VRIJE UNIVERSITEIT

MOLECULAR GENETIC STUDIES OF THE OVERPRODUCTION OF GLUCOAMYLASE IN *ASPERGILLUS NIGER*

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr E. Boeker,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der biologie
op dinsdag 5 april 1994 te 13.30 uur
in het hoofdgebouw van de universiteit, De Boelelaan 1105

door

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Dit onderzoek is mede tot stand gekomen dankzij de financiële ondersteuning van het Ministerie van Economische Zaken, in het kader van het Innovatiegerichte Onderzoek Programma Biotechnologie (IOP-B).

Aan mijn vader en moeder
Voor Coby

Fotografie : M.J.M. Boermans

Illustratie omslag : Sporulerende kolonie van *Aspergillus niger*

Het onderzoek beschreven in dit proefschrift is uitgevoerd binnen de afdeling Moleculaire Genetica en Gen Technologie van het (voormalig) Medisch Biologisch Laboratorium van de Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO) te Rijswijk. Uitgave van dit proefschrift is financieel ondersteund door een bijdrage van TNO-Voeding.

CONTENTS

CHAPTER 1

Strain improvement strategies for the overproduction of fungal proteins by filamentous fungi

I. INTRODUCTION

Filamentous fungi, which can be isolated from a large number of different natural environments (e.g. from soil, organic waste and as pathogens from plants and animals), can use a wide variety of carbon and nitrogen sources for growth. These different habitats and the competition with other (micro-)organisms also present in these habitats probably underlie the extraordinary metabolic diversity of many filamentous fungi. The multitude of biosynthetic and catabolic pathways necessary for this metabolic diversity makes filamentous fungi potential production organisms for a wide range of low molecular-weight primary and secondary metabolites, and also for intra- and extracellular hydrolytic enzymes. In effect, filamentous fungi and the enzymes they produce have been used in food and foodprocessing industry for many decades which resulted in a so-called GRAS status (Generally Recognized As Safe; U.S Food and Drug Administration) for some of the species (such as *Aspergillus niger* and *A. oryzae*).

The industrial potential of filamentous fungi has stimulated research in the development of large scale fermentation processes, down-stream processing and methods for strain improvement (see below section II), the results of which further contributed to the use of filamentous fungi in today's industry. The development of molecular genetic techniques (in particular gene transfer technology) for most "industrial" species (for reviews see Finkelstein, 1991; May, 1992) has further opened the way for industrial exploitation of these organisms. In particular, for the production of enzymes encoded by fungal, and non-fungal, genes. In this chapter an overview will be given of research carried out towards improved production of extracellular fungal proteins. In general, the proteins of interest are hydrolytic enzymes involved in the degradation of polysaccharides, the degradation products of which are used by the fungi as carbon and energy source.

II. STRAIN IMPROVEMENT STRATEGIES

The production level of the protein of interest in naturally occurring strains is, in most of the cases, too low for commercial exploitation. Therefore, strains producing the protein of interest are subjected to strain improvement programs. Traditionally, two different methods for strain improvement are used; 1) mutagenesis and 2) genetic recombination (section IIA). Recently, with the development of molecular genetic techniques and transformation systems for fungi, a third method (so-called genetic engineering) became available (section IIB).

A. Traditional Strain Improvement Strategies

The traditional approach for strain improvement consists of mutagenesis and selection. Random mutations may be induced either by UV irradiation (e.g. Vialta and Bonatelli, 1988; Berka et al., 1991), or by chemicals (e.g. Berka et al., 1988; Hara et al., 1992) including toxic substrate analogues (Gosh et al., 1991; Ward et al., 1993). Subsequently, selection of mutants with improved characteristics has to be carried out. In most cases this can only be done by analysis of a large number of putative mutant strains which makes this approach cumbersome. To facilitate preliminary identification of strains with improved production characteristics and thus to minimalize the number of mutants to be analyzed in detail, the development of a simple screening procedure on plates can improve the selection to a certain extent.

Using the approach of mutagenesis, improved production strains were isolated for a number of commercially interesting enzymes; α-amylases of *A. oryzae* (Hara et al., 1992) and proteases from *A. oryzae* and *A. sojae* (Hara et al., 1992), glucoamylase of *A. niger* (Bonatelli et al., 1991) and *A. awamori* (Smiley. 1967; Vialta and Bonatelli., 1988) and xylanases and β-xylosidases of *A. awamori* (Smith and Wood and references therein, 1991), cellulases and β-glucosidase of *Trichoderma reesei* (Bigelis and Das and references therein, 1988; Rakshit and Sahai, 1989). Although the mutational strategies described are directed towards improving levels of secreted protein, the selected strains may carry mutations

indirectly affecting protein secretion (e.g. altered fermentation characteristics, morphology). Only in a few cases of mutational improvement strategies has the basis for the improvement been studied and even then only marginally. Both, effects on the level of secretion (Bigelis and Das, 1988 and references therein) and on the level of expression (Morawetz et al., 1992) have been reported.

