosynthesis and secretion of homologous and heterologous glycoproteins. An understanding of the glycosylation of proteins is important when using filamentous fungi as hosts for heterologous protein production as the diversity in the composition of glycans associated with proteins could have major consequences with respect to their antigenicity and functionality (Speake *et al.*, 1980). Many commercially important eukaryotic proteins are glycosylated (Jeenes *et al.*, 1991). Carbohydrate structures of varying complexity are attached to the protein via *N*-glycosidic linkage to the asparagine residue in the sequence Asn-X-Ser/Thr or via *O*-glycosidic linkage, mainly to serine or threonine.

Like yeasts and mammalian cells, filamentous fungi are capable of both *N*- and *O*-linked glycosylation with varying carbohydrate structures (Archer, 1994; Kornfeld and Kornfeld, 1985; Neustroev *et al.*, 1993). The early events of N-glycan synthesis take place in the endoplasmic reticulum and are identical in yeasts, plants and higher eukaryotes (Maras *et al.*, 1997b). This is also true for fungi and the glycosyltransferase enzymes involved in the initial stages of the *N*-linked protein glycosylation pathway have been identified in *A. niger* (Speake *et al.*, 1980; Wallis *et al.*, 1999). A common precursor oligosaccharide (namely $Glc_3Man_9GlcNAc_2$) is transferred to Asn residues in nascent proteins still associated with the ribosome (Kornfeld and Kornfeld, 1985; Kukuruzinska *et al.*, 1987; Long and Rudick, 1979). Further processing occurs by removal of three glucose (Glc) residues and one mannose (Man) residue. The synthesis pathways then diverge between yeast and higher eukaryotes: another three Man residues are removed in the latter, resulting in a $Man_5GlcNAc_2$ oligosaccharide. This N-glycan then serves as an acceptor substrate for the first glycosyltransferase involved in complex carbohydrate formation, namely GlcNAc-transferase I. In yeast, however, large high-Man oligosaccharides are

formed by addition of Man residues by various mannosyltransferases (Kukuruzinska *et al.*, 1987). In filamentous fungi, N-glycans seem to be further processed by removal rather than addition of Man residues, which is closer to the situation prevailing in higher eukaryotes (Aleshin *et al.*, 1992; Chiba *et al.*, 1993; Evans *et al.*, 1990; Limongi *et al.*, 1995; Maras *et al.*, 1997a; Svensson and Larsen, 1983; Turnbull *et al.*, 1990; Upshall *et al.*, 1987). In fact, the presence of an acceptor substrate for GlcNAc-transferase I on certain glycoproteins from various *Aspergillus* and *Trichoderma* species has been reported (Chiba *et al.*, 1993; Maras *et al.*, 1997a). There may be differences in the selectivity for the various types of sugar residues, the capacity to add some types of residues (e.g. sialic acid) is absent and a tendency to add large numbers of mannose residues to the core backbone has occasionally been observed. For example a high mannose *N*-linked structure was found in recombinant *A. niger* pectate lyase expressed in *A. nidulans* (Colangelo *et al.*, 1999b) and in recombinant endopolygalacturonase I from *A. niger* (Colangelo *et al.*, 1999a). However, the "hyperglycosylation" that has been a major problem in *S. cerevisiae* appears to be less of a problem in filamentous fungi. Examples of this include human lactoferrin, the insect glycoprotein Bm86 and human tissue plasminogen activator. Lactoferrin is a glycosylated protein expressed and secreted by glandular epithelial cells and is found in high concentrations in milk of most species, functioning as protection against microbial infection and in the regulation of immune function and cellular growth promotion (Ward *et al.*, 1997). Murine lactoferrin expressed in *A. awamori* was biologically active and indistinguishable from native human breast milk lactoferrin regarding size, immunoreactivity and glycosylation. The insect glycoprotein Bm86, a cell surface glycoprotein from cells of the digestive tract of the cattle tick *Boophilus microplus,* when expressed in *A. niger* and *A. nidulans* was also glycosylated to a similar extent as the native glycoprotein (Turnbull *et al.*, 1990). In the case of human tissue plasminogen activator (t-PA) expressed in *A. nidulans* the fungus was capable of mimicking the differential glycosylation of the single chain forms leading to a doublet seen when the protein is secreted from mammalian cells (Kinghorn and Unkles, 1994; Upshall *et al.*, 1987). There is also no hyperglycosylation and this is in strong contrast to the t-PA produced in *S. cerevisiae* which is extensively hyperglycosylated

(Lemontt *et al.*, 1985; Upshall *et al.*, 1987). Regarding fungal enzymes, a comparison between the glycosylation patterns of phytase from *A. niger* and a recombinant phytase showed that the oligosaccharides released from native and recombinant phytase gave the same glycosylation pattern (Panchal and Wodzinski, 1998) and this was also the case in recombinant *Coprinus cinereus* peroxidase produced in *A. oryzae* (Limongi *et al.*, 1995). Expression of the hepatitis B virus S protein in *A. niger* also resulted in a product similar in size to the native protein (24 kDa), thereby indicating the absence of hyperglycosylation (Paper 1, this thesis).

Strategies to overcome possible hyperglycosylation problems include the *in vitro* conversion of the carbohydrate structure of heterologous proteins produced by fungi to mammalian-type oligosaccharides (Maras *et al.*, 1997b). This strategy was successfully employed to convert high-mannose carbohydrates on *T. reesei* cellobiohydrolase I to mammalian-type hybrid structures and could be employed for heterologous mammalian proteins. In yeast, the use of glycosylation mutants defective in mannan biosynthesis proved effective to reduce hyperglycosylation (Innis, 1989). This approach has not been used in filamentous fungi, but could be replicated.

In some cases overglycosylation can be desirable, as was found with heterologous *Aspergillus kawachi* glucoamylase and calf chymosin produced in *A. awamori* (Archer, 1994; Goto *et al.*, 1997). The addition of a glycosylation site to the glucoamylase resulted in an enzyme with higher digestibility of raw corn starch and higher stability in response to heat and extreme pH (Goto *et al.*, 1997). Introduction of an additional *N*-glycosylation site to chymosin led to a product with at least three-fold improved production levels, however with a reduction in specific activity (Archer, 1994).

2.4.1.8. Proteases

Proteolytic degradation is one of the major factors influencing the yield of secreted proteins, especially heterologous proteins where yields rarely reach the gram-per-litre level of many homologous enzymes (Archer and Peberdy, 1997; Van den Hombergh *et al.*, 1997c). Degradation can occur during the

secretion process and after secretion in the culture fluid as a result of host cell proteases (Jarai, 1997). Many filamentous fungi secrete considerable amounts of proteolytic enzymes, some of which are of interest in industrial applications, such as soy sauce production, *sake* brewing and milk coagulation in cheese manufacturing (Cheevadhanarak *et al.*, 1991; Gomi *et al.*, 1993; Thompson, 1991). Analysis of the *Aspergillus* proteolytic spectrum revealed the presence of a number of aspartic proteases, alkaline and semi-alkaline serine proteases and serine carboxypeptidases (Jarai, 1997; Van den Hombergh *et al.*, 1997c). Refer to Figure 2.

Four extracellular proteases with acid pH optima have been purified from *A. niger* culture filtrates (Dal Degan *et al.*, 1992; Inoue *et al.*, 1991; Krishnan and Vijayalakshmi, 1985; Krishnan and Vijayalakshmi, 1986; Van den Hombergh *et al.*, 1994). These include two aspartyl proteases, PEPA and

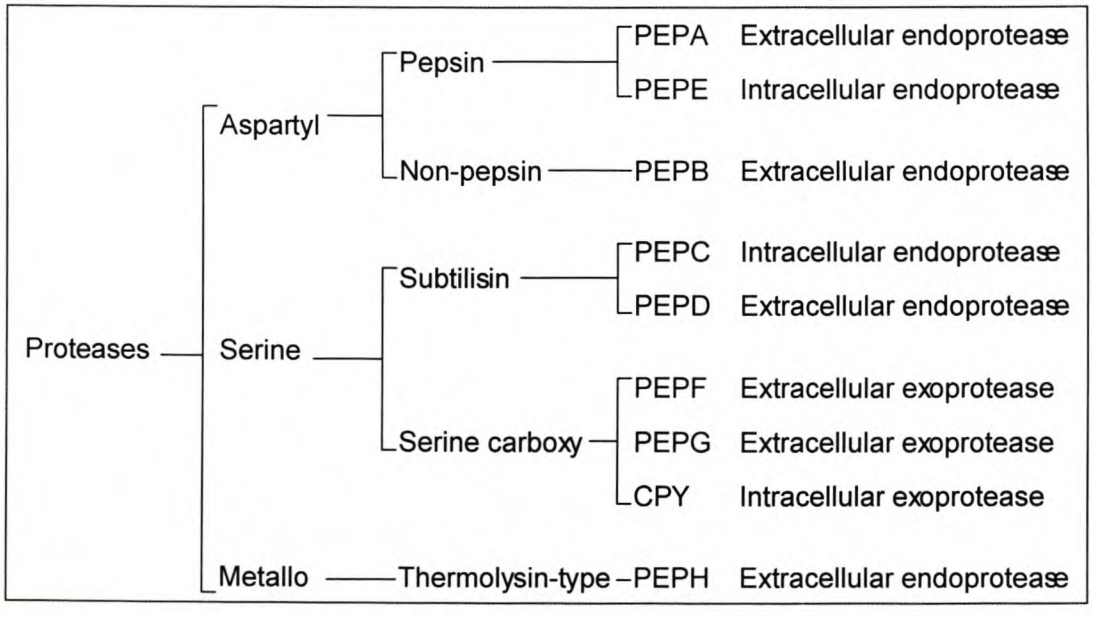

Figure 2. The proteolytic system of *A. niger* (Van den Hombergh *et al.*, 1997c).

PEPB, and two serine carboxypeptidases, PEPF (or CPDII) and PEPG (or CPDI). Aspergillopepsin A (PEPA) seems to be responsible for the majority of

extracellular acidic activities, while PEPB is a non-pepsin type acidic endopeptidase (Berka *et al.*, 1990; Gomi *et al.*, 1993; Inoue *et al.*, 1991). The *pepA*, *pepB* and *pepF* genes have been shown to be regulated by nitrogen and carbon catabolite repression, environmental pH and the presence of exogenous protein (Jarai and Buxton, 1994; Van den Hombergh *et al.*, 1994). *A. niger* also has a metalloprotease, PEPH (Van den Hombergh *et al.*, 1997c), and an alkaline protease, PEPD, which is an extracellular subtilisin-type serine protease (Jarai *et al.*, 1994a). *A. nidulans*, however, produces at least three neutral or alkaline extracellular proteases, which are produced in response to carbon-, nitrogen-, or sulphur-limiting conditions (Cohen, 1973a; Cohen, 1973b). A gene, designated *prtA,* encoding one of these alkaline extracellular serine proteases has been isolated (Katz *et al.*, 1994). In contrast to *A. niger,* only one gene (designated *prtB*) has been isolated encoding an acid protease homologous to the *A. niger* aspergillopepsin (Van Kuyk *et al.*, 2000).

In *A. niger* the proteases with acidic pH optima predominate, exhibiting the highest activity at low pH (Bartling *et al.*, 1996; Mattern *et al.*, 1992; Van den Hombergh *et al.*, 1997b). However, in *A. nidulans* neutral and alkaline proteases play a more important role and acid proteases are weakly expressed, which was indicated by the high levels of alkaline protease (*prtA*) expression and low levels of acid protease (*prtB*) expression observed in *A. nidulans* (Van Kuyk *et al.*, 2000). These differences may reflect differences in the physiology of the two species. *A. niger* rapidly acidifies the growth medium, whereas external alkalinisation occurs in *A. nidulans.* However, extracellular protease expression increases significantly upon derepression with respect to carbon and/or nitrogen source in both *A. niger* and *A. nidulans.* The molecular basis for these transcriptional control mechanisms has been elucidated with the cloning of three wide-domain regulatory genes, *creA, areA* and *pacC.* The *creA* gene encodes a negative regulator protein (CREA) that mediates carbon catabolite repression (Kelly, 1994), the *areA* gene encodes an activator protein (AREA) needed to alleviate nitrogen metabolite repression (Kudla *et al.*, 1990) and the *pacC* gene encodes a pH regulator protein which is only active at an alkaline pH and acts as an activator of alkaline target genes and a repressor of acid target genes (Tilburn *et al.*, 1995). Consensus recognition motifs for CREA and AREA

have been identified in the promoter regions of the *A. niger pepA, pepB* and *pepF* genes and mutations in the *areA, creA* and *pacC* genes resulted in reduced transcriptional levels of all extracellular proteases (Jarai and Buxton, 1994; Van den Hombergh *et al.*, 1997c).

Intracellular proteases can also be found in *Aspergillus* and thus far three proteases that are homologous to yeast vacuolar proteases have been cloned from *A. niger.* The PEPC protease is a subtilisin-type serine endoprotease homologous to the yeast vacuolar proteinase B (PRB1), PEPE is a pepsin-type aspartyl endoprotease homologous to the yeast vacuolar *pep4* gene product and CPY is a serine carboxypeptidase (Jarai *et al.*, 1994b; Van den Hombergh *et al.*, 1997c). These proteases are constitutively expressed and are probably involved in non-specific protein degradation and turnover, as well as in the activation of the precursors of several vacuolar enzymes (Jarai *et al.*, 1994b).

Various strategies have been employed to address the problem of proteolytic degradation of heterologous protein products. Many protease-encoding genes have been cloned and used to provide specific protease deletion strains and protease-deficient mutants have also been produced (Archer and Peberdy, 1997). Disruption of the gene encoding the major extracellular acid protease PEPA in *A. awamori* and *A. niger* reduced extracellular proteolytic activity to approximately 20% of that of the wild type (Berka *et al.*, 1990; Mattern *et al.*, 1992). Expression of chymosin in an *A. awamori pepA* deletion strain resulted in improved levels of extracellular chymosin production (Ward *et al.*, 1993). Proteolytic activity of a *pepB* deletion strain, however, was only reduced to 94% of that of the wild-type (Van den Hombergh *et al.*, 1997b). Deletion of *pepE* resulted in reducing the total intracellular proteolytic activity to 32% (Van den Hombergh *et al.*, 1997b). An *A. niger* strain lacking *pepA, pepB* and *pepE* was subsequently constructed and protease activity in this strain was reduced to 7% of that of the wild-type strain. This strain showed higher levels of *A. niger* pectin lyase production than the single or double protease mutants constructed and can be used to further optimise homologous and heterologous gene expression (Van den Hombergh *et al.*, 1997b).

Several protease-deficient UV mutants of *A. niger* have also been isolated and characterised (Mattern *et al.*, 1992; Van den Hombergh *et al.*, 1995; Van den Hombergh *et al.*, 1997a). These mutants have varying levels of proteolytic activity ranging from 80% to as little as 1-2% of that of the parental wild-type strain and contain mutations in structural and/or regulatory genes involved in proteolysis. When one of these strains was used to express human interleukin-6 and porcine phospholipase A2, respectively, active recombinant protein was secreted in both cases (Broekhuijsen *et al.*, 1993; Roberts *et al.*, 1992). An *A. nidulans* mutant with reduced levels of extracellular proteases has also been isolated with the mutations affecting the levels of alkaline protease gene mRNA, as well as altering the expression of other proteases (Katz *et al.*, 1996). Another strategy to construct protease deficient mutants is the antisense control strategy (Zheng *et al.*, 1998). By expressing the antisense RNA of the structural gene of carboxypeptidase (CPase), an *A. oryzae* mutant was isolated which showed (CPase) levels of 30% of that of the wild-type strain. Using this mutant as a heterologous protein expression host, a stable, higher level of human lysozyme expression could be obtained, compared with the wild type. Mutagenesis has also been employed to yield strains that acidify the medium less, thereby preventing induction of acid protease expression and degradation of heterologous protein products (Gordon *et al.*, 2000b). This kind of protease deficient, non-acidifying strain is commonly used in the expression of heterologous proteins, particularly non-fungal proteins, and was also utilised for the expression of hepatitis B viral proteins (Paper 1, this thesis; Paper 2, this thesis; Gordon *et al.*, 2000b; Wiebe *et al.*, 2001). In general, a mutagenesis-based selection programme with classical genetic strain improvement coupled to the cloning and targeted disruption of certain protease genes can provide a selection of host strains with diverse protease spectra and low proteolytic activity which can be employed in heterologous protein expression (Jarai, 1997).

Yield reduction of target heterologous proteins can be due not only to co-secreted proteases, but due to intracellular degradation (Gouka *et al.*, 1996a) and the release of intracellular proteases from hyphal disruption in older cultures (Archer *et al.*, 1992). Proteolytic activity can also be reduced by

growing cultures of recombinant *Aspergillus* strains in buffered media containing glucose as the carbon source at high concentrations to prevent depletion of preferred carbon source and acidification or alkalinisation of the medium with concomitant protease induction (Bartling *et al.*, 1996). Some problems with proteolysis may also be overcome by the inclusion of protease inhibitors in the growth media, but this is unlikely to be a cost-effective measure (Archer and Peberdy, 1997).

Apart from addressing the above-mentioned factors affecting heterologous protein production levels, other strategies for the improvement of protein production include random mutagenesis (MacKenzie *et al.*, 2000), DNA-tagged mutagenesis involving restriction enzyme-mediated integration as a mutagen (Yaver *et al.*, 2000), and physiological optimisation through temperature or substrate adjustments (MacKenzie *et al.*, 1994). Although considerable progress has been made concerning the understanding of protein overproduction, it should be considered that overcoming limitations at, for example, the level of transcription will probably reveal new limitations at other levels concerning cell physiology and protein secretion. Also, protein secretion in filamentous fungi has proven to be very expensive in relation to energy consumption. Therefore extensive protein overproduction may affect cell physiology which could in turn affect gene expression and genetic stability (Verdoes *et al.*, 1995). In conclusion, the factors influencing heterologous protein yield are complex and include biochemical and biophysical properties of the protein itself; its influence on, and modification of, the host's own cellular functions and the choice and deployment of those sequences that are necessary for efficient transcription and translation. Therefore optimisation strategies must be individually designed and assessed for each heterologous protein production system.

2.4.2. Heterologous proteins produced in *Aspergillus*

Since the development of molecular-biological techniques opened new ways to use filamentous fungi for the production of homologous and heterologous proteins, many fungal and non-fungal proteins have been expressed with varying success (Gouka *et al.*, 1997b; Kinghorn and Unkles, 1994; Verdoes

et al., 1995). A large market exists for recombinant enzymes in various industries, including the animal feed, glucose syrup, alcohol, wine, brewing, baking, fruit juice and dairy industries and this is reviewed by Archer (2000), Archer and Peberdy (1997), Bennett (1998) and Bodie *et al.* (1994). Some bacterial and fungal enzymes of commercial importance that have been produced by recombinant aspergilli are summarised in Table 4.

Table 4. Enzymes expressed in *Aspergillus*.

Enzyme	Source	Expression host	Production levels	Reference
Acid phosphatase	*Aspergillus awamori*	*A. awamori*	24.2 U	Piddington *et al.*, 1993
Acid protease	*Aspergillus oryzae*	*A. oryzae*	5236 U/g dry mycelium	Gomi *et al.*, 1993
Alkaline protease	*A. oryzae*	*A. oryzae*	41529 U/g substrate	Cheevadhanarak *et al.*, 1991
α-Amylase	Barley	*Aspergillus niger*	60 mg/l	Juge *et al.*, 1998
α-L-Arabino-furanosidase	*A. niger*	*A. niger* *Aspergillus nidulans*	2.48 U/ml 2.64 U/ml	Flipphi *et al.*, 1993 Flipphi *et al.*, 1993
Aspartic proteinase	*Rhizomucor miehei*	*A. oryzae* *A. awamori*	3.3 g/l 1.97 g/l	Christensen *et al.*, 1988 Ward *et al.*, 1993
Catalase	*A. niger*	*A. niger*	11 U/mg protein	Fowler *et al.*, 1993
Chloroperoxidase	*Caldariomyces fumago*	*A. niger*	10 mg/l	Conesa *et al.*, 2001
Cutinase	*Fusarium solani pisi*	*A. awamori*	30 – 70 mg/l	Van Gemeren *et al.*, 1996
Endoglucanase	*Cellulomonas fimi*	*A. nidulans*	~20 mg/l	Gwynne *et al.*, 1987
1,4-β-Endoxylanase	*A. awamori*	*A. awamori*	58 kU/ml	Hessing *et al.*, 1994
Δ6-Fatty acid desaturase	*Mortierella alpina*	*A. oryzae*	NR	Sakuradani *et al.*, 1999
α-Galactosidase	*A. niger*	*A. niger*	NR	Den Herder *et al.*, 1992
Glucoamylase	*A. niger*	*A. awamori* *A. niger* *A. nidulans*	4.6 g/l 7.5 g/l 1.2 g/l	Finkelstein *et al.*, 1989 Finkelstein *et al.*, 1989 Devchand *et al.*, 1989
Glucoamylase	*A. oryzae*	*A. oryzae*	29.4 U/ml	Hata *et al.*, 1991
Glucose oxidase	*A. niger*	*A. niger* *A. nidulans*	13.8 U/mg protein 5.4 U/mg protein	Whittington *et al.*, 1990
α-Glucosidase	*A. niger*	*A. nidulans*	18 mU/mg protein	Nakamura *et al.*, 1997

Table 4. Continued.

Enzyme	Source	Expression host	Production levels	Reference
β-Glucosidase Cellobiohydrolase Endoglucanase	*Trichoderma reesei*	*A. oryzae*	Co-expressed, several hundred mg/l cellulases	Takashima *et al.*, 1998
β-1,4-Endoglucanase	*T. reesei*	*A. niger*	138 U/ml	Rose and Van Zyl, 2002
Laccase	*Coprinus cinereus*	*A. oryzae*	8 – 135 mg/l	Yaver *et al.*, 1999
	Myceliophthora thermophila	*A. oryzae*	11 – 19 mg/l	Berka *et al.*, 1997
	Pycnoporus cinnabarinus	*A. niger*	70 mg/l	Record *et al.*, 2002
	Trametes villosa	*A. oryzae*	NR	Yaver *et al.*, 1996
Lignin peroxidase	*Phanerochaete chrysosporium*	*A. niger*	Enzyme inactive	Conesa *et al.*, 2000
Lipase	*Thermomyces lanuginosa*	*A. oryzae*	2.1 relative lipase units/ml	Yaver *et al.*, 2000
Manganese peroxidase	*P. chrysosporium*	*A. niger*	100 mg/l	Conesa *et al.*, 2000
		A. oryzae	5 mg/l	Stewart *et al.*, 1996
Manganese peroxidase	*Pleurotus eryngii*	*A. nidulans*	148.5 U/mg protein	Ruiz-Duenas *et al.*, 1999
Pectate lyase	*Erwinia carotovora*	*A. niger* *A. nidulans* *A. awamori*	0.4 mg/l 2.0 mg/l 0.8 mg/l	Bartling *et al.*, 1996
Pectin lyase A	*A. niger*	*A. niger*	NR	Harmsen *et al.*, 1990
Pectin methyl esterase	*A. niger*	*A. niger*	45 U/mg protein	Khanh *et al.*, 1991
Phenol oxidase	*Acremonium murorum*	*A. awamori*	0.6 g/l	Gouka *et al.*, 2001
Phytase	*A. awamori*	*A. awamori*	328.9 U	Piddington *et al.*, 1993
Polygalacturonase I	*A. niger*	*A. nidulans*	510 U/ml	Bussink *et al.*, 1992a
Polygalacturonase II	*A. niger*	*A. niger*	88 U/ml	Bussink *et al.*, 1990; Bussink *et al.*, 1992b
Polygalacturonase C	*A. niger*	*A. nidulans*	33 U/ml	Bussink *et al.*, 1992a
Taka amylase	*A. oryzae*	*A. oryzae*	12 g/l	Christensen *et al.*, 1988
Triglyceride lipase	*R. miehei*	*A. oryzae*	8810 U/mg protein	Huge-Jensen *et al.*, 1989
Vanillyl-alcohol oxidase	*Penicillium simplicissimum*	*A. niger*	NR	Benen *et al.*, 1998
Xylanase	*A. awamori*	*A. awamori*	58 kU/ml	Hessing *et al.*, 1994
	A. awamor	*A. niger*	140 kU/ml	Verdoes *et al.*, 1995
	T. reesei	*A. niger*	480 U/ml	Rose and Van Zyl, 2002

NR = Not Reported

In addition to the expression of fungal enzymes, many studies on heterologous gene expression have concerned the genes of higher eukaryotes using the expression hosts *A. nidulans, A. niger* and *A. awamori* for commercial reasons (Van den Hondel *et al.*, 1991). High level heterologous protein production facilitates the study of the structure and/or biological function of a protein. Where the availability of a certain protein may be erratic, expression in a microbial host can fulfil demand and pharmaceutically important proteins that were previously only obtained from biological samples such as serum can be obtained more easily with less risk of contaminating biological agents such as viruses. Some of the proteins from non-fungal higher eukaryotes that have been expressed in *Aspergillus* are summarised in the following section:

2.4.2.1. Chymosin

Chymosin, sometimes referred to as rennin, is a gastric aspartyl protease extracted from the fourth stomach of unweaned calves (Ward, 1989). This 35.6 kDa enzyme is used commercially for milk clotting during the cheese-making process because it specifically cleaves κ-casein and is readily heat inactivated, preventing the degradation of the whey fraction. The first filamentous fungal host used to express bovine chymosin was *A. nidulans* (Cullen *et al.*, 1987). Chymosin is naturally secreted as a zymogen precursor (prochymosin) and the amino terminal propeptide is autocatalytically cleaved at low pH. The gene encoding prochymosin was placed under transcriptional control of the *A. niger* glucoamylase gene and various fusion constructs with glucoamylase were also made to enhance secretion. Secretion of chymosin enzymatically and immunologically indistinguishable from bovine chymosin was achieved with all the constructs transformed to *A. nidulans*. With the success of chymosin expression in *A. nidulans,* the commercially important species *A. oryzae* and *A. awamori* were subsequently employed for high-level expression (Tsuchiya *et al.*, 1993; Ward *et al.*, 1990). Significant increase in yield was obtained using *A. awamori* as host with the prochymosin cDNA fused to the entire *A. awamori glaA* gene with levels reaching 140 mg active chymosin per liter culture (Ward *et al.*, 1990). One problem that was identified as a factor contributing to low yields was the production by *A. awamori* of the extracellular aspartyl protease,

aspergillopepsin, which degraded the secreted chymosin (Dunn-Coleman *et al.*, 1991). The yield was therefore further improved to 250 mg/l by deletion of the native aspartyl protease gene (*pepA*) and commercial levels of chymosin of 1 g/l were subsequently achieved in *A. awamori* by random mutagenesis of this protease deficient strain (Dunn-Coleman *et al.*, 1991). Chymosin was also successfully expressed and secreted by *A. oryzae* under control of the native glucoamylase gene promoter, however levels were lower than those achieved with *A. awamori,* reaching 150 mg/kg wheat bran in commercially used solid-state culture (Tsuchiya *et al.*, 1993; Tsuchiya *et al.*, 1994). The strains of *A. awamori* producing commercially viable amounts of chymosin were evaluated with extensive biochemical and toxicological tests (detailed in Ward, 1989) and cheese-making trails were conducted with the conclusion that this chymosin constitutes a safe and effective alternative to chymosin obtained from calves (Ward, 1991). Recombinant chymosin is now the first heterologous fungal product that has the approval of the United States Food and Drug Administration for commercial use (Kinghorn and Unkles, 1994). Various reviews outline the further details of this "success story" (Archer, 1994; Kinghorn and Unkles, 1994; Ward, 1989; Ward, 1991).

2.4.2.2. Lysozyme

Lysozyme is a 14.5 kDa enzyme that catalyses the hydrolysis of the β-1,4-glycosidic bonds of the polysaccharide of bacterial cell walls and has applications as a safe anti-bacterial, anti-inflammatory and even anti-tumoral drug (Baron and Tiraby, 1992; Tsuchiya *et al.*, 1992). Human lysozyme and hen egg-white lysozyme (HEWL) have been successfully expressed in *Aspergillus* (Archer *et al.*, 1990a; Tsuchiya *et al.*, 1992). Human lysozyme was expressed in *A. oryzae* under control of the Taka-amylase A gene (*amyB*) gene promoter, with transformants secreting active human lysozyme at levels of 1.2 mg/l (Tsuchiya *et al.*, 1992). HEWL was initially expressed in *A. niger* under control of the *A. awamori glaA* promoter and the *A. nidulans gpdA* promoter (Archer *et al.*, 1990a). Secretion was directed by the native HEWL secretion signal peptide, which was correctly processed by *A. niger.* Lysozyme levels up to 12 mg/l were secreted when expression was controlled by the starch-

inducible *glaA* promoter and 1 mg/l when controlled by the constitutive *gpdA* promoter. The specific activity of the recombinant protein was identical to that of authentic hen egg-white lysozyme and the protein was correctly folded. Strategies to improve HEWL production included increasing the copy number of the transforming vector, buffering the growth medium, optimisation of culturing conditions and fusion of HEWL to the *A. niger glaA* gene (Archer *et al.*, 1990b; Jeenes *et al.*, 1994; MacKenzie *et al.*, 1994). HEWL secretion was improved by optimising the levels of ammonium chloride and sodium phosphate buffer in the medium and maximal levels were found at culturing temperatures of 20 to 25°C in rich medium containing soya milk (MacKenzie *et al.*, 1994). Cultures incubated at 30 to 37°C showed faster growth with a more rapid acidification of the medium and lower levels of HEWL. These results suggested that protease activity, perhaps from more rapid autolysis reduced the levels of HEWL. The lower growth temperature may also enhance protein folding and passage through the secretory pathway. Implementing these optimisation strategies led to levels of 30 to 60 mg/l secreted HEWL. Further optimisation of the growth conditions in fermentation studies, influencing mycelial morphology led to yields of up to 1 g/l (Archer *et al.*, 1995; Wongwicharn *et al.*, 1999). However, some strain instability has been observed in HEWL-producing *A. niger* strains grown in glucose-limited chemostat culture where the generation of morphological mutants was pH dependent, demonstrating the importance of preserving adequate stock cultures of new transformants and avoiding sub-culturing as much as possible (Mainwaring *et al.*, 1999).

2.4.2.3. Human interleukin-6

The cytokine human interleukin-6 (hIL6) is a 23 kDa secreted glycoprotein with antiviral and B-cell proliferative properties (Kinghorn and Unkles, 1994). The biologically active protein was first successfully expressed in *A. nidulans* under control of the *A. niger glaA* promoter (Carrez *et al.*, 1990). Secretion was facilitated by both the native hIL6 secretion signal and the glucoamylase secretion signal, with the fungal secretion signal proving to be more efficient. *A. nidulans* transformants secreted up to 25 µg hIL6/l into the medium and the recombinant protein was similar in size to the native hIL6 and was not

glycosylated. Subsequently production in *A. nidulans* was improved by fusing the hIL6 gene to the entire *A. niger glaA* gene through a spacer peptide containing a KEX2-like protein processing signal (Contreras *et al.*, 1991). This approach resulted in 250 times more mature hIL6 with expression levels reaching 5 mg/l. *A. niger* and *A. awamori* have also been successfully employed to produce this protein (Broekhuijsen *et al.*, 1993; Gouka *et al.*, 1997a). Initially, no recombinant hIL6 could be detected from *A. niger* transformants and this was attributed to extracellular proteases (Carrez *et al.*, 1990). Subsequently, a protease-deficient *A. niger* transformed with several copies of a glucoamylase-hIL6 fusion construct under control of the constitutive *A. nidulans gpdA* promoter produced 15 mg/L recombinant protein (Broekhuijsen *et al.*, 1993). In the case of expression in *A. awamori,* hIL6 was fused to the catalytic domain of glucoamylase and placed under control of the inducible *A. awamori* 1,4-β-endoxylanase A (*exlA*) promoter (Gouka *et al.*, 1997a). Transformants carrying a single copy of the expression vector secreted 5-10 mg hIL6/l, which compared favourably with levels obtained from multicopy *A. nidulans* and *A. niger* transformants.