A combination of various favourable characteristics in one strain may be obtained by multiple rounds of mutagenesis. Another approach to obtain strains with new characteristics is genetic recombination. Most of the fungi with a potential for enzyme production lack a sexual cycle. Recombination can, however, be carried out by either protoplast fusion (for review see Peberdy, 1991) or somatic recombination (Bos, 1988). These recombination methods were shown to be successful improvement of for glucoamylase production in *A. niger* (Ball et al., 1978; Ilczuk and Fiedurek, 1985 and; Das and Gohsh, 1989), protease production in *A. oryzae* and *A. sojae* (Hara et al., 1992) and exoglucanase production in *T. reesei* (Sandhu and Bawa, 1992). In most of the studies intraspecific (protoplast) fusions were made between a low producing strain with attractive cultivation characteristics and a high producing strain. Although, recently also interspecific and intergeneric protoplast fusion of *Aspergillus* and *Monascus* or *Rhizopus* has been carried out (Hara et al., 1992 and references therein) strain improvement by genetic recombination is basically restricted to one (or closely related) species.

B. Strain Improvement by Genetic Engineering

The approaches for strain improvement as described in the previous section have been shown to be successful in several cases. However, in general the production level, compared to the parental strains, is only modestly increased (less than 2-fold) in a single step improvement approach. Furthermore, as indicated, several restrictions for fruitful application have to be taken into account such as additional mutations and genetic barriers. A completely new strategy for strain improvement became available with the development of gene transfer systems for fungi. Through this technology it is now possible to introduce additional gene copies encoding the protein of interest in different fungal hosts which is considered

to lead to a concomitant increase in protein production. The general approach for construction of overproducing strains by genetic engineering consist of 1) screening for and isolation of a strain which produces the protein of interest; 2) cloning the gene encoding the protein of interest from the isolated strain; 3) generation of strains carrying multiple copies of the gene; 4) analysis of protein production in transformants obtained and 5) application of additional strain improvement methods. The different steps will be illustrated with results obtained with studies aimed at the (over)production of commercially interesting fungal proteins in filamentous fungi.

1. Source of Genetic Material

The basis of the molecular genetic strain improvement strategy is the selection of a strain which produces a specific enzymatic activity. This strain may result from the screening of natural isolates (e.g. van Gorcom et al., 1990) or from a strain collection originated from a mutagenesis program of an available production strain (e.g. Christensen et al., 1988). The choice of the strain may depend on the high(est) levels of specific activity in a strain (e.g. van Gorcom et al., 1990) or on special requirements of the protein in a particular application, such as thermostability (e.g. Allison et al., 1992) or special substrates specifities (e.g. Shibuya et al., 1990; Joutsjoki and Torkkeli, 1992). The strain producing the desired enzymatic activity will be used as source of genetic material for the isolation of the gene encoding the corresponding enzyme.

2. Gene Isolation

When the source of genetic material is identified, the next step is the cloning of the gene encoding the protein of interest from the selected strain. A number of approaches concerning gene isolation and cloning have been published (for review see Timberlake, 1991; Finkelstein 1991). For the cloning of genes encoding identified enzymes, the most commonly used method is the so-called "reverse genetics". The desired enzymes are purified from the culture filtrate or mycelial

extract of the selected strain. From the purified protein a part of the amino acid sequence (N-terminus of the complete protein or tryptic fragments) is resolved by Edman degradation. Based on the identified amino acid sequence an oligonucleotides mixture is designed. These oligonucleotides are then used for screening a genomic or c-DNA library constructed from DNA or mRNA of the selected strain. In the majority of cases reported in the literature genes encoding the proteins of interest have been cloned using this approach (see also Table 1). Recently this approach has been further improved by the application of the polymerase chain reaction (PCR) technology (Sambrook et al., 1989). A (homologous) probe may be generated by amplification of a DNA fragment using degenerated oligonucleotide derived from two distinct amino acid sequences. This approach considerably simplifies subsequent screening of a genomic gene library as homologous hybridization conditions can be applied. To date the PCR approach has been used to clone the genes encoding aspartic protease from *Cryphonectria parasitica* (Choi et al., 1993) and acid protease from *A. oryzae* (Gomi et al., 1993).