2.4.2.4. Human interferon α-2

Interferon α-2 is used to treat viral disease and acts by lowering viral load by reducing viral replication (Kassianides *et al.*, 1988). The main source of the protein is human serum, making its purification extremely expensive and difficult. Biologically active human interferon α-2 was secreted from *A. nidulans* by placing the gene under the transcriptional control of the tightly regulated promoters of the *A. nidulans alcA* or the *A. niger glaA* gene (Gwynne *et al.*, 1987). Optimisation of the translation initiation sequence and direct fusion of the synthetic signal peptide to the mature interferon protein sequence yielded levels of 1 mg/l secreted interferon in shake flasks on minimal medium.

2.4.2.5. Human tissue plasminogen activator

Tissue plasminogen activator (t-PA) is a complex, large, secreted serine protease consisting of two chains (67 kDa and 30 kDa), which is important in the dissolving of blood clots (Upshall *et al.*, 1987). It is used therapeutically for

the treatment of myocardial infarction, thrombosis, pulmonary embolism and stroke, and with low serum levels of t-PA it is necessary to obtain this protein through a recombinant source (Wiebe *et al.*, 2001). The protein contains multiple cysteine residues which are involved in the formation of disulphide bonds and there are three *N*-linked glycosylation sites which have been shown to be utilised in mammalian systems. Production of this protein in *A. nidulans* under control of the *A. nidulans tpiA* promoter resulted in a correctly processed, secreted, active protein that was not hyperglycosylated (Upshall *et al.*, 1987). The highest levels of t-PA secreted into the culture medium were 100 µg/l. However, utilising the *A. nidulans* ethanol induced *alcC* gene promoter or the *A. niger* alcohol dehydrogenase (*adhA*) gene promoter resulted in transformants producing levels of 1 mg/l t-PA. For production in *A. niger*, a protease-deficient strain was utilised and t-PA was fused to the catalytic domain of the native glucoamylase protein and placed under control of the constitutive *A. nidulans gpdA* promoter or the inducible *A. niger glaA* promoter (Wiebe *et al.*, 2001). t-PA was produced in quantities of 12 to 25 mg/l, and although it was present in the two-chain form, most of the protein was not active or had low specific activity compared with human melanoma t-PA. The reasons for this were unclear, but possible factors influencing activity were the $(His)_6$ tag employed for purification purposes, incorrect glycosylation or aberrant processing. There was also evidence that t-PA was degraded by extracellular proteases during fed-batch cultivation, despite the fact that a protease-deficient strain was utilised.

2.4.2.6. Lactoferrin

Human lactoferrin (hLF) is a 78 kDa iron-binding glycoprotein present in milk, other exocrine secretions and the secondary granules of polymorphonuclear granulocytes (Ward *et al.*, 1992b). The functions proposed for lactoferrin include protection against microbial infection, cellular growth promotion and regulation of intestinal iron homeostasis. Initial heterologous expression studies were performed in *A. nidulans* and, under control of the *alcA* promoter, 5 mg/l hLF was produced of which approximately 30% was secreted into the culture medium. The recombinant protein was indistinguishable from native hLF with

respect to size and immunoreactivity and was functional by the criterion of iron-binding capacity. Subsequent expression in *A. oryzae* under control of the native α-amylase promoter yielded levels of 25 mg/l active, correctly processed protein with appropriate *N*-glycosylation (Ward *et al.*, 1992a). Commercial levels of hLF have since been obtained from *A. awamori* in excess of 2 g/l by employing the glucoamylase fusion strategy combined with a classical strain improvement programme (Ward *et al.*, 1995). This recombinant hLF was intact, biologically active in terms of its ability to bind iron and human enterocyte receptors and glycosylated to the same extent as the native hLF (Sun *et al.*, 1999; Swarts *et al.*, 2000; Ward *et al.*, 1995). The protein was found to function as a potent broad spectrum antimicrobial protein and could have potential nutritional and therapeutic applications (Ward *et al.*, 1995). Similarly, murine (mouse) lactoferrin has also been successfully expressed in *A. awamori* and will be an invaluable tool to address both the species specificity of lactoferrin action and to further clarify the *in vivo* biological role of this protein (Ward *et al.*, 1997).

2.4.2.7. Porcine pancreatic phospholipase A_2

Porcine pancreatic phospholipase A_2 has been extensively studied as a model enzyme for protein engineering and efficient heterologous protein production of this enzyme can provide sufficient quantities for structure-function studies (Roberts *et al.*, 1992). To this end, the cDNA gene encoding porcine pancreatic prophospholipase A_2 was cloned into an *A. niger* expression vector downstream of the *glaA* promoter region. Detectable PLA_2 was however only obtained when an in-frame fusion to the entire *glaA* gene was made and a protease-deficient (*pepA*$^-$) *A. niger* strain was used. This strain secreted up to 10 mg/l correctly processed, enzymatically active PLA_2.

2.4.2.8. Cattle tick cell-surface glycoprotein

The cell surface glycoprotein Bm86 from cells of the digestive tract of the cattle tick *B. microplus* has been shown to elicit a protective immunological response against the ectoparasite in vaccinated cattle, but is difficult to isolate in sufficient quantities (Turnbull *et al.*, 1990). Thus, this 83 kDa protein was expressed and secreted in *A. nidulans* and *A. niger* by using the *A. nidulans amdS* promoter

and the native Bm86 secretion signal. Bm86 was secreted from both *Aspergillus* strains as a soluble protein that was glycosylated to a similar extent as the native glycoprotein. No intracellular Bm86 was detected and the highest levels were obtained from *A. nidulans* which secreted up to 1.8 mg/l recombinant Bm86.

2.4.2.9. Antibody fragments

The high affinity and specificity of monoclonal antibodies for a particular antigen, as well as the possibility for "infinite" production, have made them a valuable tool for the pharmaceutical industry and medical or other research (Frenken *et al.*, 1998). The high cost and limited availability and stability of monoclonal antibodies has however limited their application. These limitations could be overcome by the large-scale heterologous production of stable antibodies or antibody fragments such as single chain Fv (scFv). Various expression cassettes encoding the scFv-LYS D1.3 fragment were constructed for heterologous expression in *A. awamori*, utilising the *A. niger glaA* and *A. awamori exlA* promoters and signal sequences. Production levels of 10 mg/l were obtained for both promoter systems. This level could be improved to 50 mg/l when the protein was fused to the first 514 amino acids of glucoamylase. The recombinant protein was correctly processed and contained some *O*-linked glycosylation. Subsequent expression of other scFv fragments has shown varying success, indicating that certain scFv fragments are more easily degraded than others in the *Aspergillus* mycelium and/or culture medium. However, fed-batch fermentations of a recombinant *A. awamori* producing scFv-4715 consistently yielded production levels of 200 mg/l, indicating that this approach is likely to deliver a process of large-scale, cost-effective production of scFv fragments.

2.4.2.10. Thaumatin

Thaumatin is an intensely sweet protein isolated from the fruit of the tropical plant *Thaumatococcus danielli*, native to Western Africa (Zermanek and Wasserman, 1995). In contrast to other sweeteners, thaumatin is very heat and acid stable, occurs naturally and does not disturb the balance of the amino acid

pool in the body after digestion and these unique properties could make it highly attractive to the food industry as an intense sweetener and flavour enhancer. It is also safe for diabetics and toxicological studies have established its safety as a food additive. This protein has therefore been expressed in *A. oryzae* and *A. awamori* (Faus *et al.*, 1998; Hahm and Batt, 1990). For expression in *A. oryzae,* the thaumatin II cDNA was placed under transcriptional control of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and secretion was directed by the native plant signal sequence (Hahm and Batt, 1990). In this case, the *S. cerevisiae gpd* promoter was properly recognised and regulated by *A. oryzae* and an immunoreactive 22 kDa protein indistinguishable from mature thaumatin was present in the culture medium at a concentration of 50 ng/ml. In *A. awamori* thaumatin expression was directed by the *A. niger glaA* promoter and the thaumatin cDNA was fused in-frame with the complete *A. awamori* glucoamylase gene (Faus *et al.*, 1998). Laboratory fermentations of recombinant *A. awamori* strains carrying this expression cassette yielded secreted thaumatin concentrations of 5-7 mg/l. This production level was further improved by using an *A. awamori* strain deficient in aspergillopepsin ($PEPA^-$); employing two strong fungal promoters, the *A. awamori gdhA* promoter and the *A. nidulans gpdA* promoter; constructing double transformants with different expression cassettes and utilising a synthetic gene encoding thaumatin with fungal codon usage (Moralejo *et al.*, 1999). This optimised transformant gave expression levels of nearly 100 mg/l in fermentors and has potential application at the commercial scale.

2.4.2.11. Human β2-microglobulin

Class I human leukocyte antigens (HLA) bind peptides that result from cleavage of intracellular antigens (O'Herrin *et al.*, 1996). The HLA molecule is a heterodimer consisting of the noncovalent association of a polymorphic heavy chain (α chain) and a soluble 12 kDa β_2-microglobulin (β_2m). The trimeric complex (α chain, β_2m and antigenic peptide) provides the context for recognition of peptides by cytotoxic T lymphocytes. A readily available supply of HLA class I components would be useful for assembly studies and peptide screening. Thus the β_2m has been expressed in *A. nidulans* using the

A. awamori glaA promoter. In 60-liter fermentations, 117 µg/l of recombinant β_2m was secreted into the culture medium which was biochemically similar to that obtained from human urine, and effectively interacted as a subunit of class I MHC molecules. This successful expression of biologically active β_2m suggests that fungal systems might be useful for the production of other active components of the HLA class I MHC complex.

2.4.2.12. Human mucus proteinase inhibitor

Human mucus proteinase inhibitor (MPI) is a serine antiproteinase of leukocyte elastase, chymotrypsin-like proteinases and trypsin (Mikosch *et al.*, 1996). The inhibitor is produced locally by mucosal cells, including those of the respiratory tract, where its principal physiological function is probably protection of the airway epithelium from attack by elastase. MPI therefore has applications as a therapeutic agent in destructive lung diseases such as pulmonary emphysema or cystic fibrosis and has also been shown to have antiretroviral activity, which may contribute to the infrequent oral transmission of HIV-1. In its biologically active conformation the 14 kDa protein is highly disulphide bonded. MPI was expressed in *A. niger* by utilising the promoter, signal sequence and first 514 amino acids of the *A. niger glaA* gene, creating an in-frame fusion to the human *mpi* gene via a linker containing a KEX2-like processing site. Recombinant *A. niger* secreted up to 3 mg/l MPI into the medium, which was correctly processed and folded properly. Recombinant MPI retained full inhibitory activity against chymotrypsin and leukocyte elastase.

Some other non-fungal proteins expressed in *A. niger* include bovine enterokinase (5 mg/l) (Svetina *et al.*, 2000) and bovine pancreatic trypsin inhibitor (10 to 20 mg/l) (MacKenzie *et al.*, 1998), where some molecules were however aberrantly processed. Expression of the *E. coli* enterotoxin subunit B gene in *A. nidulans* resulted in mature peptide that was, however, retained in the endoplasmic reticulum (Turnbull *et al.*, 1989).

2.4.3. Future directions for the *Aspergillus* expression system

The results described in the previous section indicate that although considerable progress has been made in the development of *Aspergillus* for the production of complex mammalian proteins, not all problems have been resolved. However, the utility of this host for effective expression of mammalian proteins has been proven with the numerous successes that have been achieved. However, despite the apparent success of expressing higher eukaryotic proteins in *Aspergillus,* the potential of this host to produce another pahrmaceutically important group of proteins, namely viral proteins, is largely unknown. Only one report has been published in a Chinese journal of the production of hepatitis B surface antigen (HBsAg) in *Aspergillus foetidus* (Liu *et al.*, 1990). No other reports have been found to date detailing the production of viral proteins in *Aspergillus*. The inherent advantages and potential of the system, however, warrant an investigation into the capability of this fungal host as a viral protein production system.

2.5. The Hepatitis B Virus

The first report of what must have been hepatitis B was that of Lürman who reported on an epidemic of hepatitis that occurred in shipyard workers in Bremen following vaccination against small pox with glycerinated lymph of human origin in 1883 (Purcell, 1994; Zuckerman, 1975). The first step in the identification of the hepatitis B virus was made in 1963 by Baruch S. Blumberg who observed an antibody that reacted with an antigen present in the blood of an Australian aborigine infected with hepatitis, and he thus called this antigen the Australia antigen (Krugman, 1985; Tiollais and Buendia, 1991). This antigen was then later (in 1968) identified as the hepatitis B virus surface antigen (HBsAg) (Tiollais and Buendia, 1991). In 1970, Dane and colleagues first identified the so-called Dane particle under the electron microscope, thus visualising the complete viral particle for the first time (Krugman, 1985).

2.5.1. Prevalence of hepatitis B

Since serological tests for the detection of hepatitis B virus (HBV) infection were first applied in the mid-1960s, the incidence of hepatitis B progressively

increased until the mid-1980s (Purcell, 1994). To date, hepatitis B is a widespread and serious liver disease and can be considered a global health problem with an estimated 2000 million HBV carriers in the world today (Figure 3) (Vryheid *et al.*, 2001). Even though there is a highly effective vaccine available, between 200 000 and 300 000 people acquire HBV infection each year in the United States alone (Maddrey, 2000). Approximately 5% to 10% of infected patients become long-term carriers of the virus. According to some estimates, of the more than 350 million people world-wide that are chronically infected with HBV, 25% to 40% will die from liver cirrhosis or primary hepatocellular carcinoma associated with the virus (Nowak *et al.*, 1996; Vryheid *et al.*, 2001). At least one million deaths per year world-wide may be attributed to HBV-induced and associated liver diseases (Vryheid *et al.*, 2001). The problem is most prominent in developing countries with 95% of chronic carriers residing in the developing world (Ayoola, 1988). In Southeast Asia and tropical Africa, chronic carriers of the virus represent 10% or more of the population, whereas they make up less than one percent in North America and western Europe. It is estimated that there are about 50 million HBV carriers in Africa alone with 60% to 99% of healthy African adults showing evidence of exposure to HBV.

With regard to southern Africa specifically, in a study performed in Swaziland in 1983 and in Namibia in 1985, 82.6% and 98.9%, amongst those tested showed exposure to HBV, respectively (Ayoola, 1988). In South Africa, more than 70% of the population has been exposed to HBV, with an estimated 10% being carriers (i.e. HBsAg positive) of the virus (Tsebe *et al.*, 2001). The carrier rate differs regionally, with higher rates of infection in rural areas (5-10%) than urban areas (<5%). In Western industrialised countries, high-risk populations consist of persons in direct contact with chronic carriers or with their blood samples (nurses, doctors, dentists), recipients of blood or blood products (haemophiliacs, patients receiving blood transfusions or dialysis treatments), prison inmates, intravenous drug abusers, homosexuals and persons with multiple sex partners. However, in Africa all susceptible groups (i.e. those who are negative for HBV markers) are at high risk of infection, with the highest risk occurring among children (Ayoola, 1988; Garrison and Baker, 1991; Tiollais and

Buendia, 1991). A pre-immunisation survey conducted in South Africa in 1999 indicated HBsAg carriage of 8.9% in 1-year-olds, increasing to 12.8% in 3-year-olds and rising to 15.7% by the age of 6 years (Vardas *et al.*, 1999).

HBV is also a highly infectious virus. In the far East, viral transmission occurs most frequently from an infected mother to her infant (Hino *et al.*, 2001). However, this mode of transmission is of little significance in Africa. Active infection is unusual during early infancy, because of protective maternal antibodies passively transferred. Clearance of such antibodies occurs within the first year and thereafter most African children are infected. Amongst African adults, blood transmission of HBV is of minor significance, rather scarification, tattooing, blood-sucking vectors, sexual intercourse and uncontrolled injections

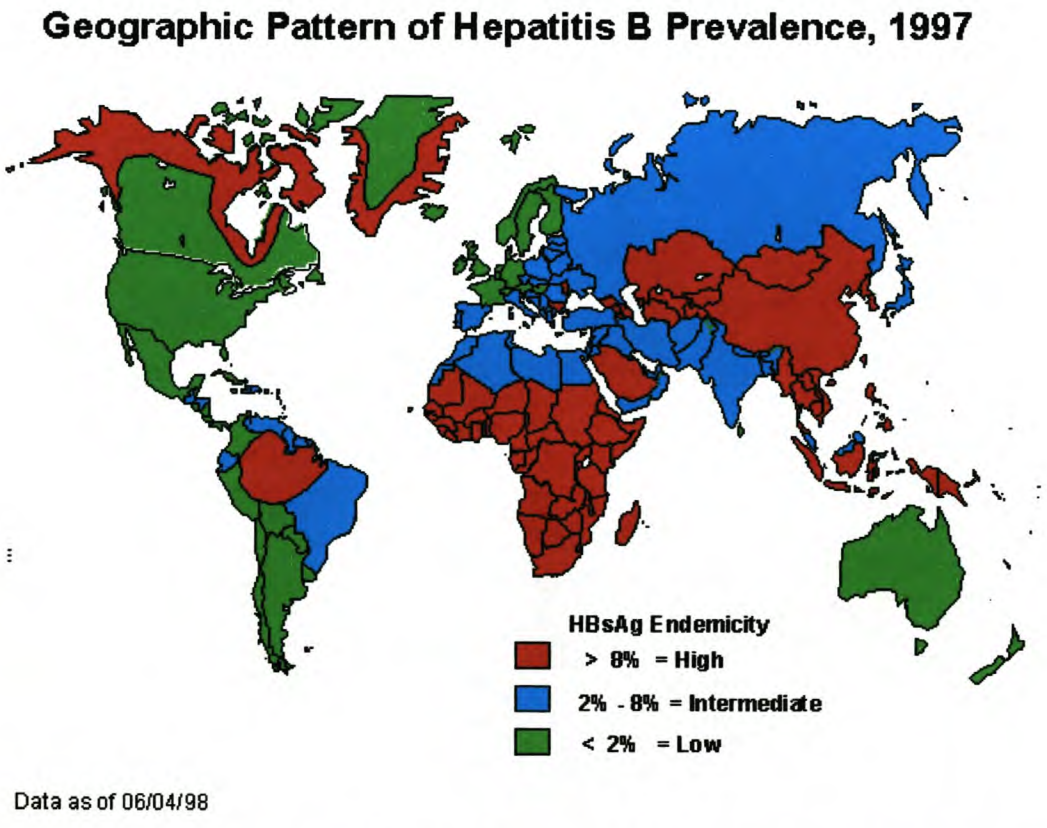

Figure 3. Geographic distribution of chronic hepatitis B infection world-wide, shown as hepatitis B surface antigen prevalence. World Health Organisation (Maddrey, 2000).

have been incriminated in transmitting HBV (Ayoola, 1988). All these, however, contribute a small proportion of HBV transmission, as the majority of infections

occur in early childhood, and this is also the case in South Africa (Vardas *et al.*, 1999). The virus can also spread rapidly within a family or small community, because it can be found in blood, saliva and semen (Tiollais and Buendia, 1991). In fact, the epidemiology of HBV is very similar to that of Acquired Immunodeficiency Syndrome (AIDS), but HBV is far more contagious than AIDS. HBV is therefore primarily a disease of infants in developing nations, whereas in Western countries it is mostly confined to adults. This fact has very important consequences for future vaccination strategies in that in the Third World, mass vaccination is necessary, but in Western countries only high-risk population groups need to be protected. Therefore, in accordance with recommendations made by the World Health Organisation (WHO), 110 countries had introduced the HBV vaccine into their Expanded Programme on Immunisation (EPI) by April 2000 (Tsebe *et al.*, 2001) (Refer to Figure 4). Of these, only 8 are African countries, despite the high prevalence of HBV infection on this continent. South Africa integrated the HBV vaccine into the EPI in April 1995 and this has already shown a positive impact in the elimination of HBsAg carrier rate in children <5 years (Aspinall and Kocks, 1998; Hino *et al.*, 2001; Tsebe *et al.*, 2001).

Figure 4. Countries using hepatitis B vaccine in their national immunisation system, 2000 (WHO).

2.5.2. Subtypes of the Hepatitis B Virus

The HBsAg possesses a number of antigenic subdeterminants (Holland, 1985; Peterson *et al.*, 1982). The *a* determinant is common to all HBV isolates, regardless of geographical origin or clinical source. It has a highly conserved amino acid sequence and is exposed on the surface of the HBV particle (Holland, 1985; Howard and Allison, 1995). The *a* determinant is thus regarded as a group-specific determinant (Eble *et al.*, 1987). Variations in the primary structure of the *a* determinant have been described and were shown to markedly change the antigenic structure of HBsAg (Tonekaboni-Seddigh *et al.*, 2000). Apart from this group-specific determinant, the HBsAg also contains either the *d* or *y* determinant and either the *w* or *r* determinant (Eble *et al.*, 1987). These are the type-specific determinants and it was found that variations at amino acid sites 122 and 160 alone defined the *d/y* or *w/r* variations, respectively (Eble *et al.*, 1987; Magnius and Norder, 1995). The 5' end of the preS1 region of *ad* subtypes also contains an additional sequence of 33 bp or 12 bp compared with *ay* subtypes (Tiollais *et al.*, 1985). There are therefore four major antigenic types of HBsAg, namely *adw, ayw, adr* or *ayr*. Subdivision of the *w* determinant into four subdeterminants and the identification of the *q* determinant has led to serological classification of HBsAg into 9 categories (or subtypes), namely *ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+* and *adrq-* (Holland, 1985; Magnius and Norder, 1995). It is generally accepted that this heterogeneity reflects genetic differences between hepatitis B viruses of different subtypes (Eble *et al.*, 1987). Thus, sequence data of viral genomes can be used to trace routes of infection, to reconstruct the phylogenetic history of viruses and to delimit genetic subtypes (Hu and Peterson, 1988). Genetic classification based on the comparison of complete genomic sequences has defined 6 genomic groups (genotypes) of HBV, designated A, B, C, D, E and F. A review of the subtypes contained in these groups as well as their distribution is given by Magnius and Norder (1995) and summarised in Table 5.

Table 5. Geographical distribution of HBV genotypes and subtypes (Magnius and Norder, 1995).

Hepatitis B Genomic Group	Subtype	Areas of high prevalence
A	adw2	North-western Europe, sub-Saharan Africa
	ayw1	Central Africa
B	adw2	Indonesia, China
	ayw1	Vietnam
C	adw2	East Asia
	adrq+	Korea, China, Japan
	adrq-	Polynesia
	ayr	Vietnam
D	ayw2	Mediterranean area
	ayw3	India
E	ayw4	West Africa
F	adw4q-	American natives, Polynesia

2.5.3. The Disease

Hepatitis B is a liver disease that can manifest itself in various ways. After a two- to six-month incubation period, HBV infection can lead to acute hepatitis and liver damage, causing abdominal pain, jaundice, elevated levels of certain enzymes in the blood, inflammation and other symptoms (Ganem and Varmus, 1987; Tiollais and Buendia, 1991). Many individuals experience mild or no liver injury and the disease remains permanently asymptomatic. Viral replication does not appear to be cytosolic, rather variation in the severity of liver damage can be attributed to differences in host immune response (Ferrari *et al.*, 1996; Ganem and Varmus, 1987). Usually, a patient with acute hepatitis will recover completely as primary infection is mostly self-limited, with a prompt, vigorous and broad-based cellular immune response resulting in clearance of viral antigens and infectivity from liver and blood and the development of lasting immunity to re-infection (Ganem and Varmus, 1987; Grob, 1998; Moradpour and Wands, 1995). However, 5% to 10% of individuals do not resolve primary infection, but develop a persistent, usually life-long, hepatic infection, possibly due to a weak immune response and thus become chronic HBV carriers (Ganem and Varmus, 1987; Grob, 1998; Tiollais and Buendia, 1991). Generally, it is from these chronic carriers that the virus is spread and also most of the mortality resulting from HBV infections occurs from chronic rather than

acute disease (Ganem and Varmus, 1987). Some chronic carriers are healthy, having limited liver damage and no symptoms (Tiollais and Buendia, 1991). Others acquire chronic persistent hepatitis, sometimes causing fatigue. In the worst cases, chronic active hepatitis develops which can lead to cirrhosis of the liver and finally hepatocellular carcinoma (HCC), a primary liver cancer, which usually does not develop until after a 30- to 50-year latency period. In fact, the hepatitis B virus is second in importance only to tobacco as a known human carcinogen. It is this association, amongst other aspects, that has fuelled the study of the structure and molecular biology of the virus.

2.5.4. Treatment of Hepatitis B

Therapy of HBV carriers can aim to either inhibit viral replication or enhance immunological responses against the virus or both (De Clercq, 1999; Nowak *et al.*, 1996). Various drugs have been developed for the treatment of viral hepatitis, such as the nucleoside analogue (-)-2'-deoxy-3'-thiacytidine (lamivudine). Lamivudine has potent inhibitory effects on HBV replication *in vivo* and was effective in lowering viral levels, but as soon as the drug is withdrawn, the virus returns. Other experimental drugs include suramin, acyclovir and adenine arabinoside (which causes a reduction in hepatitis B DNA polymerase levels) and many others, however some of these drugs were associated with severe and unacceptable toxicity or caused side-effects (De Clercq, 1999; Tabor, 1988). In fact, so far, none of the marketed antiviral drugs have been found to be effective for the treatment of viral hepatitis. Researchers have therefore studied the human immune system for clues as to the treatment of viral hepatitis. Recovery from hepatitis B virus infection is dependent on the integrated activities of the patient's interferon and immune systems (Thomas, 1988). Viral antigens are seen on the surface of the hepatocyte and the antigens, in association with the class I major histocompatibility complex (MHC) proteins, make the cell a target for cytotoxic T-cell lysis. In the early stage of acute HBV infection, alpha-interferon (alpha-IFN) is produced, resulting in the increase of MHC expression on hepatocytes and the activation of a series of enzymes which lead to inhibition of viral protein synthesis (McClary *et al.*, 2000). Recombinant α-IFN was thus produced recombinantly and used in the

treatment of chronic HBV infection and was shown to induce complete remissions in certain HBV carriers (Dusheiko *et al.*, 1988; Kassianides *et al.*, 1988; Perez *et al.*, 1988). While alpha-IFN can be toxic when given in high doses and does show substantial side-effects, it has been shown to be a promising therapy (Kassianides *et al.*, 1988; Thomas, 1988). Other therapies include the use of beta-IFNs, gamma-IFNs, interleukin II and combinations thereof which mainly play a role in stimulating and amplifying the immune response (De Clercq, 1999; Gómez *et al.*, 1988; McClary *et al.*, 2000; Ohta *et al.*, 1988; Thomas, 1988). For example, it has been demonstrated that the use of a combination of lamivudine and α-IFN is more effective than either monotherapy (Maddrey, 2000). However, HBV will often recur after cessation of therapy and thus, preventative measures such as vaccination strategies are still the only real solution to the problem of HBV.

2.5.5. Structure of the virus

The hepatitis B Virus (HBV) is a small (440Å) DNA virus classified in the family *Hepadnaviridae* (hepatropic DNA viruses), genus *Orthohepadnavirus* (Huovila *et al.*, 1992; Purcell, 1994). HBV has a double-walled structure consisting of two concentric protein coats (Figure 5) (Tiollais and Buendia, 1991). The envelope, or outer coat, contains three proteins, designated major (S), middle (M) and large (L) protein, respectively. One virion contains 300 to 400 major protein molecules and 40 to 80 middle and large protein molecules (Tiollais *et al.*, 1985). The viral particle also has a host-derived lipid composition consisting of phospholipids (principally phosphatidyl choline), free and esterified cholesterol and small quantities of triglycerides (Ganem and Varmus, 1987). The capsid, or inner coat also known as the core, is made up of a single core protein species that surrounds and interacts with the viral DNA.

The HBV genome is a partially double-stranded, relaxed-circular DNA molecule of only 3.2 kilobases (kb) in length, making it the smallest of any animal DNA virus yet encountered (Seeger and Mason, 2000). The DNA molecule displays two remarkable asymmetries that set it apart from all other viral chromosomes:

1. The two base-paired strands are not identical in length, i.e. one strand is longer than the other. The full-length strand is complementary to the viral mRNAs and, by convention, is designated as the minus strand. The shorter complementary strand, which can vary in length at the 3' position, is thus the plus strand.
2. The minus strand DNA contains proteins covalently linked to the 5' terminus, whereas the plus strand contains an oligonucleotide attached to the 5' terminus.

These asymmetries are a result of the replicative mechanism of the genome (for a review see Ganem and Varmus, 1987).

2.5.6. Genomic organisation

The HBV genome is a miracle of compactness, consisting of only four open reading frames (ORFs) all encoded by minus-strand DNA and coding for four genes, namely the *S, C, P* and *X* genes which show extensive overlapping (refer to Figure 5) (Ganem and Varmus, 1987; Tiollais and Buendia, 1991). Regulatory sequences controlling the production of viral proteins as well as the replication cycle are also situated in these coding sequences.

- Gene *S.* This gene codes for the major envelope protein (sometimes called the small protein [S]) and includes all the specifications for hepatitis B surface antigen (HBsAg) (Tiollais and Buendia, 1991). Upstream of this ORF is an in-phase reading frame with two conserved in-phase ATG codons, dividing this upstream sequence into two regions, namely the *preS1* and *preS2* region. These regions are involved in the synthesis of the other envelope proteins: the middle protein (M) is encoded by *preS2* and the *S* gene and the large protein (L) is encoded by *preS1, preS2* and the *S* gene.

- Gene *C.* This gene encodes the capsid protein and is also preceded by a short *preC* region encoding a hydrophobic peptide involved in viral particle assembly.

- Gene *P.* Parts of all the other genes make up this gene and it is believed to encode the viral polymerase (Mr~90 000), which possesses a reverse

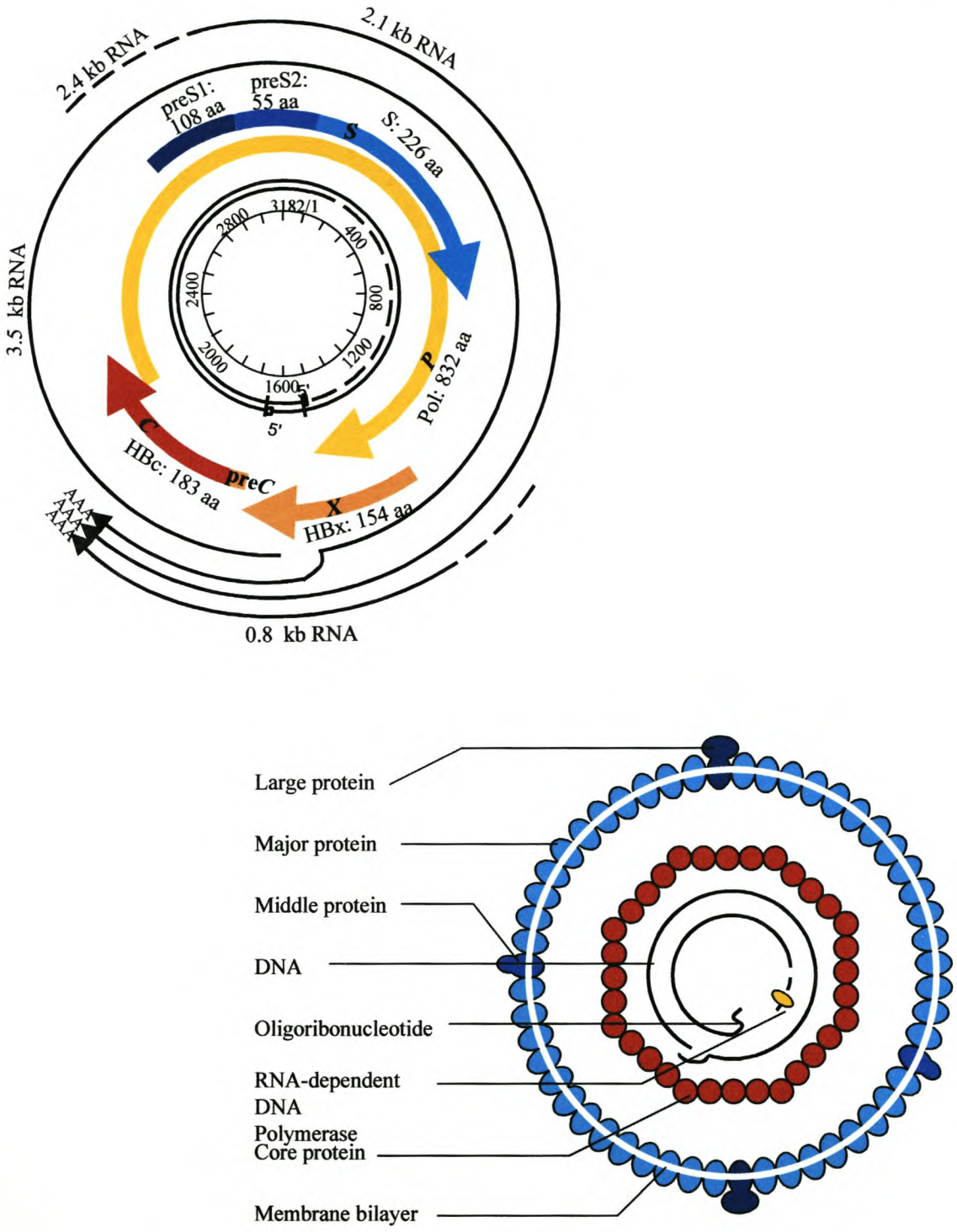

Figure 5. Schematic structure of HBV particle and genomic organisation. The ORFs for preS1preS2S, preS2S, preC/C, P and X are indicated with the encoded polypeptides. The outer circle depicts the viral transcripts with the common polyadenylation site (Caselman, 1996).

transcriptase activity and is essential to viral replication (Ganem and Varmus, 1987; Tiollais *et al.*, 1985; Tiollais and Buendia, 1991). In addition to its catalytic role for viral genome synthesis, the P protein also plays a role in encapsidation (Jeong *et al.*, 2000).

- Gene *X*. This gene covers the cohesive ends of the viral DNA strands and its protein product (HBx) of 145 to 154 amino acids (depending on the subtype) acts as a transcriptional transactivator of the HBV core gene promoter (Reifenberg *et al.*, 1999; Tiollais and Buendia, 1991). HBx is also involved in host cellular signal transduction pathways, playing a role in the deregulation of cell growth, and is therefore associated with development of liver oncogenesis (Rahmani *et al.*, 2000; Schlüter *et al.*, 1994).

It is interesting to note the remarkable parsimony underlying the coding organisation of the viral genome: every nucleotide in the genome is in at least one coding region, 50% of the sequence can be read in more than one frame and even within a frame multiple ATG codons are sometimes used to give rise to multiple related proteins (Ganem and Varmus, 1987).

2.5.7. Synthesis and assembly of viral proteins

Electron microscopy of partially purified preparations of HBV from human serum reveals three types of viral particles (Ganem and Varmus, 1987) (Figure 6):

1. 43 nm double-shelled particles, called Dane particles, which represent the intact virion.
2. 20 to 22 nm spheres, usually present in 10^3- to 10^6-fold excess over virions and termed the hepatitis B surface antigen (HBsAg) (Peterson, 1987).
3. Filaments of 20 to 22 nm in diameter and with variable length.

The latter two contain no viral DNA and are called subviral particles. The protein composition of the filaments is identical to that of the complete virion, while the spherical particles contain the major and middle proteins in about the same ratio as in the virion, but with at least 20 times less of the large protein (Tiollais *et al.*, 1985). To understand the dynamics of the formation of these various particles it is important to explore viral synthesis and assembly.

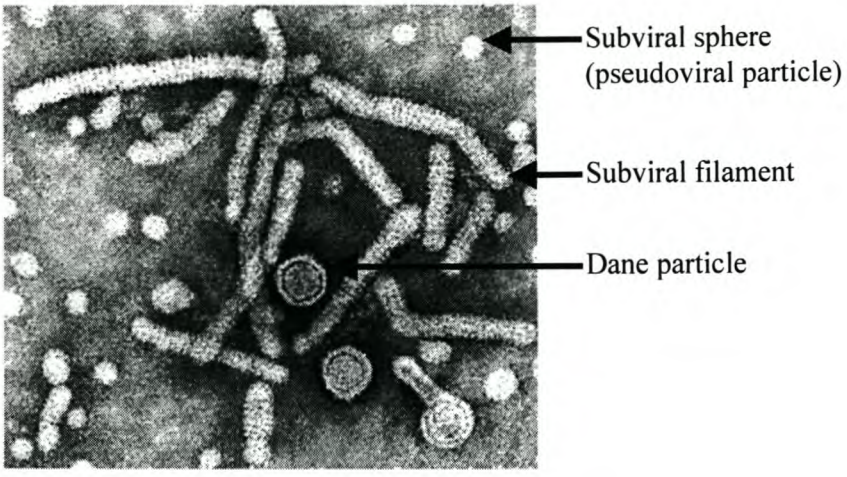

Figure 6. Electron micrograph of serum containing the three distinct morphological forms of the hepatitis B viral particles. Magnification: 252 000x (Zuckerman, 1975).

In the life cycle of HBV, the synthesis of viral proteins is tightly regulated at the transcriptional and translational level (Tiollais and Buendia, 1991). Within the nucleus of the hepatocyte, the single-stranded region of the viral DNA is completed and the genome is converted into a supercoiled DNA molecule, referred to as the covalently closed circular (CCC) DNA (Fallows and Goff, 1996). In contrast to retroviruses, the hepadnaviral genome is not integrated into the host cell chromosome (Mason *et al.*, 1983; Tagawa *et al.*, 1986). Instead, the unintegrated molecules of CCC DNA serve as template for transcription. However, various linear forms of viral DNA resulting from abortive or inefficient replication have been identified in infected liver cells. These DNA molecules then participate in nonhomologous recombination with cellular DNA thereby integrating into the host genome, sometimes resulting in chromosome translocation which has been associated with hepatocarcinogenesis (Meyer *et al.*, 1992; Yang and Summers, 1999).

Each of the HBV genes has one or more promoters regulating its activity and these promoters are in turn regulated by one or both of the viral enhancer elements that are located upstream of the core promoter (Seeger and Mason, 2000). Two major mRNA molecules exist, namely a 2.1-kb mRNA and a 3.5-kb mRNA (Tiollais *et al.*, 1985) (Refer to Figure 5). The 2.1-kb mRNA covers the

preS2 region, gene *S* and gene *X*. The 3.5-kb mRNA is actually longer than the complete genome and contains a terminal repeat of about 100 nucleotides. It encodes the capsid protein and the viral polymerase (Tiollais and Buendia, 1991). A third transcript of 2.4-kb has also been identified and this serves as the mRNA for the expression of the pre-S1 protein (Ganem and Varmus, 1987; Masuda *et al.*, 1990) and a 0.8 kb X-mRNA transcript was demonstrated after transfection of cell lines with HBV DNA (Caselman, 1996). Transcription is generally attributed to host RNA polymerases, probably RNA polymerase II, and no splicing events occur (Ganem and Varmus, 1987; Tiollais *et al.*, 1985). In fact, HBV *S* transcripts contain a region known as the posttranscriptional regulatory element (PRE), which allows for the export of HBV *S* transcripts without these transcripts becoming spliced and thus activates their transport from the nucleus to the cytoplasm without any effects on transcriptional initiation or cytoplasmic RNA stability (Huang and Yen, 1995).

2.5.7.1. The Major Protein

The *S* gene promoter directs constitutive expression of the *S* gene and it is also the protein product of this gene that is present in the highest amounts (Tiollais *et al.*, 1985). The S protein consists of 226 amino acids and is present in both unglycosylated form (24 kDa) and *N*-glycosylated form (27 kDa) (Huovila *et al.*, 1992). As mentioned, the 22 nm subviral particle consists almost solely of this protein with approximately 100 monomers making up the particle (Ganem and Varmus, 1987). The monomers are extensively cross-linked by interchain disulphide bonds and the intact particle is highly immunogenic. It has been postulated that these "dummy particles" serve to absorb neutralising antisurface antibodies during progression of infection as these non-infectious particles are produced in excessive amounts (50-300 mg HBsAg/l of serum). The assembly of this particle occurs in an intermediate compartment between the ER and the Golgi apparatus (Huovila *et al.*, 1992; Roingeard *et al.*, 1990). The 226-amino acid sequence of the S protein is sufficient for the mobilisation of cellular lipids (which make up about 25% of the particle mass) needed for subviral particle assembly, self-assembly of the 100 protein molecules, intracellular transport and secretion of the lipoprotein complex (Prange *et al.*, 1995a). The S protein

is initially synthesised as a transmembrane polypeptide and the membrane translocation process does not involve cleavage of N-terminal amino acids. Both translocation and export of the protein can proceed in the absence of any other viral gene products (Eble *et al.*, 1987).

The primary structure of the S protein can be divided into three hydrophobic and two hydrophilic regions (Berting *et al.*, 1995; Eble *et al.*, 1986). The aminoterminal part (residues 11 to 29) is able to insert into the ER membrane, thus mediating the translocation of the upstream part of the protein into the ER lumen and this region is thus called signal I (Berting *et al.*, 1995; Eble *et al.*, 1987). A second section, called signal II (residues 80 to 98), also inserts into the ER membrane and mediates translocation of the downstream sequences (Figure 7). Both signal sequences contain several conserved structural features, including a hydrophobic domain followed by specific charged residues (Eble *et al.*, 1987). The third hydrophobic area is the C-terminus of the protein and it projects into the ER lumen and is not completely in a transmembrane configuration as it can induce antibody response (Berting *et al.*, 1995; Eble *et al.*, 1987). Two further membrane spanning domains have been proposed, based on model building, but have not been examined experimentally (Ostapchuk *et al.*, 1994). Deletion of the major hydrophobic domain (N-terminal 100 aa) completely abolishes the ability to form particles and removal of amino acids 21 to 80 decreases the ability to form particles, and the particles formed are correspondingly smaller (Araki *et al.*, 1990). The first hydrophilic region (residues 120 to 160) forms the HBV-specific determinant "*a*" and projects into the ER (Berting *et al.*, 1995). The Asn found in this region (Asn-146) is the only one of the multiple potential glycosylation sites used and the initial glycosylation events occur in the ER. The potential glycosylation sites at Asn-3 and Asn-59 are not used, but it is unknown whether this reflects their failure to enter the lumen or whether they are in a conformation unfavourable for glycosylation (Eble *et al.*, 1987). Eight cysteins are also located in this region which may play a role in intramolecular or intermolecular disulphide bridge formation. The second hydrophilic domain (residues 30 to 80) also contains conserved cysteins which are essential for secretion of 22 nm particles.

Within one hour of polypeptide formation, the polypeptide chains embedded in the ER membrane in this way undergo a conformational change and/or aggregation making them no longer susceptible to protease action (Simon *et al.*, 1988). Newly synthesised S protein is translocated across to the lumen of the ER where disulphide-linked dimers are rapidly formed (Huovila *et al.*, 1992). After the transport of dimers to a post-ER/pre-Golgi compartment, the formation of oligomer crosslinks occurs and thus the subviral particle matures by budding into this post-ER/pre-Golgi compartment (Huovila *et al.*, 1992; Prange and Streeck, 1995). The particles are then rapidly exported from the cell via the constitutive pathway of vesicular transport and the Golgi where further modification of the oligosaccharides occurs (Simon *et al.*, 1988). All of the oligosaccharides on the secreted HBsAg lipoprotein particles are of the *N*-linked complex type (Huovila *et al.*, 1992).

2.5.7.2. The Middle and Large Proteins

Like the S protein, the middle (281 amino acids) and large (389 or 400 amino acids depending on the subtype) proteins are also found in two forms, either glycosylated at Asn-146 of the S sequence or unglycosylated at this site (Prange and Streeck, 1995). The M protein is also glycosylated at Asn-4. Thus the M protein is present in two forms, one of 33 kDa, containing only one glycan and one of 36 kDa, containing 2 glycans (Tiollais *et al.*, 1985). The L protein is 42 kDa (glycosylated) or 39 kDa (non-glycosylated) in size. The M protein is efficiently secreted in a similar manner to the S protein with the preS2 region on the luminal side of the ER (Prange *et al.*, 1995a) (Figure 7). The L protein is, however, not easily secreted. It has also been shown that both M and S are found in the virion and subviral lipoprotein particles, while L is found almost exclusively in the virion. The hypothesis has been made that the virion incorporates the dimeric forms of the glycoprotein before they exit from the ER while the excess S and M dimers are transported to the intermediate compartment in which maturation to lipoprotein particles occurs (Huovila *et al.*, 1992). The L protein is essential for virion formation and some L protein can also be found in the filamentous form of the subviral particles (Prange and Streeck, 1995; Prange *et al.*, 1995a). The entire preS region of the L protein is

generally not translocated across the ER membrane, but is situated on the cytosolic side where the N-terminus of the L protein is co-translationally myristoylated (Ostapchuk *et al.*, 1994; Prange and Streeck, 1995). However, the L protein can have a dual topology, playing a role in both envelopment of viral nucleocapsids and also taking part in binding to the host cell (Berting *et al.*, 1995). The preS1 domain of the L protein is initially located on the cytosolic side of the ER, but substantial alteration in the transmembrane conformation occurs posttranslationally, yielding two topologically different populations. Thus, on the complete virions 50% of the L proteins expose the preS1 domain on the surface of the virions (Figure 7). It has thus been hypothesised that the dimers associate in such a way as to form a hydrophilic channel to transport the hydrophilic preS1 domain from the lumen of the budded particles to the surface. Finally, transactivating functions have also been attributed to viral proteins translated from 3'-truncated HBV *preS/S* sequences (Caselman *et al.*, 1997). For example, deletion of that least 87 C-terminal amino acids of the M protein results in the generation of the transactivating form, which then acts as a pleiotropic activator of various cellular promoters and thereby plays a role in hepatocarcinogenesis (Caselman *et al.*, 1997; Schlüter *et al.*, 1994).

2.5.7.3. The Core Protein

Transcription of the *C* gene promoter seems to be inducible, with a transcriptional enhancer element 450 bp upstream of the *C* gene promoter (Ostapchuk *et al.*, 1994). This element exhibits a preferred activity in human hepatocytes. The 21 kDa core protein has an extremely basic segment at the 3' end which shows DNA-binding activity and HBV core antigens accumulate predominantly in the nucleus (Ganem and Varmus, 1987; Ostapchuk *et al.*, 1994). The core protein has also been shown to interact with the viral polymerase and this interaction is required for several steps in genome replication and encapsidation (Koschel *et al.*, 2000; Lott *et al.*, 2000). In some cases, the precore sequences are also expressed and these proteins are proteolytically processed and secreted into the medium, forming the so-called"e" antigen which can be found circulating in the blood during HBV

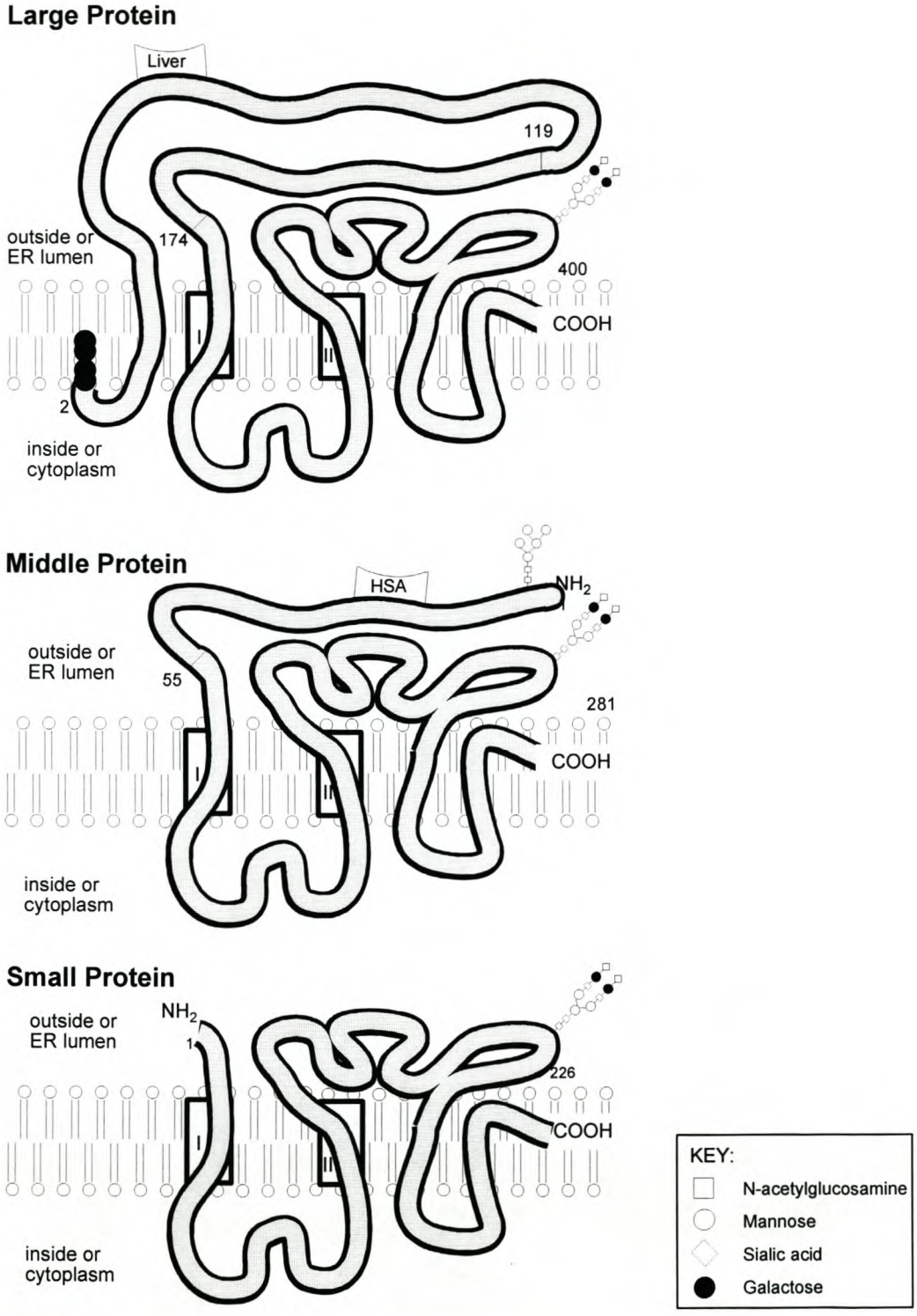

Figure 7. Hypothetical model of the three hepatitis B surface proteins. "**Liver**" indicates the hepatocyte binding site and "**HSA**", the human serum albumin binding site. The amino-terminus of the large protein is bound at Gly2 to myristic acid. **I, II**: Signal sequences. A mannose-rich glycosidic group is attached to Asn4 of the middle protein and a complex glycoside is present in approximately half of the S domain (Gerlich *et al.*, 1990).

infection (Ganem and Varmus, 1987; Ostapchuk *et al.*, 1994). The core protein also has the ability to assemble into nucleocapsids independently of any other viral gene products and these nucleocapsids are resistant to cytotoxic T-lymphocyte-induced apoptosis (Pasquetto *et al.*, 2000). Two separate regions of the core polypeptide are exposed on the surface of core particles and thus provide targets for interaction with the envelope components (Seeger and Mason, 2000). The budding of the complete virion is triggered by interactions of the cytoplasmic nucleocapsid with the preS region of the L transmembrane envelope protein (Ostapchuk *et al.*, 1994).

2.5.8. Functions of the viral envelope proteins

As has been mentioned, the S protein is the predominant constituent of the viral envelope and plays a primary role in the assembly of the viral particle (Prange and Streeck, 1995). The M protein has been found in higher concentrations in the DNA-containing HBV particles (virions) than in the subviral particles and the L protein is found almost exclusively in complete virions with low levels of L protein occurring in the filamentous subviral particles (Pontisso *et al.*, 1989; Prange *et al.*, 1995a). This suggested a role for these proteins in infectious HBV particle assembly or function (Pontisso *et al.*, 1989). The preS2 domain of the M protein possesses a polymerised human serum albumin (pHSA) binding site (localised to amino acid residues 126 to 140) and plays a role in attachment of HBV to hepatocytes using pHSA, which also has an affinity for liver cells, as a linker molecule (Heerman *et al.*, 1988; Petit *et al.*, 1992). This is a species-specific process and HBV binds only to human and other primate serum albumin (Ganem and Varmus, 1987; Gerlich, 1991). The L protein, apart from being absolutely necessary for virion formation, also plays a role in viral attachment to hepatocytes with an attachment site situated on the preS1 domain from amino acid 21 to 47 (Gerlich, 1991; Paran *et al.*, 2001; Prange and Streeck, 1995). This site is the HBV hepatocyte receptor (HBV Hep-R) binding site and attaches directly to liver plasma membranes via an 80-kDa protein receptor (Petit *et al.*, 1992; Pontisso *et al.*, 1989; Ryu *et al.*, 2000). However, the number of S proteins on the HBV envelope is much higher than that of preS1 domains and they probable play a role in attachment by forming multiple

contacts with the cell membrane (Paran *et al.*, 2001). This binding, however, is probably unstable, but is stabilised by the subsequent interaction of the preS1 epitope with its corresponding receptor. The L protein also regulates the nuclear pool of covalently closed circular viral DNA (Prange *et al.*, 1995a). The preS amino acid sequences are the least conserved coding sequences among different hepadnaviruses and have thus been implicated in host range determination. It was shown that the species-specificity of hepadnaviral infection is determined at the level of virus entry and is governed by the preS domain of the viral L protein (Ishikawa and Ganem, 1995). Variations in the preS regions could be a means of evading the host immune system (Tiollais *et al.*, 1985). The S protein may also play a direct (e.g. by mediating membrane fusion) or indirect (e.g. by allowing proper display of the preS sequences) role in viral entry (Ishikawa and Ganem, 1995).

For more detailed information on hepatitis B virus biology including replication, transcription, translation, encapsidation, infection and mechanisms of hepatocarcinogenesis see Caselman, 1996; Fallows and Goff, 1996; Ganem and Varmus, 1987; Seeger and Mason, 2000.

2.6. Hepatitis B Vaccines: Heterologous Expression of Hepatitis B Surface Antigens

Since the first successful vaccination against an infectious disease (smallpox) by Edward Jenner in 1796, the science of vaccinology has developed immensely from the classical use of whole-cell vaccines, consisting of killed or attenuated pathogens, to new vaccines based on the subunit principle and, most recently, nucleic acid vaccines (Dertzbaugh, 1998; Gregoriadis, 1998; Hilleman, 2000; Liljeqvist and Ståhl, 1999).

Prevention of hepatitis B was initially based on passive immunoprophylaxis through administration of serum immune globulin derived from pooled human serum and containing antibodies against hepatitis A virus and a varying degree of antibodies against HBV (Garrison and Baker, 1991). Significant advancement in the prevention of hepatitis B was made with the development of

vaccines. The history of hepatitis B vaccine development is summarised in Table 6 (Greenberg, 1993).

Table 6. History of hepatitis B vaccine development (Greenberg, 1993; Tsebe *et al.*, 2001).

Date	Event
1883	First recognised outbreak of HBV infection in Germany
1950s-1960s	Krugman described two types of viral hepatitis
1965	Blumberg discovered the Australia antigen (HBsAg)
1970s	Dane particle, HBV antigens and antibodies characterised
1982	Plasma-derived vaccine available for high risk groups
1984	Screening of high risk pregnant women recommended
1986	Recombinant vaccine produced by Merck Sharp Dohme licensed
1988	Universal screening of all pregnant women recommended
1989	Recombinant vaccine produced by Smith Kline Biologicals licensed
1991	Universal childhood immunisation recommended
2000	110 Countries implement routine childhood immunisation

To date, various hepatitis B vaccines have been developed using a variety of immunogens (Kobayashi *et al.*, 1992):

1. First-generation hepatitis B vaccines: HBV surface antigen (HBsAg) small particles from healthy carriers.
2. Second-generation hepatitis B vaccines: yeast-derived HBsAg S protein particles.
3. Third-generation hepatitis B vaccines: yeast or mammalian cell-derived HBsAg M protein-containing particles.

There is also constant ongoing research to improve the existing vaccines and/or make them more cost effective.

2.6.1. Plasma-derived vaccines

Hepatitis B was first transmitted to laboratory animals (chimpanzees) in the 1970s (Purcell, 1994). The virus does not grow in cell culture so that a conventional source antigen was not available (Hilleman, 1987). Although, at the time, HBV had never been isolated and serially propagated in cell culture in any practical system, the first hepatitis B vaccine was licensed in the United States of America in 1981 (Purcell, 1994). This unique vaccine, which proved

to be safe and highly efficacious, was prepared from viral envelope protein (i.e. the 22 nm subviral particle) that was purified from the plasma of chronically infected individuals (Couroucé *et al.*, 1988; Gibas *et al.*, 1988; Taylor and Stevens, 1988). Vaccines produced in this way from human sources were formalin-inactivated and required highly technical purification procedures (Barr *et al.*, 1989; Valenzuela *et al.*, 1985). With the availability of this vaccine, a hepatitis B immunisation strategy was developed in the United States in the 1980s, targeting groups at high risk of HBV infection (Katkov and Dienstag, 1995). The vaccine was, however, limited in production by the available supply of acceptable carrier plasma and the need to inactivate any infectious agents that might also be present (Barr *et al.*, 1989; Hilleman, 1987). A necessary part of the production process included a prolonged innocuity test in increasingly scarce chimpanzees (Stephenne, 1988). Furthermore, the principal source of HBsAg-positive plasma for the manufacture of vaccine was from the same population that subsequently was at high risk of contracting AIDS (Purcell, 1994). Thus, despite the overwhelming evidence for its safety, the plasma vaccine was used less than expected due to fear of transmission of HIV by the vaccine (Hilleman, 1987). In addition, the supply of antigen was so restricted and expensive that the vaccine was generally only available to particular high-risk groups, thus not being a solution to the wide-spread problem in third world countries (Barr *et al.*, 1989; Kitano *et al.*, 1987). Also, the prevailing strategy of targeting hepatitis B vaccine to specific high-risk groups was shown to be ineffective in reducing the frequency of HBV in the US, suggesting the necessity for more wide-spread immunisation programmes (Katkov and Dienstag, 1995). This, however, required a safe, inexpensive vaccine. For these reasons, yeast recombinant vaccine was developed (Hilleman, 1987).

2.6.2. Yeast-derived recombinant S protein subunit vaccines

The recombinant yeast hepatitis B vaccine represents the world's first viral subunit vaccine, the first licensed vaccine to prevent human cancer and the first recombinant-expressed vaccine (Hilleman, 2000). It has removed all the obstacles facing the use of plasma-derived vaccines and dramatically altered the scale of vaccination that can now be undertaken (Barr *et al.*, 1989). In fact,

this vaccine is now routinely used in the 110 countries that have employed the routine childhood immunisation strategy recommended by the WHO in 1991 (Tsebe *et al.*, 2001).

Development of the recombinant subunit vaccine started when Valenzuela and co-workers reported the cloning of the hepatitis B viral genome in *E. coli* and deduced the complete primary structure of the HBsAg (the main antigenic component of the viral envelope) from the DNA sequence (Valenzuela *et al.*, 1979). High level production of HBsAg-related immunogenic material was, however, not achieved in bacteria (Valenzuela *et al.*, 1982). Despite many efforts, production of vaccines in *E. coli* has not succeeded because, in *E. coli* cells, the HBsAg gene product seems to be either unstable or to cause effects deleterious to the host, or both (Miyanohara *et al.*, 1983). Therefore, Valenzuela and co-workers examined yeast as an alternative host system and constructed an autonomously replicating plasmid containing the *S* gene under the control of the *ADH1* promoter, which they transformed to *S. cerevisiae* (Valenzuela *et al.*, 1982). The protein was successfully synthesised in yeast and spontaneously assembled with yeast lipids and carbohydrates into particles having properties similar to the 22 nm particles secreted by human cells (Sato *et al.*, 1995; Valenzuela *et al.*, 1982) (Figure 8). The yield was 10 to 25 µg HBsAg/l cells. The yeast-derived particles are more variable in size than the plasma-derived particles and, unlike the plasma-derived particles, they do not contain significant quantities of a higher molecular weight glycoprotein. Despite these differences in size and glycosylation, the yeast-derived particles were immunoreactive with anti-HBsAg antibodies, which bind to a conformational determinant that is dependent on the integrity of disulphide bridges present within the viral envelope protein (Waters *et al.*, 1987). Subsequently, various other groups also expressed the HBV S protein in *S. cerevisiae*. Expression of the *S* gene under control of the constitutive *S. cerevisiae* 3-phosphoglycerate kinase (*PGK*) gene promoter yielded 50 µg immunoreactive HBsAg/l cells (Hitzeman *et al.*, 1983). Miyanohara and co-workers constructed a yeast expression vector carrying the *S* gene of HBV (subtype *adr*) under control of the repressible yeast acid phosphatase promoter which they transformed to *S. cerevisiae* (Miyanohara *et al.*, 1983). They also obtained immunogenic virus-

like particles at 2.8 mg HBsAg/l. The *adw* subtype *S* gene was cloned and expressed by McAleer and co-workers with similar results (McAleer *et al.*, 1984). The yeast-derived antigen was shown to be at least as antigenic as the

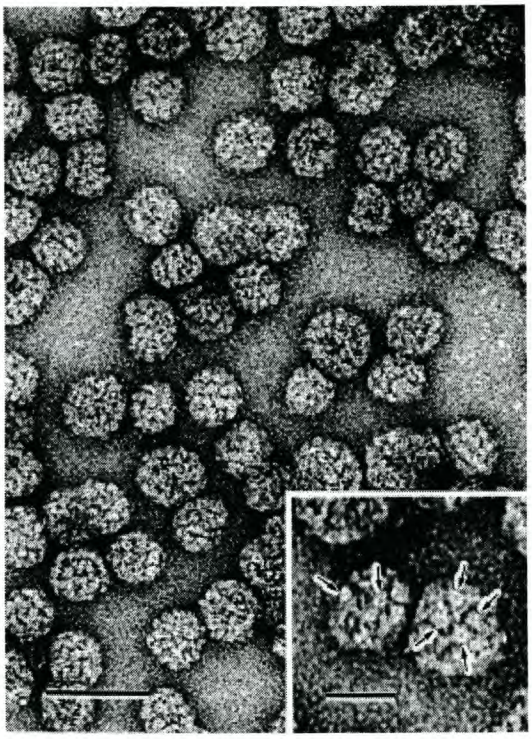

Figure 8. Negatively stained HBsAg particles isolated from recombinant yeasts. ×280000. Bar=50 nm. Inset: ×465000. Bar=20 nm. Note subunit structures with pores (arrows) (Yamaguchi *et al.*, 1998).

antigen purified from human plasma and animals vaccinated with the *adw* subtype vaccine were also protected against both subtype *adr* and *ayw* challenge. All the above expression systems did not employ yeast secretion signals and no secretion of the particles takes place from *S. cerevisiae* (Biemans *et al.*, 1992). The S protein, however, contains internal uncleaved signal sequences that direct portions of the molecule across the ER membrane. The majority of the S protein was then also found in dilated ER structures suggesting that further transport along the secretion pathway is blocked. Particle formation then occurs in the ER. The absence of *N*-linked

oligosaccharide on the yeast-derived S protein might provoke aberrant formation of disulphide bonds and thus cause the formation of aggregates that accumulate in the ER. These aggregates might be difficult to transport to the Golgi complex owing to size constraints. Thus the particles remain intracellular, however, the possibility exists that, to a limited extent, HBsAg may be secreted across the plasma membrane into the periplasm. Chen and co-workers attempted to express HBsAg in the secretion pathway of *S. cerevisiae* by using the α-factor leader sequence (Chen *et al.*, 1995). However, the yeast did not secrete the protein and the growth rate of transformants was inhibited, giving swollen, division-inhibited cells. This toxicity may be due to the complexity of the secretory pathway. The toxicity could be overcome by reducing the ammonium sulphate concentration in selective medium, but it is doubtful whether this will be viable in large-scale production of the antigen. Unlike HBsAg derived from human plasma, the yeast antigen is held together by noncovalent interactions (Wampler *et al.*, 1985). During *in vitro* purification some disulphide bonds form spontaneously between the antigen subunits, resulting in particles composed of a mixture of monomers and disulphide-bonded dimers. The fully disulphide-bonded form resembling the plasma-derived surface antigen particle is then obtained by treatment with thiocyanate. Ultrastructure analysis of *S. cerevisiae*-derived HBsAg particles showed that they are spherical to slightly ovoid with a mean diameter of 27.5 nm and consist mainly of many subunits each 4 nm in diameter with a minute central pore (Yamaguchi *et al.*, 1998). In contrast, human HBsAg particles appear mostly spherical consisting of 3 nm subunits and have a mean diameter of 21.2 nm. This difference in size is apparently due to molecular differences in polypeptide structure.

Studies were also performed on the scale-up of recombinant hepatitis B production. Controlled fed-batch fermentation comparing three recombinant *S. cerevisiae* strains expressing the *S* gene under control of the alcohol dehydrogenase (ADH1), acid phosphatase (PHO5) and glyceraldehyde-3-phosphate dehydrogenase (GPD) promoters, respectively, revealed that the highest HBsAg levels (3-10 mg/L) were obtained from the strain with the constitutive GPD promoter (Hsieh *et al.*, 1998). A fed-batch cultivation method

based on the production kinetics of batch culture enhanced HBsAg production ten times more than in batch culture (Gu *et al.*, 1991). However, in the scale-up of this culture to 9 L, ethanol accumulation occurred at a late stage and was not reutilised, inhibiting further growth. Subsequently, a continuous culture study of the expression of HBsAg was undertaken (Fu *et al.*, 1996). These studies showed that *S* gene expression varies inversely with growth rate and plasmid copy number was stable and independent of dilution rate. The S monomers were shown to dimerize in less than two minutes, but dimer oligomerisation and lipoprotein budding occur over a longer time scale (160 minutes). It seems that a constant ratio of excess S monomer to HBsAg particle may exist and that growth rate, in itself, does not affect the degree of HBsAg particle accumulation. In fact, excess level of monomer appears to act as driving force for the generation of lipoprotein particles.

In 1986 this research eventually culminated in the commercial production of two *S. cerevisiae*-derived hepatitis B vaccines based on expression of the *S* gene, namely Recombivax-HB (Merck Sharp and Dohme) and Engerix-B (SmithKline-Beecham) (Manyike *et al.*, 1992; Panda *et al.*, 1991). Recombivax-HB represents the first licensed vaccine of any kind produced by recombinant technology and thus established the precedent for new vaccines to be made using this methodology.

2.6.3. Vaccines containing middle and large proteins

The preS regions contain important antigenic determinants. This was shown by the fact that antibodies against preS occur during the resolving of HBV infections; preS sequences appear to augment the immunogenicity of S sequences in normally non-responding animals; and preS occurs on the surface of the virus and may be involved in attachment of the virus to target cells (Peterson, 1987; Yap *et al.*, 1995).

Therefore the M and L proteins have also been targeted for expression in *S. cerevisiae.*

2.6.3.1. The M Protein

As has been mentioned previously, the preS2 domain binds to polymerised human serum albumin (pHSA) and may play a role in binding of the virus to hepatocytes (Gerlich *et al.*, 1990). Antibodies to the polyalbumin receptor elicited by a vaccine carrying the preS2 region may thus directly interfere with the binding of HBV to hepatocytes and further prevent the initial step of infection (Valenzuela *et al.*, 1985). Studies showed that the preS2 region is, in fact, significantly more immunogenic than the S region, eliciting antibodies 400 times more efficiently (Itoh *et al.*, 1986; Milich *et al.*, 1985; Valenzuela *et al.*, 1985). The preS2 region contains two immunodominant regions and anti-preS2 antibodies recognise typical linear epitopes and do not distinguish between native or denatured preS2 proteins (Meisel *et al.*, 1994). Also, the preS2 region appears capable of generating T cell helper activity that can function to induce production of antibodies to the S region as well as the preS2 region, thereby circumventing S region non-responsiveness (Milich *et al.*, 1985). Hepatitis B viruses are also highly mutagenic because of errors in reverse transcription, which can result in variable specificity of surface antigens (Hilleman, 1995). All this evidence pointed to the fact that inclusion of the preS2 region could augment the effectiveness of future HBV vaccines. To this end, *S. cerevisiae* was transformed with plasmids carrying the *preS2* region as well as the *S* gene.

The M protein (encoded by *preS2-S*) has been expressed independently by several laboratories since 1985 (Gerlich *et al.*, 1988; Jacobs *et al.*, 1989; Valenzuela *et al.*, 1985). Kitano and co-workers (1987) transformed *S. cerevisiae* with a plasmid containing the sequence for the M protein of HBV subtype *adr* under control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter (Kitano *et al.*, 1987). The resultant M protein accumulated as particles of 20 to 30 nm and formed inclusion bodies in the *S. cerevisiae* cells.

Initially, it was found that the preS2 region was especially sensitive to proteolysis, however this could be solved by modifying certain base sequences or by using a protease-deficient (*pep4*) yeast host strain (Kitano *et al.*, 1987; Langley *et al.*, 1988). Two other groups performed similar experiments and obtained recombinant *S. cerevisiae* strains expressing the HBV M protein

(Imamura *et al.*, 1987; Langley *et al.*, 1988). The *preS2* gene products obtained from the yeasts assembled into the spherical forms, showed pHSA binding activity and reacted with antibodies specific for epitopes within the preS2 region. In *S. cerevisiae,* the M protein is translocated across the ER membrane, however no further processing by the yeast secretory apparatus occurs (Jacobs *et al.*, 1989). Thus, the M protein product is probably situated in the ER lumen, with some possible translocation to the Golgi. However, when a proteinase resistant *preS2-S* gene was fused to the chicken-lysozyme signal peptide and placed under control of the *GPD* promoter, *S. cerevisiae* was able to secrete HBsAg particles into the medium in the exponential growth phase (Kuroda *et al.*, 1993). Glycosylation of the M protein occurs in yeast and the yeast-produced M-containing particles differ from those of human sera in their very high content of the glycosylated M protein (Imamura *et al.*, 1987). M proteins produced in recombinant yeasts which were "wild-type" for glycosylation were highly immunogenic, however a large proportion of hypermannosylated species with extended mannose side-chains of up to several hundred thousand daltons were found (Kniskern *et al.*, 1994). This hypermannosylation was eliminated by expressing the *preS2-S* gene in a *S. cerevisiae mnn9* mutant defective for glycosylation (Ip *et al.*, 1992). The M protein particles produced by this strain are composed mainly of two major glycoproteins, GP37 and GP34 (Kobayashi *et al.*, 1992). GP37 has an *N*-linked oligosaccharide bound to Asn 4 of the high-mannose type ($Man_7GlcNac_2$ or $Man_8GlcNac_2$) and an additional *O*-linked oligosaccharide bound to the preS2 region. These oligosaccharides do not have any effect on the ability of the HBsAg M protein vaccine to elicit anti-S and anti-preS2 antibodies in experimental animals. In mice, the products of the *preS* region were shown to be more immunogenic than the product of the *S* gene (Katkov and Dienstag, 1995). However, studies carried out in humans indicated that while the vaccine containing both the S and preS2 determinants is immunogenic in healthy, immunocompetent adults and that antibodies to preS2 are detected, no dramatic advantage of preS2 vaccines over S-only vaccines was shown. The potential of preS vaccines in non-responders to S-only vaccines is promising, but still remains to be evaluated systematically.

2.6.3.2. The L protein

Further development to improve vaccine efficacy included the incorporation of the large (L) protein, coded by the *preS1-preS2-S* gene, which also plays a role in viral attachment and host range determination (Imamura *et al.*, 1987; Ishikawa and Ganem, 1995). The preS1 region has been shown to be a particularly efficient immunogen with a dominant T cell recognition site in the N-terminal residues (21-28) and B cell determinants at residues 12-32, 32-35 and 94-117 of serotype *adw* (Prange *et al.*, 1995b; Sulowska *et al.*, 1995). Thus the L protein was also expressed in *S. cerevisiae* and, like the M protein, was produced both in *N*-linked glycosylated and non-glycosylated forms (Dehoux *et al.*, 1986; Jacobs *et al.*, 1989). In addition to *N*- and *O*-linked glycoslyation, the L protein was also *N*-myristoylated at the N-terminal glycine, further confirming the entry into the endoplasmic reticulum (ER) (Biemans *et al.*, 1991). In contrast to the M protein, the L protein molecules did not readily assemble into the well-ordered structure that is typical of the major S polypeptides. Rather it is retained in the ER, provoking the proliferation of the ER resulting in the accumulation of membranous structures connected with the perinuclear cisternae.

2.6.3.3. Simultaneous expression of different HBV proteins

Subsequently, the simultaneous synthesis of various hepatitis B surface proteins in *S. cerevisiae* has been performed (Jacobs *et al.*, 1989). In yeast, transcription is directed by the yeast promoter and viral transcription initiation signals within the HBV genes are not functional. Simultaneous expression of S, M and L proteins in *S. cerevisiae* was therefore accomplished by the introduction of expression cassettes encoding the different proteins into the yeast genome. Mixed lipoprotein structures were found in yeast extracts from this transformant, with the different proteins undergoing coassembly into a single lipoprotein structure. Another *S. cerevisiae* strain was also constructed co-expressing the S, M, L and core proteins under control of the yeast *GPD* promoter (Shiosaki *et al.*, 1991). Cell lysates from this recombinant strain contained spherical particles with a diameter of 40 nm which were found to consist of core particle surrounded by envelope, thus resembling the hepatitis B

virion without DNA. While vaccines incorporating preS regions do exist, they are not in widespread use and further research is required to determine the optimal system to produce such vaccines.

2.6.4. Alternative hosts for expression of hepatitis B antigens

Already, alternative hosts for expression of hepatitis B antigens have been explored. These include the methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha,* the yeast *Kluyveromyces lactis,* as well as plant hosts (Barr *et al.*, 1989; Gellissen, 2000; Gellissen and Hollenberg, 1997; Gellissen and Veenhuis, 2001; Hadfield *et al.*, 1993; Hollenberg and Gellissen, 1997; Martinez *et al.*, 1992; Thanavala, 1996). For *P. pastoris* it has been shown in several examples that the addition of terminal mannoses occurs by α1,2 linkages instead of the α1,3 linkages frequently observed in *S. cerevisiae,* which are considered to be allergenic (Gellissen, 2000). The methylotrophic yeasts proved to be very effective in producing HBsAg, with *P. pastoris* initially producing high levels of protein (2% to 3% of the soluble protein of the cell) (Cregg *et al.*, 1987). The surface antigen also self-assembled intracellularly into 22-nm lipoprotein particles composed of approximately 100 units of a non-glycoslyated S protein and yeast lipids (Tleugabulova *et al.*, 1999). Industrial scale production of HBsAg in *P. pastoris* under control of the alcohol oxidase I (*AOX*) promoter has yielded levels of 1.3 $\pm$ 0.2 g/L (Hardy *et al.*, 2000). In this case the expression cassette was integrated into the genome of *P. pastoris* and yielded a non-glycosylated, biologically active product. This *P. pastoris*-derived HBsAg has since been employed as a safe, efficacious vaccine (HEBERBIOVAC HB, Heberbiotec S. A, Cuba). Recently, a recombinant *P. pastoris* strain has been constructed expressing the *S* gene under control of the constitutive *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase promoter, obviating the need for methanol induction thereby reducing the fermentation time for peak production levels (Vassileva *et al.*, 2001). In-frame fusion of the *S. cerevisiae* α-factor secretion signal to the *S* gene resulted in the secretion of 20 nm particles into the medium. However, the levels of secreted HBsAg particles were very low, presumably due to the inherent hydrophobicity

of the HBsAg molecule and the consequent propensity for membrane association.

H. polymorpha also produced high levels of antigen (5% to 8% of total cell protein) containing both S and L surface antigens and has also been shown to successfully express M protein (Gellissen, 2000; Janowicz *et al.*, 1991; Shen *et al.*, 1989). The proteins were also found to be glycosylated far less than in *S. cerevisiae,* making this an attractive alternative host (Janowicz *et al.*, 1991). A production strain was generated by introducing a plasmid with the *S* gene fused to the *H. polymorpha* methanol oxidase (MOX) promoter (Gellissen, 2000). The transformed strain is able to grow on glycerol and methanol as carbon sources. Currently, two *H. polymorpha*-derived hepatitis B vaccines are commercially available: Hepavax-Gene, produced by KGCC (Korea) and AgB, produced by LPC (Argentina).

In recent years, plants have gained in importance as hosts for heterologous protein production. The HBsAg has been expressed in tobacco plants (the leaf cells produce the recombinant protein) and potato plants (Domansky *et al.*, 1995; Thanavala, 1996). The transgenic tobacco-derived HBsAg was shown to retain the immunological characteristics needed to generate HBsAg-specific B and T cell responses, however the maximal level of HBsAg obtained was not sufficient to use the plants for production of a vaccine (Domansky *et al.*, 1995). Nevertheless, this technology could potentially make vaccines more readily available to countries lacking refrigeration and an elaborate infrastructure for health care, in that "edible vaccines" could be developed.

Most recently, we have evaluated the filamentous fungus *A. niger* as a heterologous host for the expression of the HBV *S* gene (Paper 1, this thesis). The fungus was shown to produce 22 nm pseudoviral particles similar to those isolated from human serum and yeast hosts expressing the *S* gene. In shake flasks, initial production levels of the pseudoviral particles were 0.4 mg/L, which compares favourably with those obtained from the yeast hosts (Cregg *et al.*, 1987; Hitzeman *et al.*, 1983; Valenzuela *et al.*, 1982). Fermentation can generate up to 20 times more dry weight of mycelium per litre than shake-flask

yields and well optimised fermentation should give yields at least five to ten times greater than shake-flask yields, indicating the potential of the *Aspergillus* system (Davies, 1994). The advantages of the *Aspergillus* system already discussed, particularly the ease of fermentation and production levels, make this host an attractive alternative for the production of hepatitis B vaccines.

Research into the development of vaccines to combat the spread of hepatitis B is still an ongoing process despite the availability of effective products. The continued necessity for cheap, effective and, particularly, convenient vaccines for the widespread use in underdeveloped countries primarily drive this quest, as vaccination provides for the only means to combat the unacceptably high prevalence of this disease world-wide.

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Chapter 3

Paper 1

Evaluation of *Aspergillus niger* as host for virus-like particle production using the hepatitis B surface antigen as a model

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3. EVALUATION OF *ASPERGILLUS NIGER* AS HOST FOR VIRUS-LIKE PARTICLE PRODUCTION USING THE HEPATITIS B SURFACE ANTIGEN AS A MODEL

Annette Plüddemann and Willem H. van Zyl*

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

3.1. Abstract

The filamentous fungus *Aspergillus niger* was transformed with the hepatitis B virus *S* gene encoding the major viral envelope protein under control of the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter. Southern analysis revealed the integration of approximately seven copies of the expression cassette on the genome, resulting in high level transcription of the *S* gene. Production of the 24 kDa S protein, as well as a 48 kDa S protein dimer, in the membrane-associated protein fraction of the recombinant *A. niger* strain was shown through Western analysis. Recombinant S protein was purified from extracts of the *A. niger* strain by sucrose gradient centrifugation and electron microscopy revealed the formation of spherical pseudoviral particles with a diameter of 22 nm. The yield of hepatitis B pseudoviral particles from mycelium of the recombinant *Aspergillus* strain was estimated to be 0.4 mg per litre of culture, which compares favourably with the reported levels initially obtained in yeast, indicating the potential of the *Aspergillus* expression system as an alternative cost-effective vaccine production system.

3.2. Introduction

Saccharomyces cerevisiae has traditionally been used as heterologous host for the production of pharmaceutical (viral) proteins, but has manifested limitations such as low product yields and hyperglycosylation of glycoproteins (Hadfield *et al.*, 1993). Since the 1980's genetic engineering procedures have been developed for the filamentous fungi, in particular aspergilli (Jeenes *et al.*, 1991; Kinghorn and Unkles, 1994; Van den Hondel and Punt, 1991). Subsequently,

various heterologous proteins of fungal and non-fungal origin have been expressed in these fungi (reviewed by Kinghorn and Unkles, 1994; Van den Hondel *et al.*, 1991). Several characteristics of filamentous fungal expression hosts give them potential advantages over *S. cerevisiae*, particularly their efficient secretion of endogenous proteins. Hyperglycosylation is less of a problem in filamentous fungi than in *S. cerevisiae,* as their glycosylation patterns more closely resemble those found in mammalian cells. For example, when produced in *S. cerevisiae*, human tissue plasminogen activator was hyperglycosylated, but this was not the case when the protein was produced in *Aspergillus nidulans* (Upshall *et al.*, 1987). Filamentous fungi are also rather robust and can grow on many organic compounds (Jeenes *et al.*, 1991; Van den Hondel *et al.*, 1991). They are known to produce and secrete enzymes in large quantities and through their use in the food and food processing industry, the species *Aspergillus oryzae* and *Aspergillus niger* have gained GRAS (Generally Regarded As Safe) status. Filamentous fungi have been extensively used in industrial processes such as organic acid and enzyme production, and therefore large-scale fermentation technology and downstream processing are already well established (Bodie *et al.*, 1994). Several proteins of pharmaceutical importance have also been successfully produced in *Aspergillus,* including tissue plasminogen activator (1 mg/l) (Upshall *et al.*, 1987), human interleukin-6 (5-10 mg/l) (Gouka *et al.*, 1997), human lactoferrin (2 g/l) (Ward *et al.*, 1995), human interferon α-2 (1 mg/l) (Gwynne *et al.*, 1987) and an industrial process for human superoxide dismutase via intracellular expression in *A. nidulans* has been developed (Davies, 1994). However, viral protein production in filamentous fungi has not been evaluated to date and the hepatitis B surface antigen provides the ideal basis for such an evaluation.

Hepatitis B is a widespread and serious liver disease and can be considered a global health problem with an estimated 2000 million HBV carriers in the world today (World Health Organisation). The problem is most prominent in developing countries with 95% of chronic carriers residing in the developing world (Ayoola, 1988). With regard to southern Africa specifically, in a study performed in Swaziland in 1983 and in Namibia in 1985, the prevalence of HBV amongst those tested was already 82.6% and 98.9%, respectively, and a high

endemicity exists in rural areas such as Kwazulu/Natal (>60%) and Kangwane (>70%) within South Africa (Ayoola, 1988; Manyike *et al.*, 1992). No effective treatment for this disease currently exists and an extensive programme of vaccination presents the only solution to curb the spread of the disease (Kassianides *et al.*, 1988). Routine infant or adolescent vaccination has been recommended by the WHO since 1991 (Vryheid *et al.*, 2001). In order to make a mass vaccination strategy feasible, large amounts of safe, affordable vaccine must be available. The hepatitis B Virus (HBV) is a small DNA virus which has a double-walled structure consisting of two concentric protein coats (Ganem and Varmus, 1987; Huovila *et al.*, 1992). The envelope, or outer coat, contains three proteins, designated the major (S) (24 kDa), middle (M) (33 kDa) and large (L) (39 kDa) protein. These proteins are encoded by the *S* gene, *preS2S* gene and *preS1preS2S* gene, respectively. All three envelope components are glycosylated, type II transmembrane proteins that form multimers stabilised by disulphide bridges formed by cysteine residues present in the S domain (Seeger and Mason, 2000). One virion contains 300 to 400 major (S) protein molecules and 40 to 80 middle and large protein molecules (Tiollais *et al.*, 1985), which together with the capsid form the 43-nm-diameter infectious viral particle (virion), referred to as the Dane particle (Ganem and Varmus, 1987). The S protein by itself and together with the larger envelope proteins spontaneously assembles into filamentous and spherical particles 20 to 22 nm in diameter, that are secreted from infected cells in 10^3- to 10^6-fold excess over virions. These pseudoviral particles, termed the hepatitis B surface antigen (HBsAg), are devoid of viral DNA and play a role in neutralising virus-eliminating immune reaction (Peterson, 1987). Hepatitis B surface antigen proteins have important functions in the viral life cycle, such as attachment and penetration, envelopment and virus maturation (Gerlich, 1991). Thus, these proteins have been targeted for heterologous expression to produce recombinant vaccines. Recombinant DNA methods have provided an alternative to the blood-origin vaccine obtained from plasma of chronic carriers and to date, yeasts (Dehoux *et al.*, 1986; Hardy *et al.*, 2000; Valenzuela *et al.*, 1982), plants (Domansky *et al.*, 1995; Mason *et al.*, 1992) and mammalian cells (Michel *et al.*, 1984) transformed with appropriate expression vectors are able to synthesise hepatitis B surface antigen (HBsAg) for immunisation purposes. The first yeast-derived

hepatitis B vaccine was produced in *S. cerevisiae* and licensed in 1986 (Barr *et al.*, 1989; Hilleman, 1987), representing the first licensed vaccine of any kind produced by recombinant technology and thus established the precedent for new vaccines to be made using this methodology.

In this paper we describe the transformation of *A. niger* with the *S* gene of a South African isolate of the hepatitis B virus subtype adw2, and the intracellular production of 22 nm pseudoviral particles.

3.3. Materials and Methods

3.3.1. Strains and media

Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1Blue (Stratagene) cultivated at 37°C in Luria-Bertani liquid medium or on Luria-Bertani agar (Sambrook *et al.*, 1989). Ampicillin for selection and propagation of resistant bacteria was added to a final concentration of 100 µg/ml. *A. niger* D15, the recipient strain for transformation, is a uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), nonacidifying (*phmA*) mutant (Gordon *et al.*, 2000). All strains used in this study are summarised in Table 1. *A. niger* D15 was cultured in *Aspergillus* minimal growth medium (Bennett and Lasure, 1991) containing 1% glucose and supplemented with 0.2% casamino acids (Difco), 0.5% yeast extract and 10 mM uridine (Sigma) in 1-liter Erlenmeyer flasks containing 250 ml medium at 30°C on a rotary shaker at 150 rpm. Transformants were selected on minimal medium without uridine containing 1.2 M sorbitol as osmotic stabiliser, 1.5% agar (Oxoid) and 10 mM acetamide as sole nitrogen source. Transformants were selected for multicopy integration of the expression cassettes on acrylamide plates (Verdoes *et al.*, 1993). Minimal medium without uridine, supplemented with 0.5% yeast extract, 0.1% casamino acids and 1% glucose, was used to culture transformants.

Table 1. Microbial strains and plasmids.

Strain/Plasmid	Genotype	Source/Reference
Bacterial strain:		
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r)][c]	Stratagene
Fungal strains:		
Aspergillus niger D15	*pyrG prtT phmA*	Gordon *et al.*, 2000
A. niger D15 (pBluescript-pyrGamdS)[a]	*pyrG*$^+$ *prtT phmA amdS*$^+$	This work
DAHB11#2	*pyrG*$^+$ *prtT phmA amdS*$^+$ *gpdA*$_P$*-S-trpC*$_T$	This work
DAHB11#3	*pyrG*$^+$ *prtT phmA amdS*$^+$ *gpdA*$_P$*-S-trpC*$_T$	This work
DAHB11#8	*pyrG*$^+$ *prtT phmA amdS*$^+$ *gpdA*$_P$*-S-trpC*$_T$	This work
Plasmids:		
pBluescript-pyrGamdS	*bla pyrG amdS*	This work
pCOR105	*bla S*	W.J. de Wet
pAN52-1	*bla gpdA*$_P$*-trpC*$_T$	Punt *et al.*, 1991
pAN52-1-S	*bla gpdA*$_P$*-S-trpC*$_T$	This work
pAHB11	*bla pyrG amdS gpdA*$_P$*-S-trpC*$_T$	This work

[a] *A. niger* D15 (pBluescript-pyrGamdS) was designated *A. niger* (control)

3.3.2. Plasmid construction and transformation of *A. niger*

Standard protocols were followed for DNA manipulations (Sambrook *et al.*, 1989). Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Tautz and Renz (1983). Restriction endonucleases and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used as recommended by the manufacturer. The open reading frame of the hepatitis B virus *S* gene was amplified from plasmid pCOR105 (donated by Prof. W.J. de Wet, Potchefstroom University, South Africa) with the aid of primers S-LEFT (5'-CTAGCCATGGAGAACATCACATCAGGATTCC-3') and S-RIGHT (5'-GATCAGATCTCCAACAGGAAGTTTTCTAAAAC-3') which contained the restriction sites *Nco*I and *Bgl*II, respectively. The resulting 814-bp PCR product was digested with *Nco*I and *Bgl*II and cloned into the *Nco*I and *Bam*H1 sites of plasmid pAN52-1 (Punt *et al.*, 1991), resulting in plasmid pAN52-1-S. In this vector the *S* gene is under the trancriptional control of the *A. nidulans gpdA* promoter and *trpC* terminator. Finally, the *A. nidulans amdS* and *A. niger pyrG* selection markers (provided by Prof. C.A.M.J.J. van den Hondel, TNO, Zeist,

The Netherlands) were introduced into pAN52-1-S at a unique *Not*1 site to generate plasmid pAHB11. A schematic representation of plasmid pAHB11 is given in Figure 1. The *A. nidulans amdS* and *A. niger pyrG* selection markers were also introduced into pBluescript (Stratagene) at the unique *Not*1 site, creating pBluescript-pyrGamdS. Fungal transformation was carried out by the protoplasting method of Punt and Van den Hondel (1992), using Novozym 234 (Sigma).

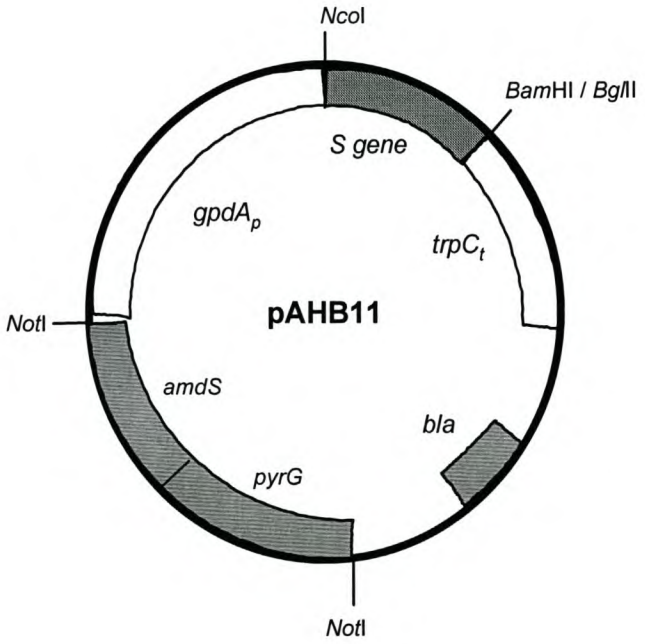

Figure 1. Schematic representation of the *Aspergillus* expression vector pAHB11 constructed in this study. The *E. coli* β-lactamase (*bla*), *A. nidulans* acetamidase (*amdS*) and *A. niger* orotidine-5'-decarboxylase (*pyrG*) genes were used as selectable markers and are indicated by the hatched boxes. $GpdA_p$ and $trpC_t$, indicated by open boxes, represent the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter and *trpC* (a trifunctional gene in tryptophan biosynthesis) gene terminator, respectively. The hepatitis B virus *S* gene is indicated by the cross-hatched box.

3.3.3. Isolation of RNA and Northern analysis

Total RNA was isolated from *A. niger* D15 transformants cultured for 40 h at 30°C as follows: Mycelium was harvested through Miracloth (Calbiochem), frozen under liquid nitrogen, ground to a fine powder with a mortar and pestle and resuspended in cold STE buffer (0.25 M Tris-HCl [pH7.2], 0.1 M NaCl,

0.01 M EDTA [pH8]). One volume phenol/chloroform/isoamylalcohol (PCI) and 0.05% sodium dodecyl-sulphate (SDS) was added and the mycelial debris was collected by centrifugation. The aqueous upper phase was collected, subjected to PCI extraction and precipitated to yield total RNA. The total RNA was used for conventional Northern blot analysis as described by Sambrook *et al.* (1989). The probes were prepared by labelling the 814-bp hepatitis B virus *S* gene and a 1350-bp fragment of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene with [α-^{32}P]dATP, using a random primed DNA labelling kit (Roche Molecular Biochemicals) as recommended by the manufacturer.

3.3.4. Isolation of genomic DNA from *A. niger* and Southern blotting

The genomic DNA was isolated from *A. niger* transformants according to La Grange *et al.* (1996), digested overnight with *Eco*RI at 37°C, separated on a 0.6% agarose gel and blotted to a Hybond-N membrane (Amersham). Southern blot analysis was carried out according to Sambrook *et al.* (1989) using the radioactively labelled 814-bp *S* gene as probe.

3.3.5. SDS-PAGE and Western blotting

Total intracellular and membrane-bound proteins were isolated from the transformants cultured for 72 h in minimal medium. Protein extraction was performed as follows: Mycelium was harvested and ground as before. Ground mycelium was suspended in protein extraction buffer (50 mM phosphate buffer [pH7.0], 1 mM $MgCl_2$, 20 µM phenylmethylsulfonyl flouride) with or without 0.1% Triton X-100, and the mycelial debris was removed by centrifugation. *S. cerevisiae*-derived Engerix-B hepatitis B virus vaccine (20µg/ml) (SmithKline Beecham Pharmaceuticals) was purchased from a local pharmacy to act as reference protein. Proteins were separated on a 12% SDS polyacrylamide gelelectrophoresis (PAGE) gel and visualised by staining with silver nitrate (Blum *et al.*, 1987). For Western blotting, proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris-HCl [pH 7.5], 192 mM glycine, 15% [vol/vol] methanol) using a semidry transfer apparatus (Hoefer). The

Chemiluminescence Western Blotting Kit (Roche Molecular Biochemicals) was used as recommended by the manufacturer. The primary antibodies were polyclonal goat anti-hepatitis B surface antigen (1:5000 dilution) (Dako) and secondary antibodies were anti-goat immunoglobulins conjugated to horseraddish peroxidase (1:10 000 dilution) (Dako). HBsAg was assayed with the Auszyme MC Dynamic kit (Abbott), using HBsAg derived from human serum as a standard. HBsAg was diluted to give levels of 0.09-1.86 ng/ml, and the absorbance at 492 nm after colour development gave a linear relationship in this range. This method is dependent on the conformational epitopes of HBsAg particles and will not detect free unassembled HBsAg proteins.

3.3.6. Dry cell weight determination

Dry weight was determined gravimetrically using nitrocellulose filters (0.45 µm, Gelman Sciences). The filters were predried in a microwave oven for 10 min and weighed. A known volume of culture was filtered and washed with an equal volume of double-distilled water. The filters were dried in a microwave oven for 10 min and weighed. Dry cell weight was determined in triplicate.

3.3.7. Purification of hepatitis B S protein

Protein extracts from mycelium were made as described above and 1 ml of the protein extract from the transformant was layered on a linear 36-ml 5-30% sucrose gradient made in 10 mM sodium phosphate (pH7.0)/0.15 M NaCl. The sucrose gradient was centrifuged in a Beckman SW28 rotor with a Beckman L7 Ultracentrifuge at 19 000 rpm for 20 h at 5°C. The gradient was subsequently fractionated into 2-ml fractions while monitoring the absorbance at 280 nm. Fractions were run on a 12% SDS-PAGE gel to determine which fractions contained the HBsAg.

3.3.8. Electron microscopy

Sucrose fractions containing HBsAg were dialysed against 50 mM sodium phosphate (pH7.0), stained with 2% uranyl acetate and visualised under 110 000x magnification by transmission electron microscopy.

3.4. Results

3.4.1. Recombinant *A. niger* expressing the hepatitis B *S* gene

The recombinant plasmid pAHB11 containing the hepatitis B *S* gene, encoding the major (S) protein of the envelope of the hepatitis B virus (subtype adw2) under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter, was constructed and transformed to *A. niger* D15. The vector pBluescript-pyrGamdS was also transformed to *A. niger* D15. Several uridine prototrophic, acetamide utilising transformants were obtained for both vectors. Transformants were purified twice on selective medium and plated on acrylamide plates. Efficient growth and sporulation on acrylamide plates reflects multicopy integration of the transforming vector (Verdoes *et al.*, 1993).

3.4.2. Gene copy number and expression

Three isolates of *A. niger* D15 transformed with pAHB11 (designated DAHB11#2, DAHB11#3 and DAHB11#8) and showing growth and sporulation on acrylamide plates were selected and cultured in minimal medium containing glucose for 40 h and analysed for *S* gene transcription by Northern blotting (Figure 2A). One *A. niger* D15 transformant containing pBluescript-pyrGamdS was also cultured as reference strain. Transformant DAHB11#8 (lane 3) showed the highest levels of *S* gene mRNA and was selected for further analysis. To determine the copy number of the integrated plasmid in this transformant genomic DNA was isolated, digested with *Eco*RI (which has only one recognition site in pAHB11) and subjected to Southern analysis using the [α-^{32}P]dATP-labelled *S* gene as probe (Figure 2B). Results indicated that about seven copies of the expression cassette integrated into the *A. niger* genome.

3.4.3. Viral protein production by *A. niger*

Transformant DAHB11#8 was cultured in glucose-containing minimal medium for 72 h. Total intracellular proteins were isolated, separated on a 12% SDS-PAGE gel and Western analysis was carried out with HBsAg-specific antibodies. A 24-kDa and a 48-kDa protein species was highlighted, which did

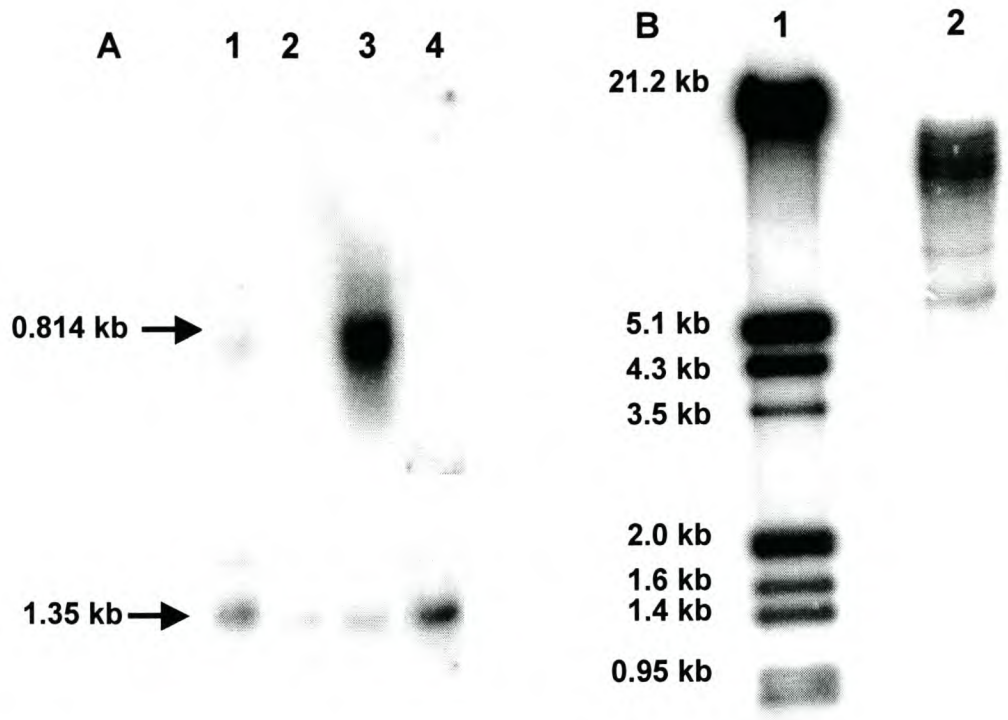

Figure 2. **(A)** Northern analysis of *S* gene mRNA transcripts from isolates of *A. niger* D15 transformed with plasmid pAHB11. Lane 1, transformant DAHB11#2; lane 2, transformant DAHB11#3; lane 3, transformant DAHB11#8; lane 4, *A. niger* (control). **(B)** Southern blot analysis to determine the number of *S* gene copies integrated into the recombinant *Aspergillus* genome. Lane 1, molecular weight marker; lane 2, DNA isolated from *A. niger* (control) digested with *Eco*RI; lane 3, DNA isolated from DAHB11#8 digested with *Eco*RI. The 814-bp *S* gene was used as an [α-^{32}P-dATP]-labelled probe in (A) and (B) and in the northern analysis a 1350-bp fragment of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene was used as internal standard.

not appear in the reference strain (Figure 3A). These proteins also appeared in the Engerix vaccine positive control, confirming that the 24 kDa protein corresponds to the S protein and the 48-kDa protein species represents a dimer of the S protein. It has been shown previously that complete reduction of HBsAg, to obtain a single band in SDS-PAGE, can only be achieved through very harsh reducing conditions [1.3 M dithiothreitol (DTT), 4% (w/v) SDS and 65% (v/v) 2-mercaptoethanol] or through the repeated reductive treatment with simultaneous lipid removal (O'Keefe and Paiva, 1995; Tleugabulova, 1998). Subsequently, cytoplasmic proteins were isolated using extraction buffer without the non-ionic detergent Triton X-100, and membrane-associated proteins were isolated by the addition of Triton X-100 to the extraction buffer. Western

analysis of these protein fractions could detect the S protein in the fraction containing the membrane-associated proteins, but not in the cytoplasmic fraction (Figure 3B).

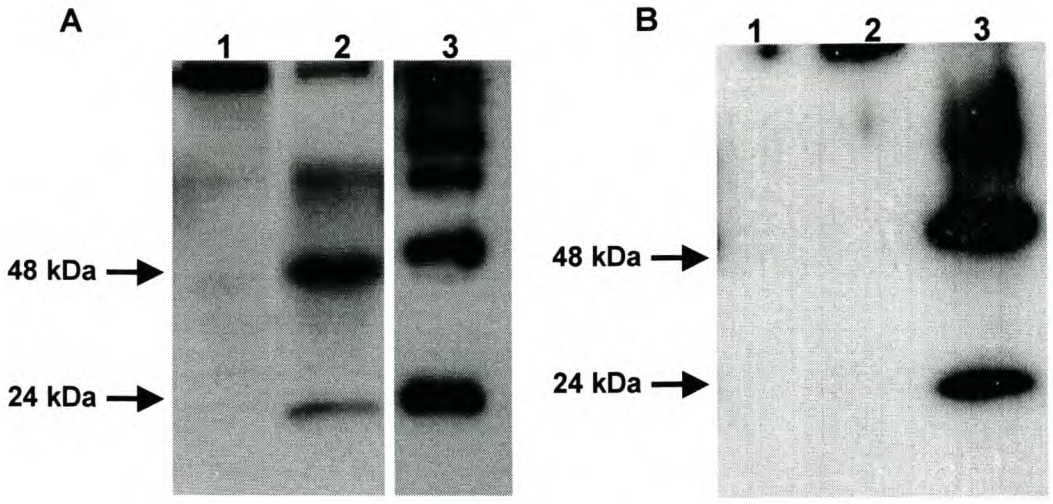

Figure 3. Detection of viral protein production by Western blot analysis. **(A)** Lane 1, *A. niger* (control); lane 2, DAHB11#8 total intracellular proteins; lane 3, yeast-derived Engerix-B hepatitis B virus vaccine. **(B)** Lane 1, *A. niger* (control); lane 2, DAHB11#8 cytoplasmic protein fraction; lane 3, DAHB11#8 membrane-associated protein fraction. Immunoblotting was done with a polyclonal goat-α-HBsAg antibody. A protein species of 24 kDa is visible in transformant DAHB11#8 corresponding to the size of the hepatitis B S protein, as well as an S protein dimer of 48 kDa.

3.4.4. Electron microscopy

The HBsAg in the fungal protein extract was subjected to sucrose gradient centrifugation for purification and removal of detergent, which is essential for the reassociation of the peptides into the immunogenic particle form (Howard *et al.*, 1986). The active fractions were dialysed, negatively stained with uranyl acetate and examined by electron microscopy (Figure 4). Aggregates of 22 nm spherical particles with a dense core were observed which did not appear in control samples. The particles have a spherical structure similar to that of HBsAg isolated from human serum and the dense core represents the characteristic central pore found on both yeast and human HBsAg particles (Yamaguchi *et al.*, 1998). Therefore, HBsAg produced by *Aspergillus* seemed to be assembled into particles similar in size and shape to the spherical pseudoviral particles found in HBV-infected patients' sera and those produced

by recombinant S-protein producing *S. cerevisiae* strains (Yamaguchi *et al.*, 1998).

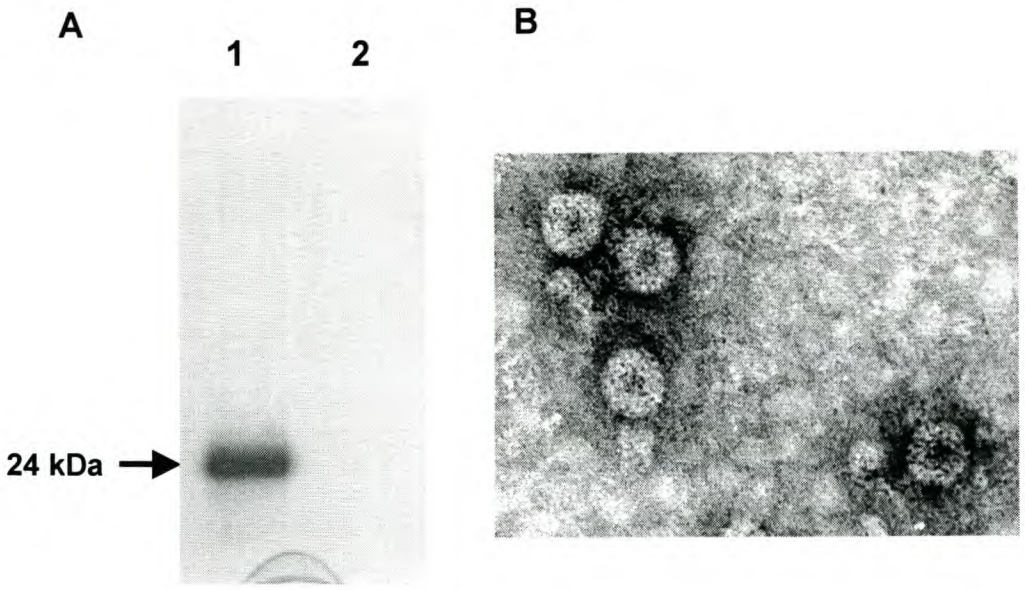

Figure 4. Protein purification and electron microscopy of pseudoviral particles. **(A)** SDS-PAGE of purified S protein. Membrane-associated protein fractions were subjected to sucrose gradient centrifugation, fractionated and dialysed. Lane 1, sucrose gradient fraction of membrane-associated proteins from transformant DAHB11#8; lane 2, corresponding sucrose gradient fraction of membrane-associated proteins from *A. niger* (control). **(B)** Electron microscopy of *Aspergillus*-derived HBsAg particles. Samples were negatively stained with 2% uranyl acetate. The bar represents 100 nm.

3.4.5. Quantification of protein production

Total intracellular protein extract including the membrane fraction was isolated from the recombinant *A. niger* strain (DAHB11#8) to determine the yield of HBsAg pseudoviral particles. The extraction was repeated six times to collect total protein and samples were assayed, using HBsAg derived from human serum as a standard. This enzyme immunoassay detects HBsAg with the aid of monoclonal antibodies against the conformational epitopes of HBsAg particles and will not detect free unassembled HBsAg proteins. The HBsAg levels in the samples were determined from the standard curve. The yield of hepatitis B pseudoviral particles from mycelium of the recombinant *A. niger* strain DAHB11#8 was determined to be 0.2 mg per gram dry mycelium, giving an estimated 0.4 mg HBsAg per litre of culture.

3.5. Discussion

Several microbial systems have been established for the heterologous production of viral proteins, specifically hepatitis B surface antigen. The hepatitis B viral genome was first cloned in *E. coli,* resulting in the deduction of the complete primary structure of the HBsAg (the main antigenic component of the viral envelope) from the DNA sequence (Valenzuela *et al.*, 1979). High level production of HBsAg-related immunogenic material was however not achieved (Valenzuela *et al.*, 1982) and, despite many efforts, production of vaccines in *E. coli* has not succeeded because the HBsAg gene product seems to be either unstable or deleterious to the host, or both (Miyanohara *et al.*, 1983). Subsequently, the yeast *S. cerevisiae* was successfully employed as an alternative host system and the hepatitis B S protein was successfully synthesised in yeast and spontaneously assembled with yeast lipids and carbohydrates into particles having properties similar to the 22 nm particles secreted by human cells (Janowicz *et al.*, 1991; McAleer *et al.*, 1984; Miyanohara *et al.*, 1983; Sato *et al.*, 1995; Valenzuela *et al.*, 1982). Other successful alternative hosts for expression of hepatitis B antigens include the methylotrophic yeasts, *Pichia pastoris* and *Hansenula polymorpha,* the yeast *Kluyveromyces lactis,* as well as plant hosts (Barr *et al.*, 1989; Hadfield *et al.*, 1993; Martinez *et al.*, 1992; Mason *et al.*, 1992; Thanavala *et al.*, 1995). The inherent advantages of the *Aspergillus* expression system such as ease of cultivation, the properties of expressed proteins and high-level protein production could prove advantageous in the production of vaccines.

In this study the hepatitis B virus S protein has been successfully expressed intracellularly in the filamentous fungus *A. niger.* The *S* gene encoding the major envelope protein was placed under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter of *A. nidulans* (Punt *et al.*, 1991) and transformed to a protease deficient, non-acidifying *A. niger* strain. This strain has been previously shown to effectively produce heterologous proteins (Gordon *et al.*, 2000; Wiebe *et al.*, 2001). Various transformants were generated and evaluated on plates containing acrylamide as sole nitrogen source. Selection on acrylamide therefore allowed for the

identification of transformants containing multiple copies of integrated vector (Verdoes *et al.*, 1993). One of these transformants (designated DAHB11#8) that showed the highest level of *S* gene transcription was studied further. Analysis of genomic DNA from this transformant revealed about seven copies of integrated vector, indicating that the high copy number of integrated vector may contribute to the level of transcription, as well as the level of protein production. Previous studies have shown that transcription and protein production levels in *Aspergillus* can be determined by the site of integration and/or vector copy number (Christensen *et al.*, 1988; Davies, 1991; Ward *et al.*, 1990). Western analysis of the total intracellular proteins with HBsAg-specific antibodies showed the presence of the 24 kDa S protein in this transformant. The HBsAg-specific antibodies also highlighted a 48-kDa protein. This protein species is also present in the yeast-derived Engerix-B vaccine sample, indicating that this is an S protein dimer.

To determine the cellular location of recombinant S protein, cell extracts were separated into the cytoplasmic protein fraction and the membrane-associated proteins. The S protein could only be detected in the membrane-associated protein fraction, which is consistent with the transmembrane characteristic of the protein (Seeger and Mason, 2000). The presence of the S protein in the membrane-associated protein fraction suggested spontaneous aggregation of S protein monomers with host membrane molecules into pseudoviral particles. The S protein was subsequently purified by sucrose gradient centrifugation. This process further indicated that the S protein was present in the extract as part of a pseudoviral particle, as the banding in a sucrose gradient is characteristic of HBsAg particles (Janowicz *et al.*, 1991). The presence of HBsAg particles was verified by electron microscopy. These *Aspergillus*-derived recombinant pseudoviral particles have a diameter of about 22 nm and are similar to the HBsAg particles observed in HBV positive human serum (Ganem and Varmus, 1987), and those obtained from yeast expressing the *S* gene which occur in a range of sizes with a mean diameter of 17 nm (Valenzuela *et al.*, 1982). We conclude that the particles made in *Aspergillus* retain the capacity for self-association and thus have the physical properties of

serum-derived HBsAg and recombinant yeast HBsAg, both of which are highly immunogenic in the particle form.

The yield of hepatitis B pseudoviral particles from mycelium of the recombinant *Aspergillus* strain DAHB11#8 was determined to be 0.2 mg per gram dry mycelium, giving an estimated 0.4 mg HBsAg per litre of culture. This value compares favourably with the reported levels initially obtained in *S. cerevisiae* which were respectively 10 to 25 µg HBsAg/l cells (Valenzuela *et al.*, 1982) and 50 µg HBsAg/l cells (Hitzeman *et al.*, 1983), and in wild-type *Pichia pastoris*, where levels of 0.14 mg/l were initially reported (Cregg *et al.*, 1987). This shows the potential of the *Aspergillus* expression system, as production levels can be expected to improve upon optimisation of the system. To the authors' knowledge this is the first report of a viral protein expressed in *A. niger* with concomitant pseudoviral particle formation. Given the emerging versatility of filamentous fungi for the production of proteins of pharmaceutical interest, this work paves the way for the evaluation of *Aspergillus* as an effective host for the expression of other viral proteins for the production of subunit vaccines to address the increasing threat of diseases caused by viruses such as HIV and rotavirus in Africa.

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Chapter 4

Paper 2

Expression of the hepatitis B virus *S* and *preS1preS2S* genes in *Aspergillus*: problems and prospects

4. EXPRESSION OF THE HEPATITIS B VIRUS *S* AND *PRES1PRES2S* GENES IN *ASPERGILLUS*: PROBLEMS AND PROSPECTS

Annette Plüddemann and Willem H. van Zyl

Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa

4.1. Abstract

The hepatitis B virus *S* and *preS1preS2S* genes encoding the major (S) and large (L) envelop proteins, respectively, were cloned into *Aspergillus* expression vectors for intracellular and extracellular expression. Both the viral genes were placed under control of the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) and inducible *Aspergillus niger* glucoamylase (*glaA*) promoters. Fusion constructs between the respective viral genes and the catalytic domain of the *A. niger* glucoamylase (*glaA2*) were also made to explore secretion of the viral protein. The various constructs were transformed to laboratory strains of *A. niger* and *Aspergillus awamori.* The majority of transformants carried single integrated copies of the respective expression vectors, and both the *gpdA* and *glaA* promoters directed *S* gene transcription. Two transformants showed transcription of the *glaA2-S* fusion gene. No full-length *preS1preS2S* gene transcripts were obtained in any of the recombinant strains transformed with this viral gene. One *A. niger* transformant carrying the *S* gene under control of the *gpdA* promoter, which had the highest vector copy number and the highest levels of transcription, revealed intracellular production of heterologous S protein and pseudoviral particle formation (detailed in Paper 1, this thesis). None of the other transformants tested showed production of heterologous viral protein and no secretion of S protein could be obtained. This work clearly indicates that factors such as the choice of strain and promoter, as well as the composition of the expression construct, influence the success of each new attempt at heterologous protein expression in *Aspergillus.* The most important insight that emerged from this study was that the *glaA2* fusion strategy was ineffectual in promoting or enhancing secretion due to the inherent spontaneous aggregation properties of the viral proteins.

4.2. Introduction

Since the 1980s, the filamentous fungus *Aspergillus* has been developed as an alternative host to the commonly employed yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris,* for the production of heterologous proteins (Kinghorn and Unkles, 1994; Romanos *et al.*, 1992). Efficient transformation protocols and various expression vectors have been developed for *Aspergillus* (Turner, 1994; Van den Hondel and Punt, 1991). Transformation generally occurs by random integration into the chromosome (Ballance and Turner, 1985; Tilburn *et al.*, 1983). Two of the promoters most commonly employed are the constitutive *Aspergillus nidulans* glyceraldehyde-3-phosphate (*gpdA*) promoter and the strong inducible *Aspergillus niger* glucoamylase (*glaA*) promoter (Fowler *et al.*, 1990; Punt *et al.*, 1988). To identify transformants, the *A. niger* orotidine-5'-phosphate decarboxylase (*pyrG*) gene is commonly used to complement the corresponding mutation in *pyrG*$^-$ auxotrophic host strains (Goosen *et al.*, 1987). In addition, the *A. nidulans* acetamidase-encoding *amdS* gene has been used as a dominant marker and also to identify transformants carrying multiple copies of the expression vector (Wernars *et al.*, 1985). One copy of the *amdS* gene is sufficient to allow the fungus to grow on acetamide as sole carbon and/or nitrogen source, however growth on acrylamide is only possible if several copies of the gene are present (Van den Hondel *et al.*, 1992). These tools have since been employed for the heterologous production of various fungal and non-fungal proteins in *Aspergillus* (Timberlake and Marshall, 1989; Van den Hondel *et al.*, 1991; Van den Hondel *et al.*, 1992).

Several proteins of pharmaceutical interest have been expressed in *Aspergillus* with varying degrees of success (Gouka *et al.*, 1997b; Kinghorn and Unkles, 1994; Verdoes *et al.*, 1995). Biologically active proteins successfully produced in this host include human interleukin-6 (15 mg/l) (Broekhuijsen *et al.*, 1993), human interferon α2 (1 mg/l) (Gwynne *et al.*, 1987), human lactoferrin (2 g/l) (Ward *et al.*, 1995), antibody fragments (200 mg/l) (Frenken *et al.*, 1998), human tissue plasminogen activator (1 mg/l) (Upshall *et al.*, 1987) and human mucus proteinase inhibitor (3 mg/l) (Mikosch *et al.*, 1996). Various heterologous expression experiments consistently show that, in general, the

yields of most heterologous proteins are low compared to homologous proteins and in most cases do not exceed levels of a few milligrams per litre. Some of the factors affecting heterologous gene expression include the site of vector integration and copy number (Verdoes *et al.*, 1994), transcriptional control (i.e. the promoter used) (Hanegraaf *et al.*, 1991; Verdoes *et al.*, 1993), codon bias (Ballance, 1986; Gouka *et al.*, 1996), secretion efficiency (Punt *et al.*, 1994; Van den Hondel *et al.*, 1992), molecular chaperone availability (Punt *et al.*, 1998), glycosylation processes (Sorensen *et al.*, 1996; Wang *et al.*, 1996) and the presence of proteases (Archer and Peberdy, 1997; Van den Hombergh *et al.*, 1997). Proteolytic degradation has been shown to be one of the major factors influencing the yield of these heterologous proteins, since *Aspergillus* strains produce a number of aspartic proteases, alkaline and semi-alkaline serine proteases and serine carboxypeptidases (Van den Hombergh *et al.*, 1997). Some of the problems experienced with the heterologous expression of non-fungal proteins have been addressed with a variety of strategies. Some of these strategies include codon optimisation (Gouka *et al.*, 1996), the generation of fusion constructs to improve secretion (Gouka *et al.*, 1997b; Jeenes *et al.*, 1993; Spencer *et al.*, 1998), overexpression of chaperone proteins (Ngiam *et al.*, 2000; Punt *et al.*, 1998) and the isolation of protease deficient mutants (Archer and Peberdy, 1997). While some of the problems encountered with the expression of pharmaceutical proteins have been addressed, for each new protein the optimal expression strategy needs to be evaluated.

This paper addresses some of the challenges encountered in the heterologous production of the hepatitis B virus *S* and *preS1preS2S* genes in *A. niger* and *A. awamori.*

4.3. Materials and Methods

4.3.1. Strains and media

Recombinant plasmids were constructed and amplified in *Escherichia coli* XL1Blue (Stratagene) cultivated at 37°C in Luria-Bertani liquid medium or on Luria-Bertani agar (Sambrook *et al.*, 1989). Ampicillin for selection and propagation of resistant bacteria was added to a final concentration of

100 µg/ml. *A. niger* D15, the recipient strain for transformation, is a uridine auxotrophic (*pyrG*), protease-deficient (*prtT*), nonacidifying (*phmA*) mutant (Gordon *et al.*, 2000). The *A. awamori* T3 strain is a uridine auxotroph (*pyrG*) derived from *A. awamori* CBS115.52 (Gouka *et al.*, 1995). *Aspergillus* strains were cultured in minimal growth medium (Bennett and Lasure, 1991) containing 1% glucose and supplemented with 0.2% casamino acids (Difco), 0.5% yeast extract and 10 mM uridine (Sigma) in 1-liter Erlenmeyer flasks containing 250 ml medium at 30°C on a rotary shaker at 150 rpm. Transformants were selected on minimal medium without uridine containing 1.2 M sorbitol as osmotic stabiliser, 1.5% agar (Oxoid) and 10 mM acetamide as sole nitrogen source. Transformants were selected for multicopy integration of the expression cassettes on acrylamide plates (Verdoes *et al.*, 1993). Minimal medium without uridine, supplemented with 0.5% yeast extract, 0.1% casamino acids and 1% glucose, was used to culture transformants. For induction of the glucoamylase promoter, minimal medium containing 5% maltodextrin was used.

4.3.2. Plasmid construction and transformation of *A. niger* and *A. awamori*

Standard protocols were followed for DNA manipulations (Sambrook *et al.*, 1989). Restriction endonuclease-digested DNA was eluted from agarose gels by the method of Tautz and Renz (1983). Restriction endonucleases and T4 DNA ligase were purchased from Roche Molecular Biochemicals and used as recommended by the manufacturer. Vectors pCOR105 and pCOR103 (donated by Prof. W.J. de Wet, Potchefstroom University, South Africa) served as template for the PCR amplification of the hepatitis B virus *S* and *preS1preS2S* genes, respectively. Primers were designed to include pentaHis tag sequences and the dibasic "Lys-Arg" proteolytic cleavage site where required. The sequences of the primers designed for the amplification of the 814-bp *S* gene and the 1433-bp *preS1preS2S* gene are summarised in Table 1.

Table 1. Primers used in this study.

Primer	Sequence
S-Nco-LEFT	5'-CTAGCCATGGAGAACATCACATCAGGATTCC-3'
S-Kex2-LEFT	5'-ATGTGATATCC**AAGCGC**ATGGAGAACATCACATCAGG-3'
S-Blg2-RIGHT	5'-GATCAGATCTCCAACAGGAAGTTTTCTAAAAC-3'
S-Hind-RIGHT	5'-GATCAAGCTTGGGCTGCAGGTCGACT-3'
S-Bgl2-His-RIGHT	5'-GATCAGATCTTAATGGTGATGGTGATGAATGTATTCCCAGAGACAAAAG-3'
S-Hind-His-RIGHT	5'-GATCAAGCTTAATGGTGATGGTGATGAATGTATTACCCAGAGACAAAAG-3'
S1S2S-Nco-LEFT	5'-CTAGCCATGGGAGGTTGGTCATCAAAACC-3'
S1S2S-Kex2-LEFT	5'-ATGTGATATCC**AAGCGC**ATGGGAGGTTGGTCATCA-3'

Restriction enzyme sites are underlined and the proteolytic cleavage site is highlighted.

Construction of the expression vectors was carried out as follows: three *Aspergillus* vectors were used as basis for all the constructions, namely pAN52-1, pAN52-10 and pAN56-8 (kindly provided by Prof. C.A.M.J.J. van den Hondel, TNO, Zeist, The Netherlands). Vector pAN52-1 carries the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter, pAN52-10 carries the inducible *A. niger* glucoamylase (*glaA*) promoter and pAN56-8 contains the *A. niger glaA* promoter and secretion signal fused to a sequence encoding truncated *A. niger* glucoamylase (GLA G2, the catalytic domain) (Figure 1). All the vectors contain the *trpC* terminator. The *S* and *preS1preS2S* genes were PCR-amplified using the appropriate primers with or without the pentaHis tag, digested with *Nco*I and *Bgl*II and cloned into the unique *Nco*I, *Bam*H1 sites in pAN52-1. Similarly, the viral genes were amplified, digested with *Nco*I and *Hin*dIII and cloned into the corresponding unique sites in pAN52-10. To fuse the *S* and *preS1preS2S* genes to the truncated *A. niger* GLA G2 form (514 aa) lacking the starch-binding domain (Broekhuijsen *et al.*, 1993), the viral genes were amplified using primer S-Kex2-LEFT and S1S2S-Kex2-LEFT, respectively, in combination with primer S-Hind-RIGHT and cloned into the unique *Eco*RV and *Hin*dIII sites in plasmid pAN56-8. The GLA G2 and hepatitis B sequences were thus separated by the dibasic proteolytic cleavage site "Lys-Arg", which results in cleavage of the fusion protein into both separate proteins by a Kex2-like protease (Broekhuijsen *et al.*, 1993). Finally, the *A. nidulans amdS* and *A. niger pyrG* selection markers (provided by Prof. C.A.M.J.J. van den Hondel, TNO, Zeist, The Netherlands)

were introduced into all the expression vectors at the unique *Not*I site. The resulting vectors are summarised in Table 2. A schematic representation of the cloning procedure and the various plasmid constructs is given in Figure 1. The *A. nidulans amdS* and *A. niger pyrG* selection markers were also introduced into pBluescript (Stratagene) at the unique *Not*I site, creating pBluescript-pyrGamdS. Fungal transformation of *A. niger* and *A. awamori* was carried out by the protoplasting method of Punt and Van den Hondel (1992), using Novozym 234 (Sigma).

4.3.3. Isolation of RNA and Northern analysis

Total RNA was isolated from *A. niger* and *A. awamori* transformants cultured for 40 h at 30°C as follows: Mycelium was harvested through Miracloth (Calbiochem), frozen under liquid nitrogen, ground to a fine powder with a mortar and pestle and resuspended in cold STE buffer (0.25 M Tris-HCl [pH7.2], 0.1 M NaCl, 0.01 M EDTA [pH8]). One volume phenol/chloroform/ isoamylalcohol (PCI) and 0.05% sodium dodecyl-sulphate (SDS) was added and the mycelial debris was collected by centrifugation. The aqueous upper phase was recovered, subjected to PCI extraction and precipitated to yield total RNA. The total RNA was used for conventional Northern blot analysis as described by Sambrook *et al.* (1989). The probes were prepared by labelling the 814-bp hepatitis B virus *S* gene and a 1350-bp fragment of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene with [α-^{32}P]dATP, using a random primed DNA labelling kit (Roche Molecular Biochemicals) as recommended by the manufacturer.

4.3.4. Isolation of genomic DNA from *A. niger* and Southern blotting

The genomic DNA was isolated from *Aspergillus* transformants according to La Grange *et al.* (1996), digested overnight with *Eco*RI at 37°C, separated on a 0.6% agarose gel and blotted to a Hybond-N membrane (Amersham). Southern blot analysis was carried out according to Sambrook *et al.* (1989) using the radioactively labelled 814-bp *S* gene as probe.

Table 2. Microbial strains and plasmids.

Strain/Plasmid	Genotype	Source/Reference
Bacterial strain:		
Escherichia coli XL1-Blue	*recA1 endA1 gyrA96 thi-1hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r)]^c	Stratagene
Fungal strains:		
Aspergillus niger D15	*pyrG prtT phmA*	Gordon *et al.*, 2000
A. niger D15 (pBluescript-pyrGamdS)[a]	*pyrG*$^+$ *prtT phmA amdS*$^+$	This work
DAHB11	*pyrG*$^+$ *prtT phmA amdS*$^+$ *gpdA*$_P$*-S-trpC*$_T$	This work
DAHB14	*pyrG*$^+$ *prtT phmA amdS*$^+$ *gpdA*$_P$*-preS1preS2S-His-trpC*$_T$	This work
DAHB15	*pyrG*$^+$ *prtT phmA amdS*$^+$ *glaA*$_P$*-S-trpC*$_T$	This work
DAHB17	*pyrG*$^+$ *prtT phmA amdS*$^+$ *glaA*$_P$*-S-His-trpC*$_T$	This work
DAHB18	*pyrG*$^+$ *prtT phmA amdS*$^+$ *glaA*$_P$*-preS1preS2S-His-trpC*$_T$	This work
DAHB19	*pyrG*$^+$ *prtT phmA amdS*$^+$ *glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-S-trpC*$_T$	This work
DAHB20	*pyrG*$^+$ *prtT phmA amdS*$^+$ *glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-preS1preS2S-trpC*$_T$	This work
Aspergillus awamori T3	*pyrG*	Gouka *et al.*, 1995
AWAHB11	*pyrG*$^+$ *amdS*$^+$ *gpdA*$_P$*-S-trpC*$_T$	This work
AWAHB15	*pyrG*$^+$ *amdS*$^+$ *glaA*$_P$*-S-trpC*$_T$	This work
AWAHB17	*pyrG*$^+$ *amdS*$^+$ *glaA*$_P$*-S-His-trpC*$_T$	This work
AWAHB20	*pyrG*$^+$ *amdS*$^+$ *glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-preS1preS2S-trpC*$_T$	This work
Plasmids:		
pBluescript-pyrGamdS	*bla pyrG amdS*	This work
pCOR103	*bla preS1preS2S*	W.J. de Wet
pCOR105	*bla S*	W.J. de Wet
pAN52-1	*bla gpdA*$_P$*-trpC*$_T$	Punt *et al.*, 1991
pAN52-10	*bla glaA*$_P$*-trpC*$_T$	C.A.M.J.J. van den Hondel
pAN56-8	*bla glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-trpC*$_T$	C.A.M.J.J. van den Hondel
pAHB11	*bla pyrG amdS gpdA*$_P$*-S-trpC*$_T$	This work
pAHB12	*bla pyrG amdS gpdA*$_P$*-preS1preS2S-trpC*$_T$	This work
pAHB13	*bla pyrG amdS gpdA*$_P$*-S-His-trpC*$_T$	This work
pAHB14	*bla pyrG amdS gpdA*$_P$*-preS1preS2S-His-trpC*$_T$	This work
pAHB15	*bla pyrG amdS glaA*$_P$*-S-trpC*$_T$	This work
pAHB16	*bla pyrG amdS glaA*$_P$*-preS1preS2S-trpC*$_T$	This work
pAHB17	*bla pyrG amdS glaA*$_P$*-S-His-trpC*$_T$	This work
pAHB18	*bla pyrG amdS glaA*$_P$*-preS1preS2S-His-trpC*$_T$	This work
pAHB19	*bla pyrG amdS glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-S-trpC*$_T$	This work
pAHB20	*bla pyrG amdS glaA*$_P$*-glaA*$_{SS}$*-glaA*$_{G2}$*-preS1preS2S-trpC*$_T$	This work

[a] *A. niger* D15 (pBluescript-pyrGamdS) was designated *A. niger* (control)

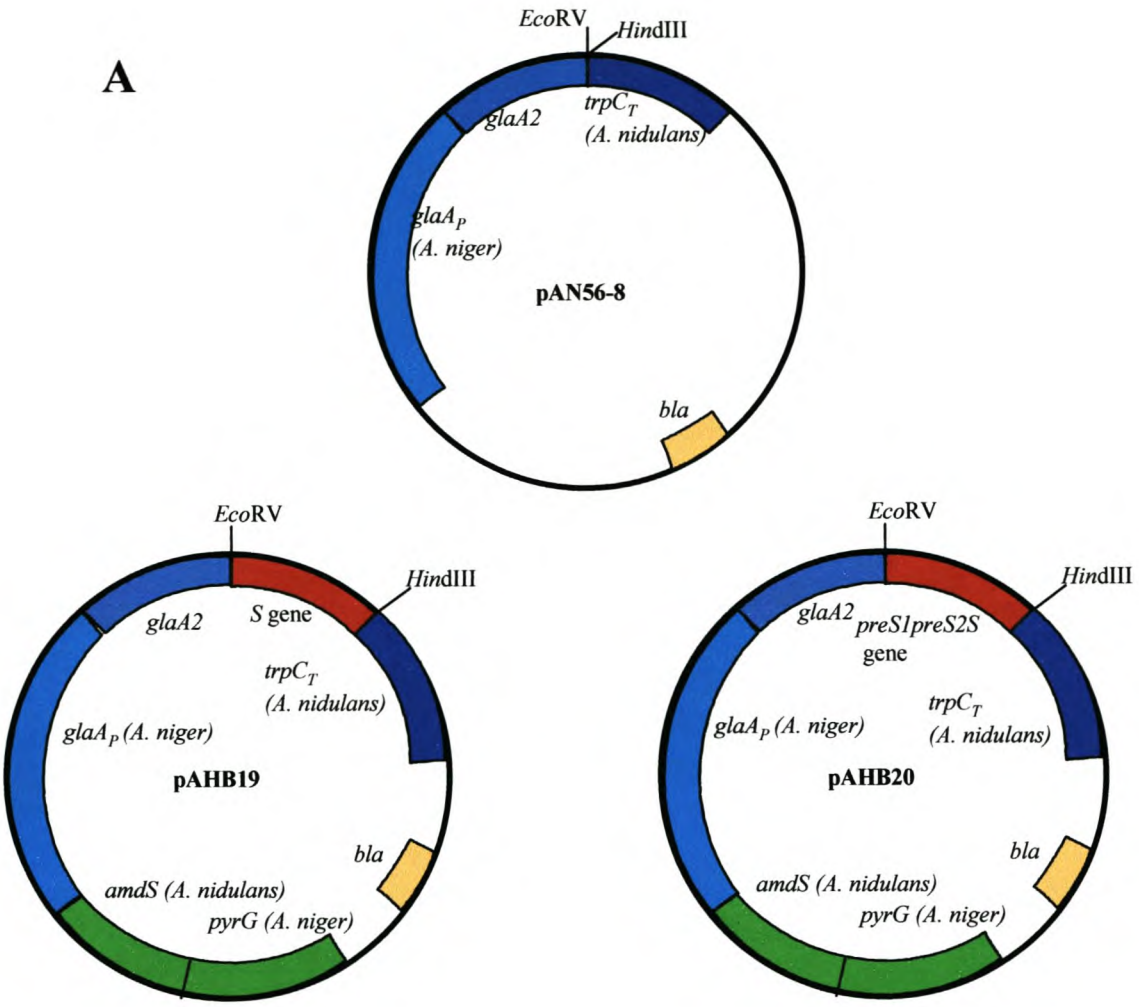

Figure 1. Schematic representation of the *Aspergillus* expression vectors constructed in this study. pAN56-8 **(A)**, pAN52-1 **(B)** and pAN52-10 **(C)** are the initial *Aspergillus* expression vectors into which the viral genes and fungal marker genes were cloned. The viral gene expression vectors constructed are grouped below the respective *Aspergillus* expression vector from which they were derived. The *E. coli* β-lactamase (*bla*), *A. nidulans* acetamidase (*amdS*) and *A. niger* orotidine-5'-decarboxylase (*pyrG*) genes were used as selectable markers. $gpdA_p$, $glaA_p$, *glaA2* and $trpC_t$ represent the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter, the *A. niger* glucoamylase gene promoter, the catalytic domain of the *A. niger* glucoamylase and the *trpC* (a trifunctional gene in tryptophan biosynthesis) gene terminator, respectively. The hepatitis B virus *S* and *preS1preS2S* genes are indicated in red.

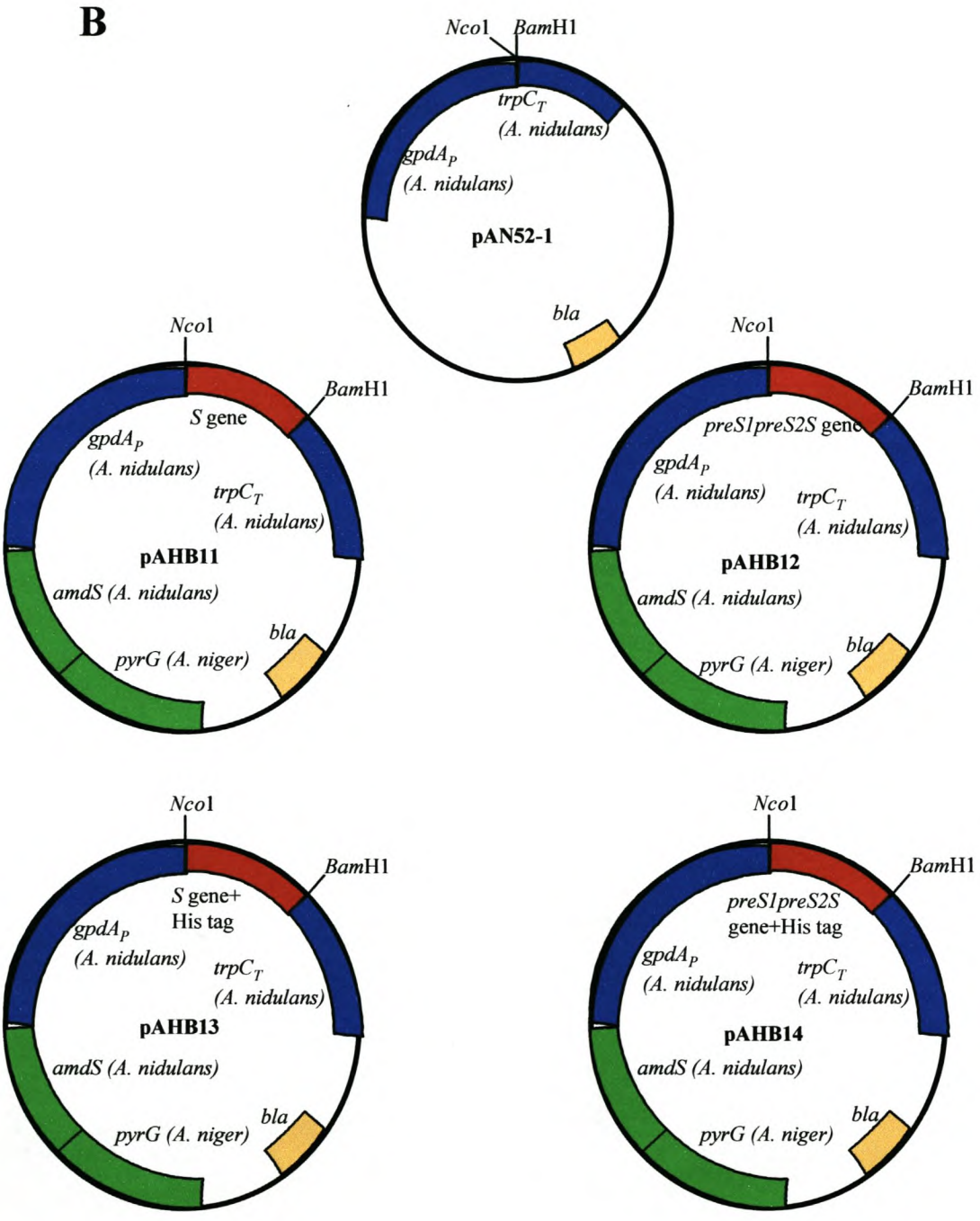

Figure 1. Schematic representation of the *Aspergillus* expression vectors constructed in this study (continued).

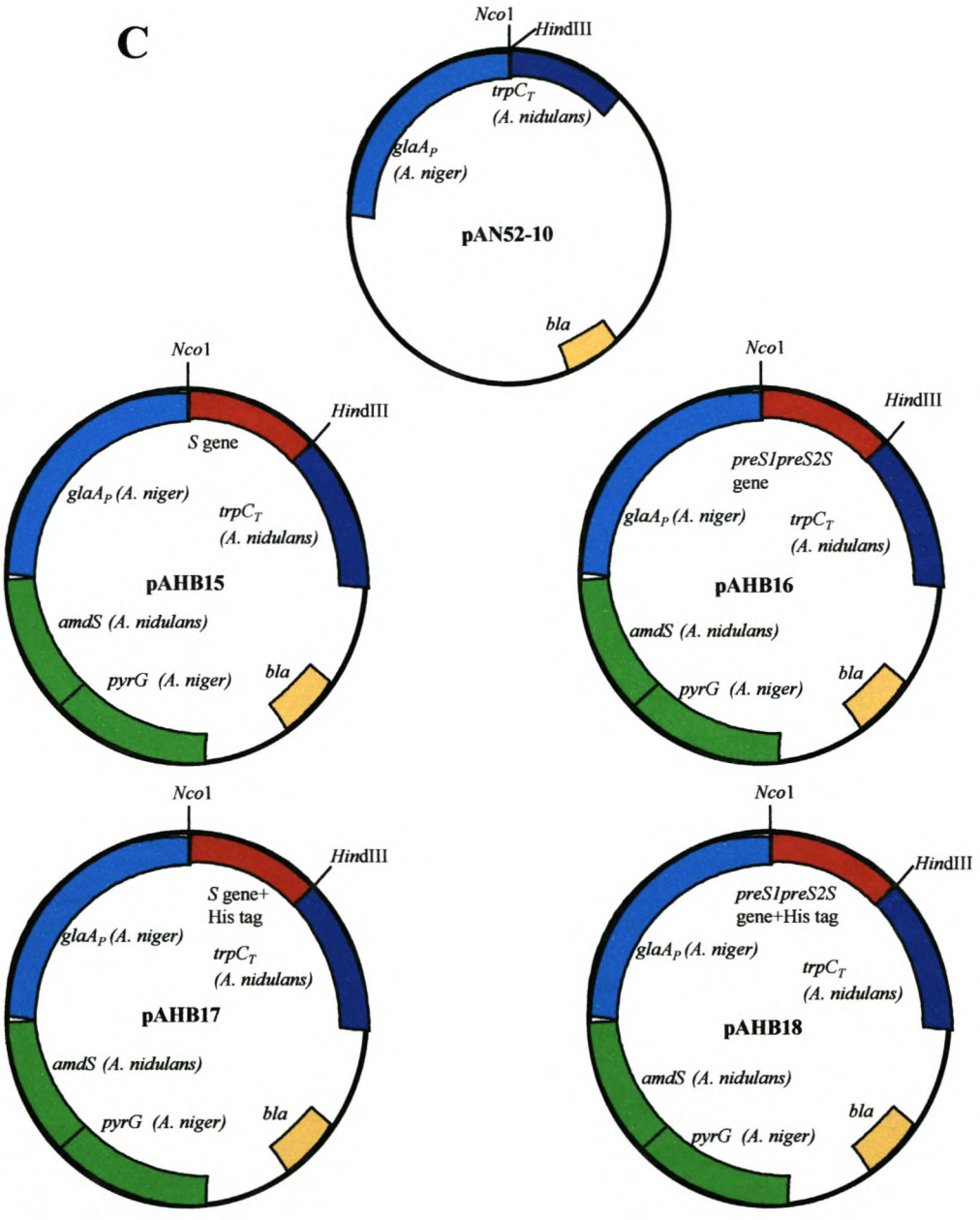

Figure 1. Schematic representation of the *Aspergillus* expression vectors constructed in this study (continued).

4.3.5. SDS-PAGE and Western blotting

Total intracellular proteins were isolated from the transformants cultured for 72 h in minimal medium containing 1% glucose or 5% maltodextrin as before. Extracellular proteins were recovered from the growth medium after filtering of the mycelium by filtration and concentration of the medium by a Diaflo Ultrafilter PM10 concentrator (Amicon Division of W.R. Grace and Co., Danvers, Mass.). Intracellular protein extraction was performed as follows: Mycelium was harvested and ground as before. Ground mycelium was suspended in protein extraction buffer (50 mM phosphate buffer [pH7.0], 1 mM $MgCl_2$, 20 µM phenylmethylsulfonyl flouride) with or without 0.1% Triton X-100, and the mycelial debris was removed by centrifugation. *S. cerevisiae*-derived Engerix-B hepatitis B virus vaccine (20 µg/ml) (SmithKline Beecham Pharmaceuticals) was purchased from a local pharmacy to act as reference protein. Proteins were separated in a 12% SDS polyacrylamide gelelectrophoresis (PAGE) gel and visualised by staining with silver nitrate (Blum *et al.*, 1987). For Western blotting, proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris-HCl [pH 7.5], 192 mM glycine, 15% [vol/vol] methanol) using a semi-dry transfer apparatus (Hoefer). The Chemiluminescence Western Blotting Kit (Roche Molecular Biochemicals) was used as recommended by the manufacturer. The primary antibodies were polyclonal goat anti-hepatitis B surface antigen (1:5000 dilution) (Dako) and secondary antibodies were anti-goat immunoglobulins conjugated to horseradish peroxidase (1:10 000 dilution) (Dako). Primary antibodies raised against glucoamylase were provided by P. Punt (TNO, Zeist, The Netherlands) and used at a dilution of 1:20 000. Anti-mouse antibodies conjugated to alkaline phosphatase served as secondary antibodies and detection was performed with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) (Promega). For Western analysis with penta-His antibodies (Qiagen) the primary antibody was used at a dilution of 1:1000 as recommended by the manufacturer and anti-mouse horseraddish peroxidase conjugated antibodies served as secondary antibodies at a final concentration of 40 mU (Roche Molecular Biochemicals).

4.3.6. Protease susceptibility assay

Samples of the intracellular fraction from DAHB11#8 containing recombinant hepatitis B S protein were incubated in 5% maltodextrin medium in which *A. niger* D15 had been cultivated for 48 h. Incubation times at 37°C were 1 h, 2 h, 3 h and overnight. Samples from the various time points were run on a 12% SDS-PAGE gel and subjected to Western analysis as described previously using the goat anti-hepatitis B surface antigen primary antibodies.

4.4. Results

4.4.1. Recombinant *Aspergillus* strains expressing the hepatitis B genes.

All ten different expression vectors (pAHB11 to pAHB20) were transformed to *A. niger* D15 and *A. awamori* T3. However, not all transformation experiments yielded transformants and a summary of the number of uridine prototrophic, acetamide utilising transformants obtained for each expression vector in both *Aspergillus* strains is given in Table 3. The vector pBluescript-pyrGamdS was also transformed to *A. niger* D15 and this strain served as a prototrophic reference strain. More transformants were obtained for the hepatitis B *S* gene, encoding the major (S) protein of the envelope of the hepatitis B virus (subtype adw2), than for the *preS1preS2S* gene, encoding the large (L) protein. In total, 33 *A. niger* D15 transformants were obtained carrying the *S* gene under the transcriptional control of either the constitutive *gpdA* promoter or the inducible *glaA* promoter, and 6 transformants were obtained carrying the *S* gene fused to the 3' end encoding region of the catalytic domain of the highly expressed *A. niger* glucoamylase. In contrast, only nine transformants were obtained carrying the *peS1preS2S* gene, of which only one was under the control of the *gpdA* promoter. Six transformants were also obtained for the *glaA-preS2preS2S* fusion construct. The *A. awamori* T3 transformation frequency was much lower and only 18 transformants carrying hepatitis B viral genes were obtained for this strain. All the transformants were purified twice on selective medium and subsequently plated on acrylamide plates. Efficient growth and sporulation on acrylamide plates reflects multicopy integration of the transforming vector (Verdoes *et al.*, 1993).

Table 3. Number of transformants obtained.

Plasmid	Plasmid Components	Strain Transformed	Number of Transformants Obtained	Transformants Analysed Further	
				Number	Designation
pAHB11	*bla pyrG amdS gpdA$_P$-S-trpC$_T$*	*A. niger* D15	10	3	DAHB11#2 DAHB11#3 DAHB11#8
		A. awamori T3	6	3	AWAHB11#1 AWAHB11#4 AWAHB11#5
pAHB12	*bla pyrG amdS gpdA$_P$-preS1preS2S-trpC$_T$*	*A. niger* D15	0	0	-
		A. awamori T3	0	0	-
pAHB13	*bla pyrG amdS gpdA$_P$-S-His-trpC$_T$*	*A. niger* D15	0	0	-
		A. awamori T3	0	0	-
pAHB14	*bla pyrG amdS gpdA$_P$-preS1preS2S-His-trpC$_T$*	*A. niger* D15	1	1	DAHB14#1
		A. awamori T3	0	0	-
pAHB15	*bla pyrG amdS glaA$_P$-S-trpC$_T$*	*A. niger* D15	10	4	DAHB15#1 DAHB15#3 DAHB15#4 DAHB15#8
		A. awamori T3	5	3	AWAHB15#1 AWAHB15#3 AWAHB15#4
pAHB16	*bla pyrG amdS glaA$_P$-preS1preS2S-trpC$_T$*	*A. niger* D15	0	0	-
		A. awamori T3	0	0	-
pAHB17	*bla pyrG amdS glaA$_P$-S-His-trpC$_T$*	*A. niger* D15	13	4	DAHB17#2 DAHB17#6 DAHB17#7 DAHB17#13
		A. awamori T3	4	0	-
pAHB18	*bla pyrG amdS glaA$_P$-preS1preS2S-His-trpC$_T$*	*A. niger* D15	8	5	DAHB18#1 DAHB18#6 DAHB18#7 DAHB18#8 DAHB18#9
		A. awamori T3	0	0	-
pAHB19	*bla pyrG amdS glaA$_P$-glaA$_{SS}$-glaA$_{G2}$-S-trpC$_T$*	*A. niger* D15	6	2	DAHB19#1 DAHB19#2
		A. awamori T3	0	0	-
pAHB20	*bla pyrG amdS glaA$_P$-glaA$_{SS}$-glaA$_{G2}$-preS1preS2S-trpC$_T$*	*A. niger* D15	6	4	DAHB20#1 DAHB20#2 DAHB20#5 DAHB20#6
		A. awamori T3	3	0	-

4.4.2. Gene copy number and expression

With regard to *A. niger* D15, three isolates transformed with pAHB11, 1 isolate transformed with pAHB14, 4 isolates transformed with pAHB15, 4 isolates transformed with pAHB17, 5 isolates transformed with pAHB18, 2 isolates transformed with pAHB19 and 4 isolates transformed with pAHB20 that showed growth and sporulation on acrylamide plates were selected (Refer to Table 3).

These transformants were cultured in minimal medium containing glucose (in the case of the *gpdA* promoter) or maltodextrin (in the case of the *glaA* promoter) for 40 h and analysed for *S* and *preS1preS2S* gene transcription by Northern blotting (Figure 2A,B). One *A. niger* D15 transformant containing pBluescript-pyrGamdS was also cultured to act as reference strain. With regard to *A. awamori* T3, three isolates transformed with pAHB11 (designated AWAHB11#1, AWAHB11#4 and AWAHB11#5) and three isolates transformed with pAHB15 (designated AWAHB15#1, AWAHB15#3 and AWAHB15#4) were selected and cultured in the same manner and analysed for *S* gene transcription by Northern blotting (Figure 2B). The untransformed parental strain acted as reference strain in this case.

Transcription levels varied significantly between the different transformants, with some showing no *S* gene transcripts. Some of the transformants carrying the *preS1preS2S* gene showed an 814-bp transcript similar to that of the transformants carrying the *S* gene, but no full-length 1433-bp *preS1preS2S* transcripts could be detected. Both transformants (DAHB19#1 and DAHB19#2) carrying the truncated glucoamylase-S protein fusion showed transcripts of the expected size for the fusion protein (2814 bp). In strain DAHB19#1 a second transcript of 814 bp was also detected. No hepatitis B viral gene transcript could be detected in any of the transformants carrying the truncated glucoamylase-preS1preS2S protein fusion (DAHB20) (data not shown). Transformant DAHB11#8 (Figure 2A, lane 3) showed the highest levels of *S* gene mRNA transcripts and was selected for further analysis, which is detailed in Paper 1 (this thesis).

To determine the copy number of the integrated plasmid in some of the *A. niger* D15 transformants genomic DNA was isolated, digested with *Eco*RI (which has only one recognition site in the expression vectors) and subjected to Southern analysis using the [α-^{32}P]dATP-labelled *S* gene as probe (Figure 3). Results indicated that most of the transformants contained single copies of the expression cassette integrated into the *A. niger* genome, but DAHB11#8, DAHB15#1, DAHB15#3 and DAHB15#4 contained multiple copies.

Subsequent Southern analysis of DAHB11#8 revealed the integration of approximately seven copies (refer to Paper 1, this thesis).

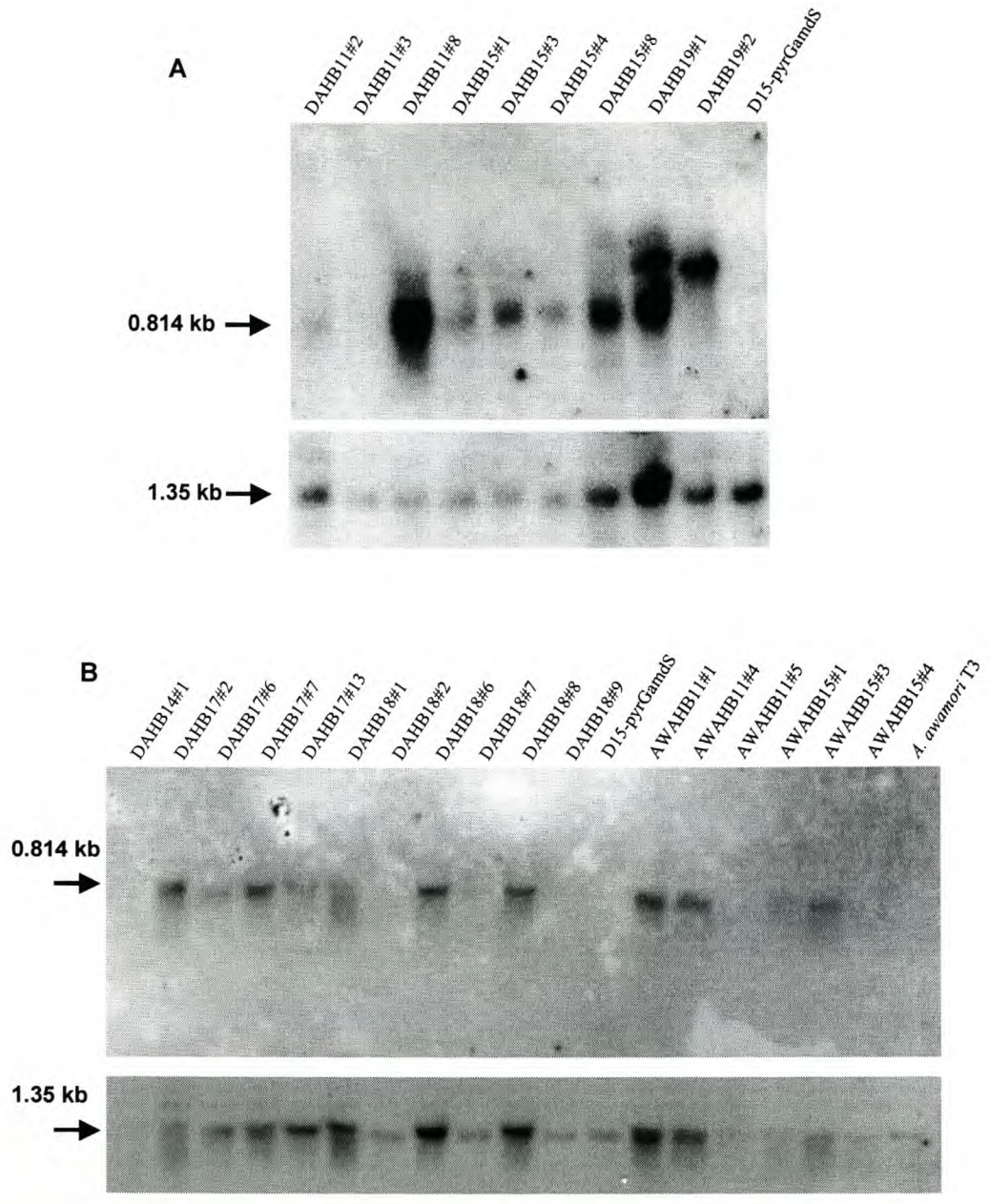

Figure 2. **(A)** Northern blot analysis of *S* gene mRNA transcripts from isolates of *A. niger* D15 transformed with plasmids pAHB11, pAHB15 and pAHB19. **(B)** Northern blot analysis of *S* and *preS1preS2S* gene transcripts from isolates of *A. niger* D15 transformed with plasmids pAHB14, pAHB17 and pAHB18 and *A. awamori* T3 transformed with plasmids pAHB11 and pAHB15. Refer to Table 2 for the genotypes of the various strains. The 814-bp *S* gene was used as an [α-^{32}P-dATP]-labelled probe in (A) and (B) and a 1350-bp fragment of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene was used as internal standard (bottom panel of A and B).

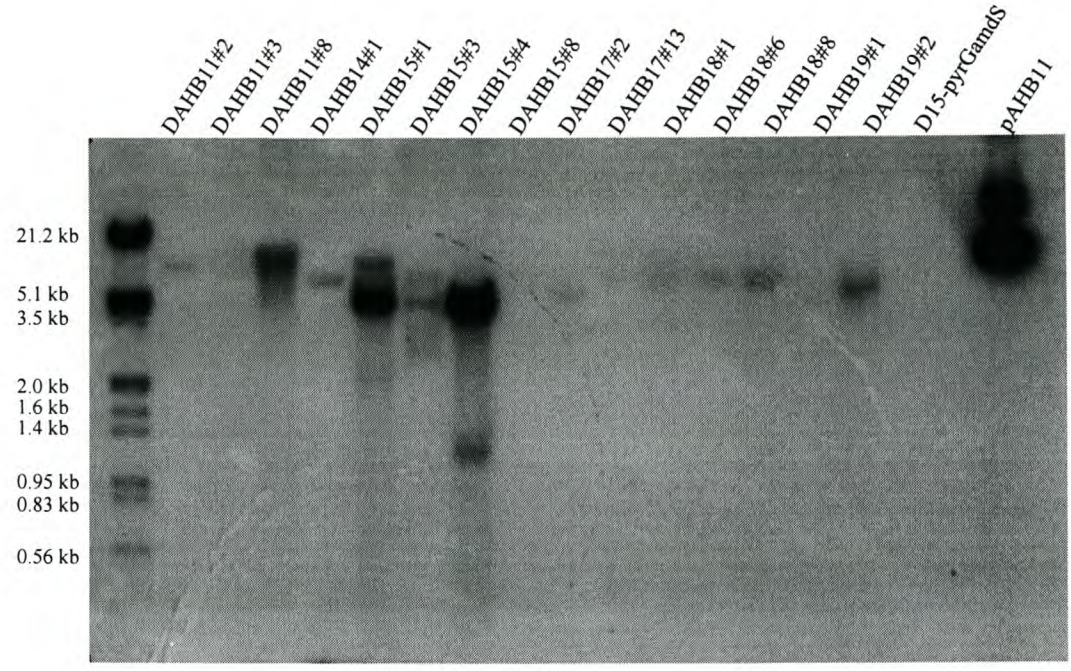

Figure 3. Southern blot analysis to determine the number of *S* gene copies integrated into the recombinant *A. niger* D15 genome. Refer to Table 2 for the genotypes of the various strains. DNA isolated from the transformants was digested with *Eco*RI. Plasmid pAHB11 served as reference DNA. The 814-bp *S* gene was used as an [α-^{32}P-dATP]-labeled probe. The sizes of the lambda marker DNA fragments are indicated.

4.4.3. Protein production analysis

Transformants carrying the viral gene under control of the *gpdA* promoter were cultured in glucose-containing minimal medium and transformants carrying the viral gene under control of the *glaA* promoter were cultured in maltodextrin medium for induction of the glucoamylase promoter. Total intracellular proteins were isolated, separated on 12% SDS-PAGE gels and Western analysis was carried out with HBsAg-specific antibodies. In the intracellular protein fraction of DAHB11#8 a 24-kDa protein species was highlighted, which did not appear in the reference strain (Figure 4). This protein also appeared in the Engerix vaccine HBsAg protein, confirming that the 24 kDa protein corresponds to the S protein. However, none of the other transformants showed any production of recombinant hepatitis B S or L protein in the intracellular fraction (Figure 4 and additional data not shown). Additional Western analysis of samples from the constructs carrying a penta-His tag (DAHB14, DAHB17 and DAHB18) with anti-

penta-His antibodies also did not reveal the presence of recombinant S or L protein (data not shown).

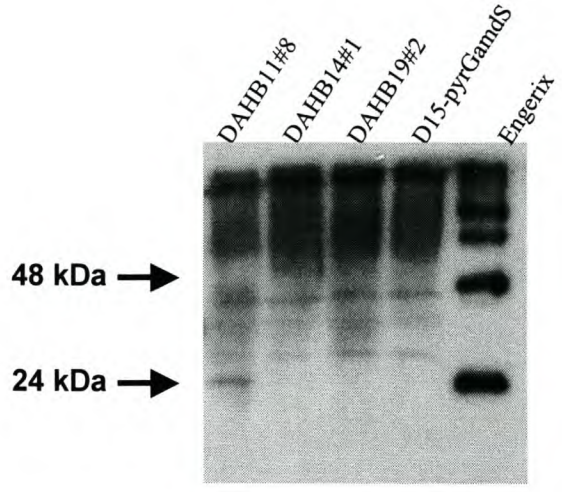

Figure 4. Detection of intracellular viral protein production by Western blot analysis. Engerix-B is a sample of commercially available hepatitis B virus vaccine which acted as reference protein. Immunoblotting was done with a polyclonal goat-α-HBsAg antibody. A protein species of 24 kDa is visible in transformant DAHB11#8 corresponding to the size of the hepatitis B S protein.

Transformant DAHB19#2 carrying the truncated glucoamylase-S protein fusion was further analysed for extracellular S protein production. Extracellular fractions of DAHB19#2 were obtained by culturing the strain for 65 h and 112 h, filtering the mycelium and subsequently concentrating the growth medium ten times. These samples were then subjected to SDS-PAGE and Western analysis as was performed for intracellular fractions. No extracellular S protein production could be detected (Figure 5). Western analysis of the DAHB19 transformants with antibodies specific for glucoamylase revealed the presence of the two forms (the larger G1 form and the smaller G2 form lacking the starch binding domain) (Boel *et al.*, 1984a; Boel *et al.*, 1984b) of the enzyme in the medium, indicating that the promoter was induced (Figure 6A). In the intracellular fraction, several breakdown products that did not appear in the reference strain, were observed (Figure 6B). This result may indicate that in the transformants carrying the truncated glucoamylase-S protein fusion, passage through the secretory pathway may be impaired, resulting in the degradation of

glucoamylase and/or the fusion protein. A protease susceptibility assay was also performed with recombinant hepatitis B virus S protein from transformant DAHB11#8 and this revealed that the S protein levels diminished somewhat after one hour, and that the protein was completely degraded after overnight incubation in the medium in which *A. niger* D15 had been previously cultured (Figure 7).

With regard to the *A. awamori* T3 transformants, even though some of the transformants showed *S* gene transcription, none of them produced any detectable recombinant S protein (Figure 8).

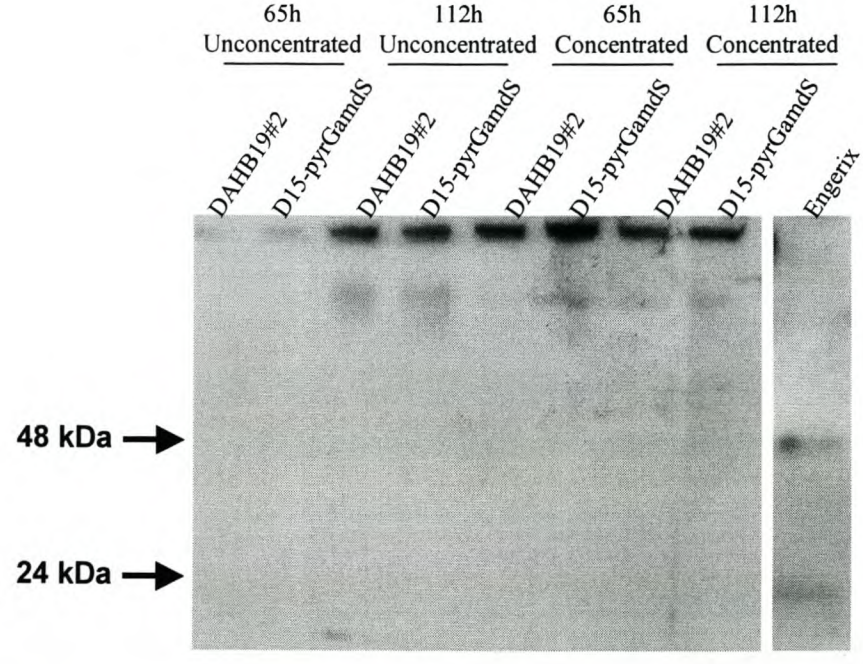

Figure 5. Western blot analysis of concentrated and unconcentrated extracellular fractions of transformant DAHB19#2. Samples were taken from the transformant and the prototrophic reference strain (D15-pyrGamdS) after 65 h and 112 h and part of the sample was concentrated ten times. Engerix vaccine served as HBsAg reference protein. Immunoblotting was done with a polyclonal goat-α-HBsAg antibody.

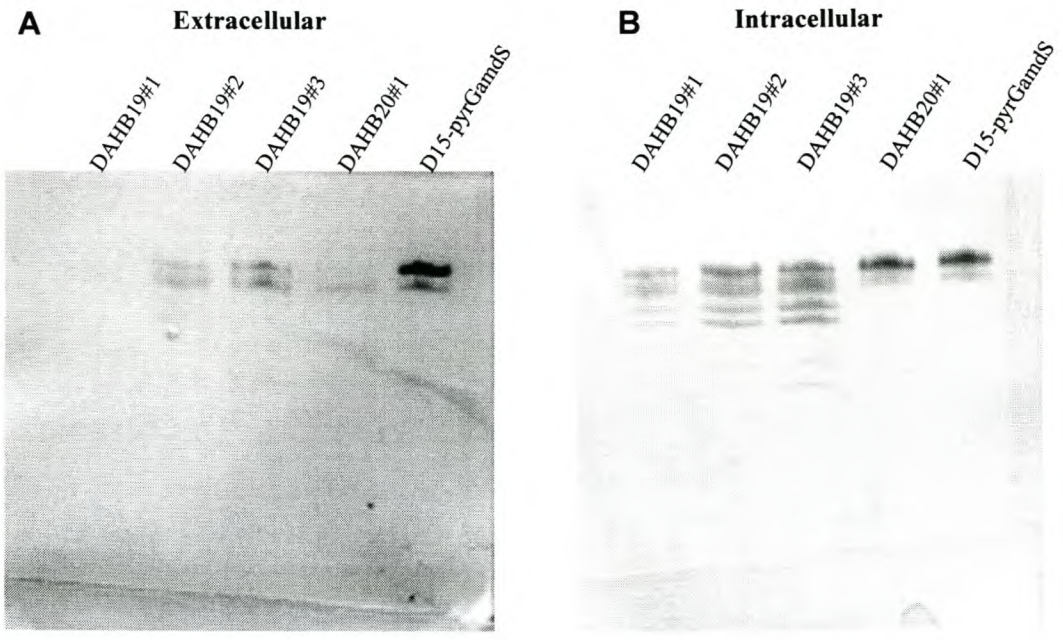

Figure 6. Western blot analysis of extracellular **(A)** and intracellular **(B)** protein fractions of three DAHB19 transformants and one DAHB20 transformant. Immunoblotting was done with a mouse-α-glucoamylase antibody.

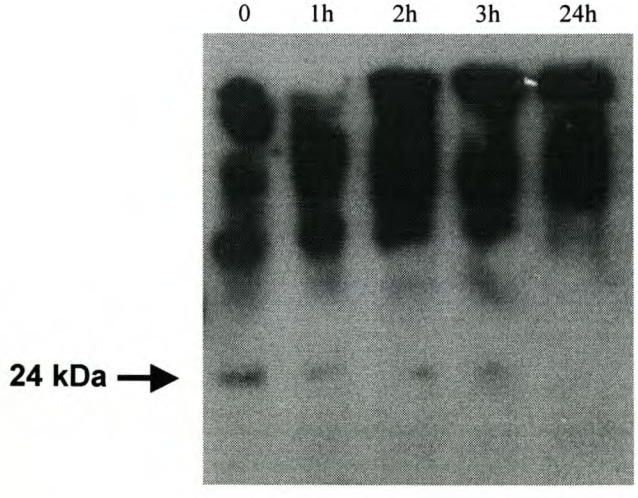

Figure 7. Protease susceptibility assay. Protein samples from the recombinant hepatitis B S protein-producing *A. niger* D15 (DAHB11#8) were incubated for 1 h, 2 h, 3 h and 24 h in medium samples in which *A. niger* D15 had been previously cultured. Subsequently the samples were subjected to Western analysis using a polyclonal goat-α-HBsAg antibody.

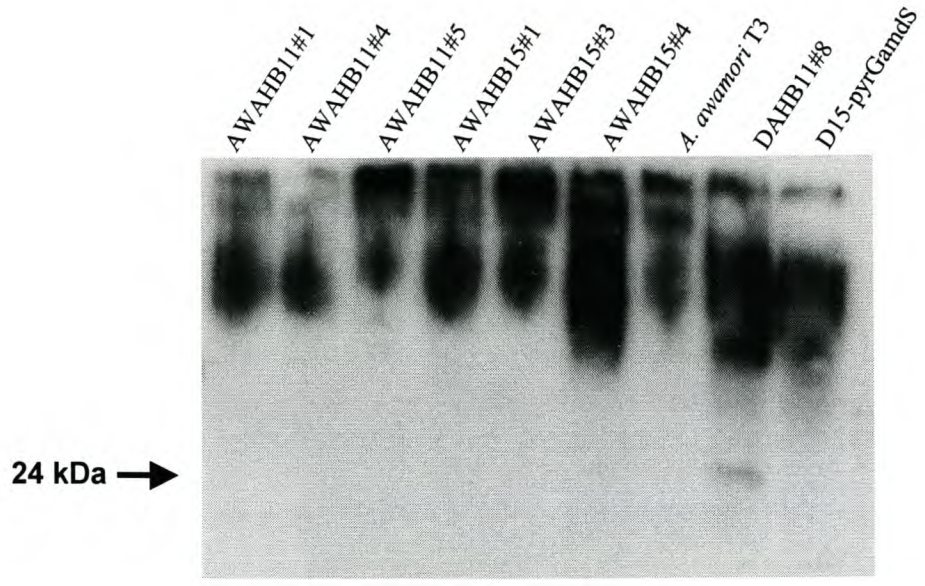

Figure 8. Western blot analysis of total intracellular proteins from *A. awamori* transformants. Intracellular proteins from the S protein-producing strain DAHB11#8 served as positive control. Immunoblotting was done with a polyclonal goat-α-HBsAg antibody.

4.5. Discussion

In recent years, *A. niger* and *A. awamori* have been extensively studied for their ability to produce several proteins of pharmaceutical interest, with varying degrees of success. Some success stories include the high level production of biologically active human lactoferrin in *A. awamori* (2 g/l) (Ward *et al.*, 1995), as well as the secretion of active human interleukin-6 by *A. niger* and *A. awamori* (Broekhuijsen *et al.*, 1993; Gouka *et al.*, 1997a). However, many problems have also been encountered in the expression of heterologous proteins by *Aspergillus*. Proteolytic degradation has been shown to be one of the major factors influencing the production of heterologous proteins (Van den Hombergh *et al.*, 1997). *A. niger* produces various extracellular proteases, four of which have acid pH optima, and the major extracellular protease of *A. awamori* is also an acid protease (Berka *et al.*, 1990; Van den Hombergh *et al.*, 1997). Other factors strongly influencing expression levels include site of vector integration,

mRNA stability and secretion efficiency (Gouka *et al.*, 1997a; Verdoes *et al.*, 1994).

In this study, various gene constructs were made to explore the intracellular and extracellular expression of the hepatitis B *S* and *preS1preS2S* genes in *Aspergillus.* The two promoters employed have both been shown to be strong promoters that have previously been successfully used for the overproduction of various heterologous proteins (e.g. Archer *et al.*, 1990; Broekhuijsen *et al.*, 1993; Carrez *et al.*, 1990; Ward *et al.*, 1990). The *A. nidulans gpdA* promoter directs the constitutive production of a key enzyme in glycolysis and gluconeogenesis and expression signals from this promoter have been shown to be very strong (Punt *et al.*, 1990). The *A. niger glaA* promoter is induced to high levels when the fungus is grown on starch, maltose or maltodextrin, but is repressed in the presence of xylose, making this an ideal inducible promoter (Santerre Henriksen *et al.*, 1999). Northern analysis showed that both promoters were effective in directing transcription of the *S* gene, but transcription levels varied significantly between the different transformants (Figure 2A, B). In some transformants no *S* gene transcript could be detected and the highest levels were obtained for transformant DAHB11#8, carrying the *S* gene under transcriptional control of the *gpdA* promoter. The various transformants analysed carrying the *S* gene under control of the *glaA* promoter consistently showed lower transcript levels. Two factors generally determine transcription levels in recombinant aspergilli, namely the copy number of the integrated plasmid and the site of integration (Christensen *et al.*, 1988; Davies, 1991; Ward *et al.*, 1990). The position of integration of a foreign gene in the chromosome can influence expression levels as heterochromatic DNA affects transcriptional activity (Thompson and Gasson, 2001). The complex regulatory mechanisms found in eukaryotes also results in a greater variation in promoter strengths, which could influence a downstream heterologous gene. Transformant DAHB11#8 was previously shown to contain approximately seven integrated vector copies, which probably contributed to the high levels of gene expression (Paper 1, this thesis). However, three transformants carrying the *S* gene under control of the *glaA* promoter (namely DAHB15#1, DAHB15#3 and DAHB15#4) also contained multiple copies of integrated vector, but transcript

levels from these transformants never reached those of DAHB11#8. This could be attributed to the site of integration, particularly since it has been shown that vector copies generally integrate in only one chromosome (Verdoes *et al.*, 1993), but the reason for lower transcript levels was more likely the combination of strain and promoter choice. The activity of the *glaA* promoter is pH dependent and appears to be activated by acidification of the medium (Withers *et al.*, 1998). As the *A. niger* strain employed in this study is a non-acidifying mutant, this could account for the rather low levels of *S* gene transcription obtained from this promoter. This hypothesis is further underscored by the observation that no glucoamylase could be detected in the culture supernatant of *A. niger* D15 on a Coomassie Brilliant Blue stained SDS-PAGE gel (data not shown) and was only detectable at low levels with Western analysis. *A. niger* N402, the parent strain of *A. niger* D15, has been shown to secrete 50 mg/l of glucoamylase, which is easily visible on a Coomassie Brilliant Blue stained SDS-PAGE gel (Verdoes *et al.*, 1993). Thus it is possible that the lack of detectable S protein in the intracellular fractions of the transformants carrying the *S* gene under control of the *glaA* promoter was due to the lower transcript levels resulting from a weakly induced promoter.

Regarding transcription of the *preS1preS2S* gene, none of the transformants carrying any of the constructs with this gene showed transcripts of the expected size of 1433 bp. Some transformants showed low levels of transcript corresponding to the size of the *S* gene. The reasons for this are unclear, but the presence of the smaller transcript may possibly be attributed to splicing of the mRNA transcript. Although hepatitis B viral transcripts in infected cells generally remain unspliced, a few spliced transcripts (generated by the removal of one or two introns) have been described in transfected cells and in the livers of infected individuals (Fallows and Goff, 1996; Günther *et al.*, 1997; Obert *et al.*, 1996). An example of alternative mRNA splicing has been identified in the *phoA* gene of *A. nidulans* which encodes two cyclin-dependent kinases (Bussink and Osmani, 1998). However, this kind of alternative pre-mRNA splicing (splicing or non-splicing of an intron containing one of two alternative in-frame translation initiation codons) has not been well documented in fungi. Whatever the reason for the absence of transcripts, the lack of recombinant L

protein production by *Aspergillus* transformants can be directly attributed to the lack of mature *preS1preS2S* transcripts.

Regarding extracellular protein production, one of the most promising strategies that has been widely employed to facilitate the secretion of heterologous non-fungal proteins involves the construction of gene fusions to genomic sequences encompassing the complete or partial coding region of a highly expressed fungal gene such as glucoamylase (Ward *et al.*, 1990). The object of these fusion constructs is to facilitate the passage of the target protein through the secretory pathway and this strategy has been proven to be effective in many cases (Contreras *et al.*, 1991; Mikosch *et al.*, 1996; Spencer *et al.*, 1998; Tsuchiya *et al.*, 1994; Ward *et al.*, 1990). Therefore an in-frame fusion was made between the *S* gene and the catalytic domain of glucoamylase to explore the possible secretion of hepatitis B S protein by *Aspergillus*. The ubiquitous KEX2 protease cleavage site was introduced between the *S* gene and the glucoamylase domain to facilitate the release of mature S protein (Contreras *et al.*, 1991). However, despite relatively high transcription levels, no extracellular S protein could be detected in the transformants carrying this construct. This lack of secreted heterologous S protein could be attributed to the presence of extracellular proteases, which is further indicated by the protease susceptibility assay which showed that heterologous S protein produced intracellularly by strain DAHB11#8 was completely degraded after overnight incubation in *A. niger* D15 culture medium.

In anticipation of the common problem of proteolytic degradation, strains deficient in extracellular protease activity are commonly employed, and thus in this study the *A. niger* strain was a protease mutant which also does not acidify the medium upon culturing, thereby reducing the induction of the acid proteases (Gordon *et al.*, 2000). However, although acid proteases predominate in *A. niger,* the fungus also produces an alkaline protease (PEPD) which may play a role in the degradation of the heterologous viral protein (Jarai *et al.*, 1994). Furthermore, the total protease activity in *A. niger* D15 was reduced by mutagenesis, not by specific protease gene deletions (Mattern *et al.*, 1992). Therefore, although protease activity in this strain is strongly reduced, it is not

absent. Aberrant processing through the secretory pathway may also contribute to the lack of secreted S protein. This was indicated by the presence of various breakdown products in the intracellular fraction of DAHB19 transformants when they were analysed with antibodies specific for the glucoamylase moiety. In the viral life cycle, the hepatitis B S protein is inserted into the ER membrane and subsequently translocated to the ER lumen where disulphide-linked dimers are rapidly formed (Huovila *et al.*, 1992). These dimers are transported to a post-ER/pre-Golgi compartment where the formation of oligomer crosslinks occurs, resulting in the formation of pseudoviral particles, which are then exported from the cell via vesicular transport (Huovila *et al.*, 1992; Prange and Streeck, 1995). In *Aspergillus* transformants carrying the glucoamylase-S protein fusion construct, this process is probably impaired, since dibasic cleavage by the Kex2p is known to occur in the Golgi complex (Brigance *et al.*, 2000) and the presence of the glucoamylase moiety will prevent assembly of the viral proteins in the ER. Therefore passage of these proteins through the secretory pathway is most likely blocked, resulting in the intracellular degradation of the heterologous S protein.

The food-grade industrial host *A. awamori* was also transformed with vectors carrying the *S* and *preS1preS2S* genes, but none of the transformants produced heterologous viral protein. This can most likely be attributed to the generally low transcription levels obtained from these transformants. The host is also not a protease-deficient strain and thus proteolysis may also contribute to the lack of heterologous protein production. When the sweet-tasting protein thaumatin was expressed in *A. awamori* it was degraded by host proteases during secretion and folding (Moralejo et al., 1999). However, expression in a protease-deficient mutant of *A. awamori* resulted in high-level production of this protein (Moralejo et al., 2000). Overall, far less transformants were obtained in *A. awamori* than in *A. niger*. In this study, only one of the 54 *A. niger* transformants obtained could be shown to produce heterologous viral S protein. In contrast, only 18 *A. awamori* transformants were obtained. Since the level of heterologous protein production is determined by various factors including the vector copy number and site of integration (Davies, 1991; Ward et al., 1990), it

is possible that if more transformants were generated, one of them may successfully produce heterologous S protein

This work clearly indicates that when evaluating the expression of a new protein in a recombinant host such as *Aspergillus* it is imperative to employ a variety of approaches, specifically regarding choice of promoter and host strain. Furthermore it is essential to obtain various transformants for each construct to screen for an efficient heterologous protein producing strain.

4.6. Acknowledgements

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Chapter 5

General Discussion and Conclusions

5. GENERAL DISCUSSION AND CONCLUSIONS

Since the filamentous fungus *Aspergillus* was first used for the production of heterologous proteins in the 1980s, there have been numerous impressive successes (e.g. commercial production of chymosin by *Aspergillus awamori* [Ward, 1989]), but also some less successful attempts. This work details the evaluation of *Aspergillus* for the expression of hepatitis B virus surface antigens and encompasses both successful and unsuccessful approaches for heterologous production, which have also furthered our understanding of the utilisation of *Aspergillus* as a heterologous host. From the extensive body of literature available it is evident that filamentous fungi, particularly aspergilli, have emerged as viable, interesting and, in some cases, preferred hosts for the production of heterologous proteins.

The heterologous expression of hepatitis B virus surface antigens in yeast has been proven to be an effective method of producing safe, affordable, immunogenic vaccines which are now routinely used world-wide (Fu *et al.*, 1996; Gellissen, 2000; Hardy *et al.*, 2000). Hepatitis B is a wide-spread and serious, potentially fatal liver disease which has a high endemicity in southern Africa (Vryheid *et al.*, 2001). South Africa implemented the routine infant HBV immunisation programme recommended by the World Health Organisation in 1995 (Hino *et al.*, 2001; Tsebe *et al.*, 2001) and as more countries implement this programme, the need for a cheap and effective vaccine will increase. As industrial fermentation systems have been established for the filamentous fungus *Aspergillus,* it may be the ideal organism for the production of an alternative cost-effective vaccine. With only one published report of the expression of hepatitis B major (S) protein in *Aspergillus foetidus* in Chinese (Liu *et al.*, 1990), limited information was available on the use of filamentous fungi for the expression of viral proteins. We therefore further investigated the potential of *Aspergillus,* particularly the GRAS status organism *Aspergillus niger*, as a host for the production of the hepatitis B major (S) and large (L) proteins.

5.1. Expression of the hepatitis B *S* gene

The hepatitis B virus *S* gene encodes the major viral envelope protein which is 24 kDa in size and spontaneously assembles with other viral envelope proteins into pseudoviral particles devoid of DNA (Ganem and Varmus, 1987). This particle assembly also occurs in the heterologous expression of the *S* gene in various yeast hosts (Cregg *et al.*, 1987; Janowicz *et al.*, 1991; Valenzuela *et al.*, 1982). To investigate the intracellular and extracellular expression of the hepatitis B virus *S* gene in *Aspergillus*, numerous vector constructs were made. These included placing the gene under the transcriptional control of two different promoters, including a pentaHis tag to aid purification and detection and adding a carrier moiety to possibly enhance secretion. These vectors were transformed to laboratory strains of *A. niger* and *A. awamori*. *A. niger,* which is routinely used in the industrial production of citric acid, has been afforded GRAS (Generally Regarded As Safe) status and many non-fungal heterologous proteins have been successfully expressed in this host. Numerous transformation experiments were undertaken, generating a total of 39 *A. niger* and 15 *A. awamori* transformants carrying the hepatitis B *S* gene. One of these *A. niger* transformants showed the intracellular high-level production of recombinant S protein that was similar in size (24 kDa) to the native protein. Furthermore, 22 nm hepatitis B pseudoviral particles were isolated from this transformant at levels of 0.4 mg/l culture.

Various factors have been shown to affect heterologous protein expression in *Aspergillus*. These may include transcriptional control, site of DNA integration, intron splicing, codon bias, secretion efficiency, proteases, glycosylation and the availability of chaperones. Some of these factors and their possible effects in the expression of the *S* gene can be summarised as follows:

5.1.1. Choice of promoter

One of the first aspects to consider during the construction of an expression vector is the choice of promoter. The two main types of promoters are constitutive and inducible promoters and in this work we placed the viral gene under control of the constitutive *Aspergillus nidulans* glyceraldehyde-3-

phosphate dehydrogenase (*gpdA*) promoter and the inducible *A. niger* glucoamylase (*glaA*) promoter. These promoters have been used extensively in the production of various proteins with the *A. nidulans* promoter directing high level transcription in various *Aspergillus* hosts and the *glaA* promoter showing high levels of induction in the presence of starch, maltose or maltodextrin (Punt *et al.*, 1988; Fowler *et al.*, 1990). Both promoters effectively directed transcription of the hepatitis B *S* gene in the numerous *A. niger* and *A. awamori* transformants obtained. Transcript levels obtained from the *glaA* promoter constructs were generally lower than those obtained from the *gpdA* promoter in both hosts. The generation of heterologous transcript can however not be equated with successful protein expression and this was evident in this work. Although many of the transformants generated showed the presence of *S* gene transcripts, only one with the *S* gene under control of the *gpdA* promoter (designated DAHB11#8), exhibiting high levels of transcription, and was finally shown to produce appreciable levels of recombinant viral protein. Therefore, in this case, the constitutive *gpdA* promoter was more effective.

5.1.2. Site of vector integration and copy number

In this work, the stable recombinant *Aspergillus* strains were generated by the random integration of the circular vector into the chromosome. This is a common feature of *Aspergillus* transformation where integration shows no dependence on extensive homology between the transforming DNA and the host chromosomal sequences (Ballance and Turner, 1985). The site of integration may influence the levels of heterologous gene transcription as different regions on the chromosome are transcribed with different efficiencies (Verdoes *et al.*, 1994). Southern analysis also revealed that the number of integrated copies of *S* gene carrying vector differed significantly between the various transformants, ranging from a single copy to as many as seven. Transformant DAHB11#8 had the highest vector copy number, suggesting the conclusion that vector copy number determined the higher transcription levels, which in turn led to the production of heterologous S protein. However, a combination of promoter choice, site of integration and copy number may have determined the higher levels of transcription.

Of the 54 *Aspergillus* transformants obtained, only one produced appreciable levels of heterologous S protein, highlighting the importance of the generation and screening of numerous transformants.

5.1.3. Secretion

The extracellular production of heterologous protein is frequently desirable as this simplifies purification procedures. In the yeast hosts, heterologous production of S-protein containing pseudoviral particles has generally been achieved intracellularly (Fu *et al.*, 1996; Gellissen, 2000; Hardy *et al.*, 2000). To investigate the secretion of hepatitis B S protein by *Aspergillus*, the *S* gene was fused to the catalytic domain of the highly expressed and efficiently secreted glucoamylase (*glaA*) gene. The two regions were separated by the dibasic KEX2 proteolytic cleavage site (Lys-Arg), which is recognised in *Aspergillus* by a KEX2-like protease. This fusion strategy was previously shown to be very effective in the enhanced secretion of proteins such as chymosin (Ward *et al.*, 1990), human interleukin (Contreras *et al.*, 1991) and human lactoferrin (Ward *et al.*, 1992). However, no secretion of S protein could be detected in any of the *A. niger* transformants carrying this construct. Intracellular degradation products were detected, suggesting that secretion of the protein was somehow blocked. The most probable explanation for this can be found in the assembly and secretion process of the S protein in infected mammalian liver cells. In the viral life cycle, the hepatitis B S protein is co-translationally inserted into the endoplasmic reticulum (ER) membrane and then translocated to the ER lumen where disulphide-linked S protein dimers are rapidly generated (Huovila *et al.*, 1992). The dimers are subsequently assembled into pseudoviral particles in a post-ER/pre-Golgi compartment and are then exported out of the cell via vesicular transport. Since the Kex2p is located in the Golgi complex, dibasic cleavage of the glucoamylase moiety from the S protein would not occur in the ER, thereby probably preventing the formation of the S protein dimers and thus inhibiting pseudoviral particle assembly. This would lead to the accumulation of glucoamylase-S fusion protein in the ER, which can then be targeted for degradation by the cell. Therefore, although the fusion protein strategy has been proven to be very effective for many heterologous proteins, it is clear that

in the case of this viral protein, which spontaneously assembles into dimers and oligomers, the presence of a foreign protein moiety impedes rather than enhances protein production. This result has highlighted the necessity of evaluating effective expression strategies for each individual heterologous protein to be expressed, taking into particular consideration the inherent properties of the protein.

5.1.4. Proteases

One of the disadvantages of *Aspergillus* as a host for the production of heterologous proteins has been the presence of various (secreted) proteases (Mattern *et al.*, 1992; Van den Hombergh *et al.*, 1997). To overcome this problem, protease-deficient host strains have been developed, which have been effective in alleviating the problem of extracellular degradation (Mattern *et al.*, 1992; Van den Hombergh *et al.*, 1997). In this study, a protease-deficient *A. niger* strain was employed which does not acidify its culture medium (Gordon *et al.*, 2000). In this strain, proteolytic activity is virtually absent at non-acidic pH and it has been previously effectively employed for the production of human interleukin-6 (Punt *et al.*, 2002). However, an *in vitro* proteolytic degradation assay involving the overnight incubation of S protein in *A. niger* culture medium revealed that the protein was degraded by residual proteolytic activity. Therefore, although the primary stumbling block in the secretion of S protein was the aberrant assembly in the ER, proteolysis may have contributed to the lack of heterologous extracellular S protein. This problem could potentially be addressed by fermentation strategies, which have shown that fungal morphology plays a role in the production of extracellular proteases (Xu *et al.*, 2000). The extracellular protease activity of *A. niger* varies with pellet size and decreases dramatically when the morphology changes from free mycelia to pellets.

Fermentation conditions have been shown to play an important role in the levels of heterologous protein production by *Aspergillus.* Factors such as the growth medium composition (Archer *et al.*, 1995; Hellmuth *et al.*, 1995), pH (Mainwaring *et al.*, 1999), temperature (MacKenzie *et al.*, 1994), aeration (Wongwicharn *et al.*, 1999b), dilution rate (Wongwicharn *et al.*, 1999a) and

agitation (Amanullah *et al.*, 1998) influence the morphology and productivity of the fungal strain. One example where the manipulation of fermentation conditions played a role in the levels of heterologous protein production is in the expression of hen egg white lysozyme (HEWL) in *A. niger.* In standard expression medium, the secreted levels were found to be maximal at 20-25°C and markedly reduced at 30-37°C (MacKenzie *et al.*, 1994). However, in richer medium generally higher levels were obtained and maximal levels were achieved at 37°C. Formation of the heterologous enzyme also increased with increasing O_2 supply and by optimising levels of ammonium chloride and sodium phosphate buffer in the medium (Wongwicharn *et al.*, 1999b; MacKenzie *et al.*, 1994). There was also a very clear distinction between conditions and morphology that maximised protein secretion and those that maximised HEWL synthesis (Wongwicharn *et al.*, 1999a). Fermentation optimisation improved HEWL levels from an initial 12 mg/l to production levels of 1 g/l (Archer *et al.*, 1990; Archer *et al.*, 1995; Wongwicharn *et al.*, 1999b). With regard to heterologous HBsAg production, optimising fermentation conditions improved HBsAg yields by *S. cerevisiae* 1000-fold, from 10-25 µg/l (Valenzuela *et al.*, 1982) to 3-10 mg/l (Hsieh *et al.*, 1988). Similarly, the production levels of recombinant HBsAg by *A. niger* could potentially also be improved by investigating the effect of the above-mentioned variables and thereby optimising fermentation conditions.

5.2. Expression of the *preS1preS2S* gene

The 1433-bp *preS1preS2S* gene encodes the 42 kDa large envelope protein of the hepatitis B virus (Tiollais *et al.*, 1985). This protein contains additional antigenic epitopes and has been successfully expressed in *S. cerevisiae*, however without concomitant pseudoviral particle formation (Dehoux *et. al.*, 1986). For expression in *Aspergillus,* this gene was also cloned into *Aspergillus* expression vectors under control of the *gpdA* and *glaA* promoters, with or without the addition of the glucoamylase catalytic domain or pentaHis tag. These vectors were transformed to *A. niger* and *A. awamori*, but only 18

transformants were obtained. Full-length *preS1preS2S* transcript could not be detected in any of these transformants and no recombinant viral protein was produced. A smaller transcript similar in size to the *S* gene transcript was detected in some transformants, which may be the result of mRNA splicing. Although viral transcripts in infected cells generally remain unspliced, a few spliced transcripts have been described in liver cells (Fallows and Goff, 1996). However, mRNA splicing has not been well documented in fungi.

5.3. Conclusions

The following can therefore be concluded from the data presented in this study:

1. The *S* gene of the hepatitis B virus subtype adw2 was successfully expressed in *A. niger* under control of the constitutive *gpdA* promoter.

2. The recombinant fungal strain produced hepatitis B pseudoviral particles 22 nm in size which are very similar to those observed in human serum and those obtained from recombinant yeast.

3. The particle production levels were somewhat higher (0.4 mg/l) than those initially obtained in *S. cerevisiae* (0.05 mg/l) (Valenzuela *et al.*, 1982) and *P. pastoris* (0.14 mg/l) (Cregg *et al.*, 1987).

4. The pseudoviral particles were located in the intracellular, membrane-associated fraction. No secretion of viral particles was detected.

5. The commonly employed strategy of fusing the heterologous gene to a highly expressed and secreted carrier (such as the catalytic domain of glucoamylase) to enhance secretion was unsuccessful due to the inherent aggregation properties of the major (S) protein.

6. Heterologous production of the hepatitis B large (L) protein could not be obtained due to the absence of full-length *preS1peS2S* gene transcript.

7. *A. awamori* was not an effective host for the production of hepatitis B viral proteins.

This study has clearly shown that the filamentous fungus *Aspergillus* is an effective alternative host for the production of hepatitis B viral protein and has the ability to produce pseudoviral particles. As the initial levels of viral particle production were somewhat higher than those obtained in yeast, it is tempting to speculate that upon optimisation of production, levels may exceed those obtained from yeast, making for a potentially more cost effective vaccine. More importantly, however, this work paves the way for the investigation of *Aspergillus* as a potential host for the heterologous production of proteins from viruses such as HIV-1 and rotavirus for the development of subunit vaccines to address the increasing health risk posed by these viruses, especially in Africa.

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Appendix

Provisional Patent

SPOOR & FISHER

JOHANNESBURG

PROVISIONAL PATENT SPECIFICATION

COUNTRY	:	SOUTH AFRICA
APPLICATION NUMBER	:	2001/9777
DATE OF FILING	:	28 NOVEMBER 2001
NAME OF APPLICANT	:	UNIVERSITY OF STELLENBOSCH
NAMES OF INVENTORS	:	VAN ZYL, WILLEM HEBER; PLUDDEMANN, ANNETTE
TITLE OF INVENTION	:	A FUNGUS STRAIN FOR PRODUCING VIRAL COAT PROTEINS AND A METHOD OF PRODUCING THE FUNGUS STRAIN

FILE REF : PA131996/P
DATE : 12 December 2001

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT

AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION NO.		S & F REFERENCE
21 01	2001/9777	PA131996/P

FULL NAME(S) OF APPLICANT(S)

71	UNIVERSITY OF STELLENBOSCH

ADDRESS(ES) OF APPLICANT(S)

	VICTORIA STREET, STELLENBOSCH, 7600, WESTERN CAPE, SOUTH AFRICA

TITLE OF INVENTION

54	A FUNGUS STRAIN FOR PRODUCING VIRAL COAT PROTEINS AND A METHOD OF PRODUCING THE FUNGUS STRAIN

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIM IS:

COUNTRY:	NUMBER:	DATE:
NIL	NIL	NIL

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

21	01	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO.~~

21	01	

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ 1. A single copy of a provisional specification of 15 pages.
- ☒ 2. Drawings of 4 sheets.
- ☐ 3. Publication particulars and abstract (Form P.8 in duplicate).
- ☐ 4. A copy of Figure of the drawings (if any) for the abstract.
- ☐ 5. Assignment of invention.
- ☐ 6. Certified priority document.
- ☐ 7. Translation of the priority document.
- ☐ 8. Assignment of priority rights.
- ☐ 9. A copy of the Form P.2 and the specification of S.A. Patent Application No .
- ☐ 10. Declaration and power of attorney on Form P.3.
- ☐ 11. Request for ante-dating on Form P.4.
- ☐ 12. Request for classification on Form P.9.
- ☒ 13. Form P.2 in duplicate.
- ☐ 14. Other.

74	ADDRESS FOR SERVICE: SPOOR & FISHER, SANDTON

Dated: 28 November 2001

S.L. Cloete

SPOOR & FISHER
PATENT ATTORNEYS FOR THE APPLICANT(S)

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REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG
REGISTRAR OF PATENTS

SPOOR & FISHER

FORM P.1

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT

AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION NO.			S & F REFERENCE
21	01		PA131996/P

FULL NAME(S) OF APPLICANT(S)

71	UNIVERSITY OF STELLENBOSCH

ADDRESS(ES) OF APPLICANT(S)

	VICTORIA STREET, STELLENBOSCH, 7600, WESTERN CAPE, SOUTH AFRICA

TITLE OF INVENTION

54	A FUNGUS STRAIN FOR PRODUCING VIRAL COAT PROTEINS AND A METHOD OF PRODUCING THE FUNGUS STRAIN

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIM IS:

COUNTRY:	NUMBER:	DATE:
NIL	NIL	NIL

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

21	01	

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- ☒ 2. Drawings of 4 sheets.
- ☐ 3. Publication particulars and abstract (Form P.8 in duplicate).
- ☐ 4. A copy of Figure of the drawings (if any) for the abstract.
- ☐ 5. Assignment of invention.
- ☐ 6. Certified priority document.
- ☐ 7. Translation of the priority document.
- ☐ 8. Assignment of priority rights.
- ☐ 9. A copy of the Form P.2 and the specification of S.A. Patent Application No .
- ☐ 10. Declaration and power of attorney on Form P.3.
- ☐ 11. Request for ante-dating on Form P.4.
- ☐ 12. Request for classification on Form P.9.
- ☒ 13. Form P.2 in duplicate.
- ☐ 14. Other.

74	ADDRESS FOR SERVICE: SPOOR & FISHER, SANDTON

Dated: 28 November 2001

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REGISTRAR OF PATENTS

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FORM P.6

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

PROVISIONAL SPECIFICATION

(Section 30(1) – Regulation 27)

OFFICIAL APPLICATION NO.			LODGING DATE	
21	01		22	28 NOVEMBER 2001

FULL NAMES OF APPLICANTS

71	UNIVERSITY OF STELLENBOSCH

FULL NAMES OF INVENTORS

72	VAN ZYL, WILLEM HEBER PLUDDEMANN, ANNETTE

TITLE OF INVENTION

54	A FUNGUS STRAIN FOR PRODUCING VIRAL COAT PROTEINS AND A METHOD OF PRODUCING THE FUNGUS STRAIN

FIELD OF INVENTION

The present invention relates to a method of providing a recombinant fungus strain.

More particularly the invention relates to a method of producing a fungus strain with the capability of producing viral coat proteins and to a recombinant DNA sequence for use in transforming a fungus strain to produce viral coat proteins.

BACKGROUND TO INVENTION

Hepatitis B is a serious, potentially lethal liver disease caused by the hepatitis B virus (HBV), which affects 400 to 500 million people world-wide (Moradpour and Wands, 1995). The disease is especially prevalent in underdeveloped areas of the world, such as Africa, where 60% to 99% of healthy adults show evidence of exposure to HBV (Moradpour and Wands, 1995; Ayoola, 1988). No effective treatment for this disease currently exists and an extensive programme of vaccination presents the only solution to curb the spread of the disease (Kassianides et al., 1988; Dehoux et al., 1986). With the establishment of mandatory childhood immunisation programmes against Hepatitis B, there is a growing demand for a safe, affordable vaccine.

Hepatitis B surface antigen proteins have important functions in the viral life-cycle, such as attachment and penetration of human liver cells, envelopment and virus maturation from the liver cells. These proteins spontaneously assemble into virus-like particles devoid of viral DNA. It has been postulated that these “dummy particles”, which also elicit an immune response, serve to absorb virus-neutralising antisurface antibodies during progression of infection as these non-infectious particles are produced in excessive amounts and are

released into the blood (Ganem and Varmus, 1987). Thus these proteins have been targeted for heterologous expression to produce recombinant vaccines.

Recombinant DNA methods have provided an alternative to the blood-origin vaccine obtained from plasma of chronic carriers and to date, bacteria, yeasts and mammalian cells, transformed with appropriate expression vectors containing the hepatitis B virus *S* gene, are able to synthesise hepatitis B surface antigen (HBsAg) for immunisation purposes (Dehoux et al., 1986; Valenzuela et al., 1982; Michel et al., 1984). Currently, a commercial yeast-derived hepatitis B vaccine has been licensed and is in general use (Hilleman, 1987; Barr et al., 1989).

Since the 1980's genetic engineering procedures have been developed for the filamentous fungi, in particular aspergilli (Kinghorn and Unkles, 1994). Subsequently, various heterologous proteins of fungal and non-fungal origin have been expressed in these fungi (Gwynne, 1992). The use of *Saccharomyces cerevisiae* as heterologous host for the production of pharmaceutical (viral) proteins has already highlighted limitations, such as low product yields and hyperglycosylation of glycoproteins.

Several characteristics of filamentous fungal expression hosts give them potential advantages over *Saccharomyces cerevisiae*, particularly their efficient secretion of endogenous proteins. Filamentous fungi are also rather robust and can grow on many organic compounds (Jeenes et al., 1991; Van den Hondel et al., 1991). They are known to produce and secrete enzymes in large quantities and through their use in the food and food processing industry, the species *Aspergillus oryzae* and *Aspergillus niger* have gained GRAS (Generally Regarded As Safe) status. Hyperglycosylation is also less of a problem in filamentous fungi than in *Saccharomyces cerevisiae,* as their glycosylation patterns more closely resemble those found in mammalian cells. Filamentous

fungi have been extensively used in industrial processes such as organic acid and enzyme production, and therefore large-scale fermentation technology and downstream processing are already well established (Bodie et al., 1994; Xu et al., 1989). Viral protein production in filamentous fungi has not been evaluated to date.

Traditionally, *Aspergillus niger* strains have been used for the production of citric acid on an industrial scale using cheap sugar syrups as substrate (Xu et al., 1989). An *Aspergillus*-derived hepatitis B vaccine could therefore provide a cost-effective alternative to the yeast-derived vaccine.

A need therefore exists to produce a fungus strain with the capability of producing viral coat proteins; to assemble the viral coat proteins into virus-like particles (VLPs); and to provide a recombinant DNA sequence for use in transforming a fungus strain to produce viral proteins.

SUMMARY OF INVENTION

According to a first embodiment of the invention, there is provided a method of producing a fungus strain which is capable of producing viral coat proteins, the method including the step of transforming a fungus strain with a DNA sequence including a gene encoding a hepatitis B virus S protein.

The DNA sequence may include a suitable promoter for promoting expression of the S protein gene in the transformed fungus. The promoter may be the *gpdA* promoter DNA sequence from *Aspergillus nidulans.*

A terminator sequence may be the *trpC* DNA sequence from *Aspergillus nidulans.*

Fungus marker genes may also be inserted into the transformed fungus strain. The marker genes may be selected from the *Aspergillus niger pyrG* gene and the *Aspergillus nidulans amdS* gene.

The fungus strain may be an *Aspergillus* strain.

The gene encoding the viral coat protein may be the *S* gene from a virus such as hepatitis B virus subtype adw2.

The gene encoding the hepatitis B virus S protein may be carried in a DNA vector. The vector may be the *Aspergillus / Escherichia coli* vector pAHB11.

According to a second embodiment of the invention there is provided a viral coat protein expressed by the fungus strain described above.

The viral coat protein may be the major S protein of hepatitis B virus subtype adw2.

According to a third embodiment of the invention there is provided one or more virus-like particles (VLPs) assembled from at least one viral coat protein expressed by a fungus strain described above.

According to a further embodiment of the invention there is provided an *Aspergillus* fungus transformed with vector pAHB11.

According to yet a further embodiment of the invention there is provided a method for fungally producing a hepatitis B virus S protein which comprises:

Causing expression in an *Aspergillus* fungus of DNA containing the coding sequence for the hepatitis B S protein preceded by a translation start signal codon and followed by a translation stop signal codon; and

Recovering the resulting hepatitis B virus S protein produced within the fungus.

The expression may result in the formation of virus-like particles.

BRIEF DESCRIPTION OF THE FIGURES

The invention will now be described by way of example with reference to the accompanying schematic drawings, diagrams and representations.

The drawings, diagrams and representations illustrate the method according to the invention for transforming an *Aspergillus* strain so that it is capable of producing hepatitis B virus S protein and virus-like particles.

Figure 1 shows the construction of the *Aspergillus* expression vector, plasmid pAHB11;

Figure 2A shows the presence of multiple copies of the HBV S protein expression cassette integrated into the *Aspergillus* genome;

Figure 2B shows the presence of *S* gene mRNA transcript in the *Aspergillus* transformant carrying integrated HBV S protein expression cassettes;

Figure 2C shows the presence of S protein in intracellular protein extract of the *Aspergillus* transformant carrying integrated HBV S protein expression cassettes;

Figure 3 shows the presence of HBV virus-like particles in intracellular extracts of the *Aspergillus* transformant carrying integrated HBV S protein expression cassettes.

Figure 4 shows the standard curve compiled to determine the levels of HBsAg production by the *Aspergillus* transformant.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The term "a fungal expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes the fungal *gpdA* promoter and *trpC* terminator DNA sequences, preferably a *gpdA* promoter DNA sequence of *Aspergillus nidulans* FGSC4 (Glasgow wild-type: Clutterbuck, 1986; Punt et al., 1988) and a *trpC* terminator DNA sequence of *Aspergillus nidulans* (Punt et al., 1987).

The term "HBV S protein expression cassette" is used to denote a recombinant DNA molecule according to the invention which includes a *S* gene, preferably the *S* gene from hepatitis B virus (HBV) subtype adw2 (donated by prof. W.J. de Wet, Potchefstroom University, South Africa), and fungal *gpdA* promoter and *trpC* terminator DNA sequences, preferably a *gpdA* promoter DNA sequence of *Aspergillus nidulans* and a *trpC* terminator DNA sequence of *Aspergillus nidulans* resident on the *Aspergillus* / *Escherichia coli* integrative vector pAHB11.

The term "fungal marker fragment" is used to denote a recombinant DNA molecule according to the invention which includes a *pyrG* gene of *Aspergillus niger* and a *amdS* gene of *Aspergillus nidulans* (donated by Prof. C.A.M.J.J. van den Hondel, Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research Institute, Zeist, The Netherlands).

STEP 1

The construction of a fungus expression vector containing the *Aspergillus nidulans gpdA* promoter and *trpC* terminator DNA regions, the *pyrG* and *amdS*

fungal marker fragment and the hepatitis B virus S gene, called plasmid pAHB11, is shown in Figure 1. The plasmid pAN52-1 carrying the *Aspergillus nidulans gpdA* promoter and *trpC* terminator (Genbank Accession Number Z32697, donated by Prof. C.A.M.J.J. van den Hondel) was digested with restriction enzymes *Nco*1 and *Bam*H1 to linearise the vector. The hepatitis B virus *S* gene was amplified from plasmid pCOR105 (donated by Prof. W.J. de Wet, Potchefstroom University, South Africa) using the PCR technique with the aid of oligodeoxyribonucleotide DNA primers designed by conventional techniques, which contained the restriction sites *Nco*1 and *Bgl*II, and cloned as a 814-bp *Nco*1-*Bam*H1/*Bgl*II DNA fragment into the *Nco*1-*Bam*H1 restriction enzyme sites of plasmid pAN52-1, generating plasmid pAN52-1-S. The two following synthetic oligodeoxyribonucleotides were used for the amplification of the hepatitis B virus *S* gene:

S-left 5'-CTAGCCATGGAGAACATCACATCAGGATTCC-3'

S-right 5'-GATCAGATCTCCAACAGGAAGTTTTCTAAAAC-3'

Plasmid pAN52-1-S was subsequently digested with the restriction enzyme *Not*1 and the fungal marker fragment containing the *pyrG* and *amdS* genes (donated by Prof. C.A.M.J.J. van den Hondel) was ligated into the *Not*1 site to generate plasmid pAHB11.

STEP 2

Plasmid pAHB11 was introduced into *Aspergillus niger* D15 (donated by Prof. C.A.M.J.J. van den Hondel) by the protoplasting method of Punt and Van den Hondel (1992), using Novozyme 234 (Sigma Chemical Co. St. Louis, USA). Selection for successful transformants was performed on medium without uridine and containing acetamide as sole nitrogen source. The *amdS* gene enables *Aspergillus niger* to utilise acetamide as sole nitrogen source (on minimal media without nitrate containing 1% glucose, 10 mM acetamide, 15 mM CsCl and trace elements). *Aspergillus niger* D15 is also a *pyrG*$^-$ strain and can therefore not grow in the absence of uridine. Transformants carrying the *pyrG* gene however are able to grow in the absence of uridine.

The presence of the *S* gene in the *Aspergillus* genome was determined by isolating chromosomal DNA from a stable *S* gene transformant. The genomic DNA was isolated according to La Grange et al. (1996) and digested overnight with the restriction enzymes *Eco*RI and *Nru*1 (which only cut once in pAHB11) and used for conventional Southern blot analysis with the 814-bp *S* gene as probe (Sambrook et al., 1989). The copy number of the *S* gene in the recombinant *Aspergillus* strain was determined to be at least 6 copies (Figure 2A).

The presence of the 814-bp *S* gene mRNA transcripts was confirmed by isolating total RNA from the recombinant *Aspergillus* strain cultured for 40 h at 30°C in minimal medium with nitrate, 0.5% yeast extract, 0.1% casamino acids ,1% glucose and trace elements.

Total RNA was isolated as follows: mycelium was harvested through Miracloth (Calbiochem, USA), frozen under liquid nitrogen, ground to a fine powder with a mortar and pestle and resuspended in cold STE buffer (0.25M Tris-HCl pH7.2 / 0.1M NaCl / 0.01M EDTA). One volume phenol/chloroform/isoamylalcohol (PCI; 24:24:1) as well as 0.05% sodium dodecyl-sulphate (SDS) was added and the mycelial debris was collected by centrifugation. The aqueous upper phase was collected, subjected to PCI extraction and precipitated to yield total RNA. The total RNA was used for conventional Northern blot analysis using the radioactively-labelled 814-bp *S* gene as probe (Figure 2B). The *Aspergillus nidulans* 1350-bp glyceraldehyde-3-phosphate dehydrogenase gene (donated by Prof. C.A.M.J.J. van den Hondel) was used as an internal standard to verify RNA integrity.

The presence of hepatitis B virus S protein was determined by extracting total intracellular and membrane-bound proteins from the recombinant *Aspergillus niger* strain cultured for 72 h in minimal medium as before.

Protein extraction was performed as follows: mycelium was harvested and ground as before. Ground mycelium was suspended in protein extraction buffer (50 mM phosphate buffer pH7.0 / 1 mM $MgCl_2$ / 20 µM PMSF / 0.1% TritonX-100) and the mycelial debris was removed by centrifugation. Proteins were

separated on a 12% SDS polyacrylamide gelelectrophoresis (PAGE) gel, transferred to a PVDF membrane (Hybond-P; Amersham Pharmacia Biotech, UK) and subjected to standard Western blotting using the Chemiluminescence Western Blotting Kit (Roche Diagnostics, Germany) as recommended by the manufacturer. The primary antibodies were polyclonal goat anti-Hepatitis B surface antigen (Dako, USA) which were used at a 1:5000 dilution and the secondary antibodies were goat immunoglobulins coupled to horseraddish peroxidase (Dako, USA) which were employed at a dilution of 1:10 000 (Figure 2C).

STEP 3

To determine whether the hepatitis B S protein monomers spontaneously aggregate into virus-like particles (VLPs), the total intracellular protein extract including the membrane fraction of the recombinant *Aspergillus* strain was subjected to sucrose gradient centrifugation as described by Mason et al. (1992). The gradient was fractionated and fractions were run on a 12% SDS-PAGE gel to identify fractions containing the hepatitis B S protein. The fraction containing the highest concentration of hepatitis B S protein was dialysed against 50 mM sodium phosphate buffer over night, negatively stained with uranyl acetate and visualised by transmission electron microscopy (Figure 3). Particles of 22 nm in size were observed which resemble hepatitis B virus virus-like particles isolated from human serum of infected individuals (Yamaguchi et al., 1998).

STEP 4

To determine the yield of hepatitis B virus-like particles from the recombinant *Aspergillus* strain the total intracellular protein extract including the membrane fraction was isolated as before. The extraction was repeated six times to yield total protein, and samples were assayed with the Auszyme MC Dynamic kit (Abbott Diagnostics, USA), using the positive control provided (HBsAg derived from human serum) as a standard.

This enzyme immunoassay detects HBsAg with the aid of monoclonal antibodies. Beads coated with mouse monoclonal antibody to Hepatitis B Surface Antigen (Anti-HBs) are incubated with the samples, appropriate controls and mouse monoclonal Anti-HBs Peroxidase (horseraddish) Conjugate (Anti-HBs:HRPO). During the incubation period, any HBsAg present is bound to the solid phase antibody and simultaneously bound by the Anti-HBs:HRPO. Unbound material is aspirated and washed and o-Phenylenediamine (OPD) solution containing hydrogen peroxide is added to the bead, which develops a yellow-orange colour in proportion to the amount of HBsAg which is bound to the bead. Absorbance of the samples and controls is then determined at a wavelength of 492 nm. The positive control was diluted to give HBsAg levels of 0.09-1.86 ng/ml, and the absorbance at 492 nm after colour development gave a linear relationship in this range (Figure 4). The HBsAg levels in the samples were determined from this standard curve. This method is dependent on the conformational epitopes of HBsAg particles and will not detect free unassembled HBsAg proteins.

Mycelium dry weight was determined gravimetrically using nitrocellulose filters (0.45 µm, Gelman Sciences). The filters were predried in a microwave oven for 10 min and weighed. A known volume of culture was filtered and washed with an equal volume of double-distilled water. The filters were dried in a microwave oven for 10 min and weighed. Dry cell weight was determined in triplicate.

The yield of hepatitis B VLPs from mycelium of the recombinant *Aspergillus* strain was determined to be 0.2 mg per gram dry mycelium, giving an estimated 0.4 mg HBsAg per litre of culture. This value compares favourably with the reported levels initially obtained in *Saccharomyces cerevisiae* which were respectively 10 to 25 µg HBsAg/L cells (Valenzuela et al., 1982) and 50 µg HBsAg/L cells (Hitzeman et al., 1983), and in wild-type *Pichia pastoris*, where levels of 0.14 mg/L were reported (Cregg et al., 1987). This indicates the potential of the *Aspergillus* expression system, as production levels can be expected to improve upon optimisation of the system.

The method of the invention has been found to be particularly effective for transforming an *Aspergillus* fungal strain into a microorganism capable of producing hepatitis B virus S protein and assembly of virus-like particles.

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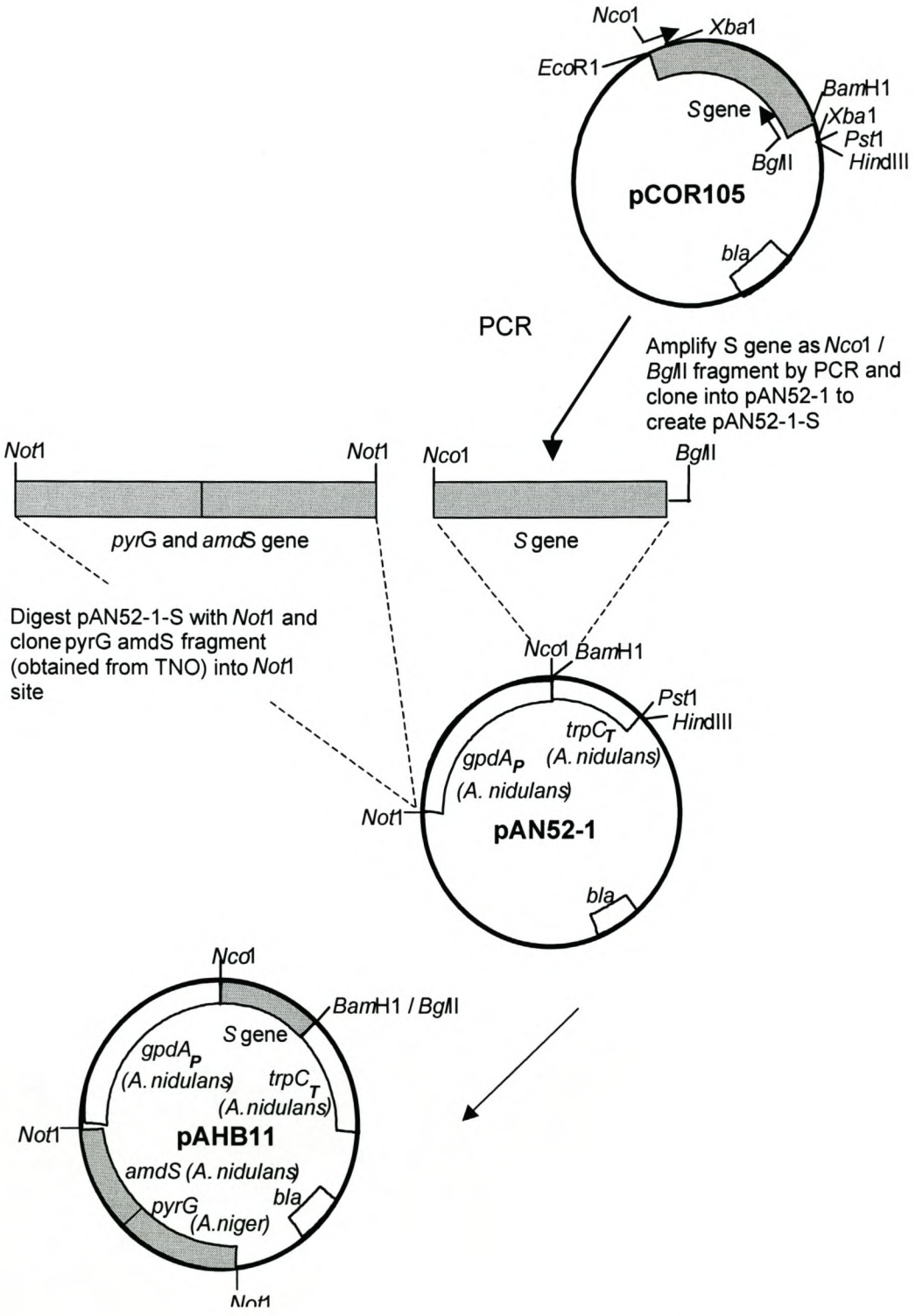

Figure 1: Construction of the *Aspergillus* expression vector pAHB11.

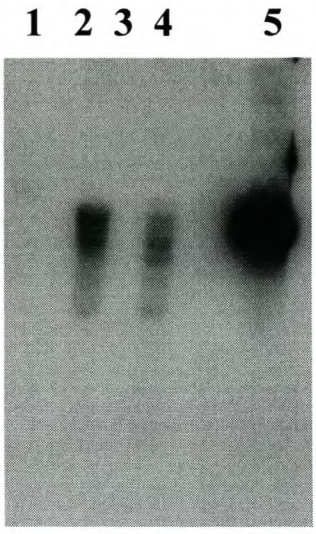

Figure 2A: Southern blot analysis to determine the number of *S* gene copies integrated into the *Aspergillus* genome. Lane 1: DNA isolated from the negative control (*Aspergillus niger* D15), digested with *Eco*R1, lane 2: DNA isolated form *Aspergillus niger* D15 transformed with pAHB11, digested with *Eco*R1, lane 3: DNA isolated from the negative control, digested with *Nru*1, lane 4: DNA isolated from *Aspergillus niger* D15 transformed with pAHB11, digested with *Nru*1 and lane 5: plasmid pAHB11 digested with *Nru*1. The *S* gene was detected with ^{32}P-labelled *S* DNA probe.

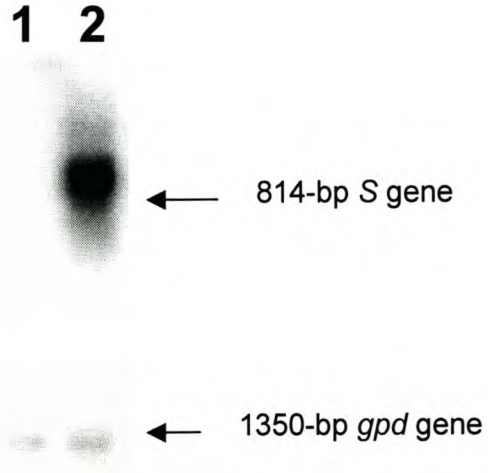

Figure 2B: Northern analysis of *S* gene mRNA transcrpts of *Aspergillus niger* D15 transformed with pAHB11. The 814 bp *S* gene was used as an [α-^{32}P-dATP]-labelled probe and the *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene was used as internal standard. Lane 1: *Aspergillus niger* D15 negative control. Lane 2: Transformant with pAHB11.

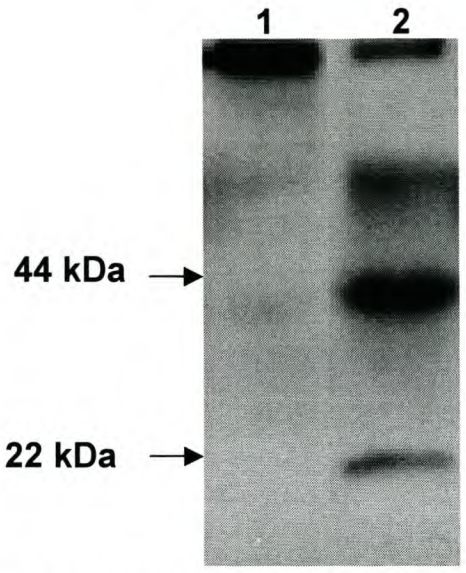

Figure 2C: Western blot analysis of total intracellular proteins isolated after culturing transformants for 72 h at 30°C. Intracellular proteins were separated in a 12% SDS-PAGE gel and transferred to a PVDF membrane by electroblotting. Lane 1: *Aspergillus niger* D15 negative control. Lane 2 : *Aspergillus niger* D15 transformed with pAHB11. Immunoblotting was done with a polyclonal goat-α-HBsAg antibody. A protein species of about 22 kDa is visible in the pAHB11 transformant (lane 2) corresponding to the size of the hepatitis B S protein, as well as a S protein dimer of 44 kDa.

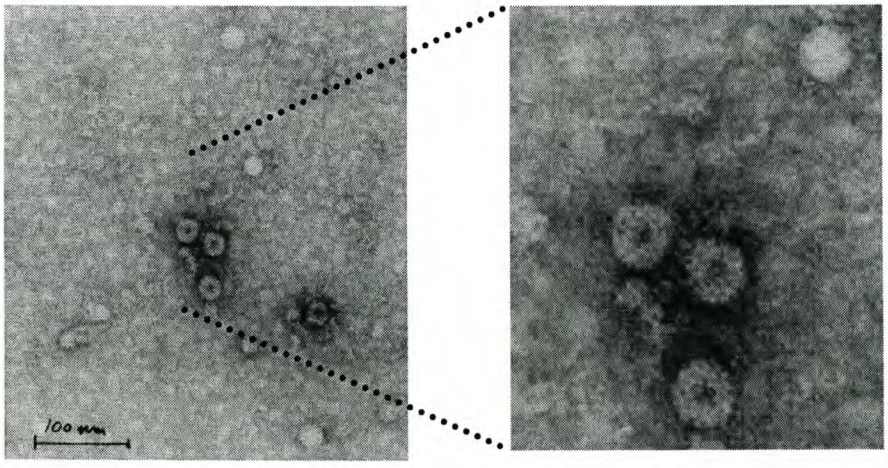

Figure 3. Transmission electron microscopy (magnification: x135 000) of purified Hepatitis B S protein to show presence of virus-like particles.

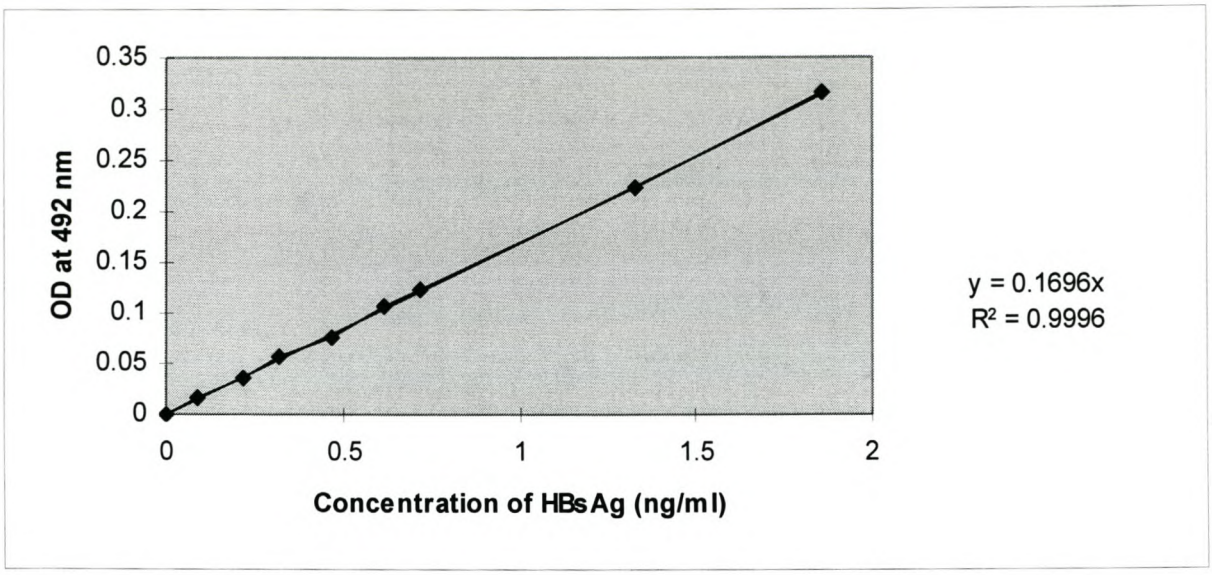

Figure 4. Standard curve of the correlation between Absorbance at 492 nm and the concentration of purified HBsAg (Subtype ad) in the detection of HBsAg by the Auszyme MC Dynamic assay.