When the exact amino acid sequence for the protein of interest is not known, similar strategies can also be used based on sequence data from corresponding genes from other strains or species. This approach is particularly useful when the gene is a member of a gene family of which one of the members has already been cloned from the same species (e.g. Kusters-van Someren, 1991; Bussink et al., 1992) or is homologous to a cloned gene from a related species (e.g. Shibuya et al., 1990; Bussink et al., 1991a). In that case the whole gene can be used as probe for screening.

A completely different gene cloning approach is based on immunochemical screening. For this approach cDNA is synthesized from isolated poly(A)$^+$ RNA and is used to construct an expression library in phage lambda. The expressed protein of interest, present in a plaque(s), is identified in a Western blot-like procedure by probing a filter to which the produced protein is bound with antibodies raised against this protein. With this approach, amongst others, the α-L arabino-furanosidase A gene (Flipphi et al., 1993) and lignin peroxidase encoding genes (Saloheimo et al., 1989) have been cloned (see also Table 1).

Cloning of genes can also be achieved by direct complementation. This

approach is feasible when a recipient strain (mutant strain or another species) lacking the enzymatic activity of interest and a simple selection or screening method is available. Following this approach the invertase genes of *Neurospora crassa* and *A. niger* have been cloned by complementation, in a *Saccharomyces cerevisiae* mutant (Carú et al., 1989) and in a sucrose non-utilising species of *Trichoderma* (Bergès et al., 1993), respectively.

3. Gene Expression

Overproducing strains are generally constructed by the introduction of multiple copies of the gene of interest. Direct selection for strains carrying multiple copies of the cloned gene is not possible in most cases. Therefore, initially indirect selection of multi-copy strains is carried out by using an (additional) selection marker. This marker gene is inserted on the same vector as the gene of interest or in case of cotransformation present on a separate vector. Three kinds of selection marker can be used: auxotrophic markers, resistance markers (conferring e.g. hygromycinB or phleomycin resistance) and a dominant nutritional marker (e.g. *amdS)* (for review see Finkelstein, 1991). In all cases transformants are obtained in which the introduced sequences are stably integrated at the genome by either homologous or heterologous recombination. The efficiency of transformation and the number of integrated plasmids depends on the recipient strain, the selection marker and the method of selection. In particular the use of the *amdS* gene as selection marker results in transformants carrying multiple copies of the transformed sequences (Finkelstein, 1991 and references therein).

In most of the initial studies the genes of interest are expressed using their own transcription control sequences (see Table 1). However, in some cases the expression level obtained in strains carrying multiple copies of the wild-type gene was not, or only modestly, increased. This may be a result of the relatively low efficiency of these expression control sequences or of other gene control mechanisms. Improvement of the expression level of the gene of interest can be achieved by placing it under control of the expression signals from a highly

expressed gene. In general, two types of efficient transcription control sequences are used: from constitutively expressed genes (e.g. *gpdA* and *pki*; Table 1) or from genes with a regulated expression (e.g. *amy, glaA and cbh1*; Table 1).

4. Protein (over)production

In many cases, introduction of multiple copies of a gene of interest has resulted in a considerable increase in protein production (Table 1). The improvement factor is significantly higher than the factor obtained with classical strain improvement strategies. Although the improvement factor for the best producing transformant from each study is indicated, strains with an increased production level could be easily found amongst a set of transformants.

In most studies the transformants obtained were only analyzed for protein production, whereas in a limited number of studies the number of gene copies was also determined. To evaluate whether these two parameters are correlated, available data about production level and copy number have been displayed graphically in Figure 1. From this figure it is clear that, in general, an increase in the number of gene copies results in a higher production level. Taken together, the results indicate a more or less linear dose-response relation with the small number of gene copies (< 5), whereas no dose-response relation is observed with higher copy numbers. With higher copy numbers the production levels are in nearly all cases lower than expected from the copy number of the gene. The results represented in figure 1 also indicate a considerable variation in production level in transformants with a comparable number of gene copies suggesting that besides the number of gene copies, also e.g. the site of integration may affect the expression of the introduced genes.

In a few cases where the level of mRNA was analyzed in the overproducing strains this analysis indicated that the levels of protein production correlated with the observed mRNA levels. Combining the available data concerning copy number, mRNA level and level of protein production these results indicate that in all cases overproduction is limited at the level of transcription.

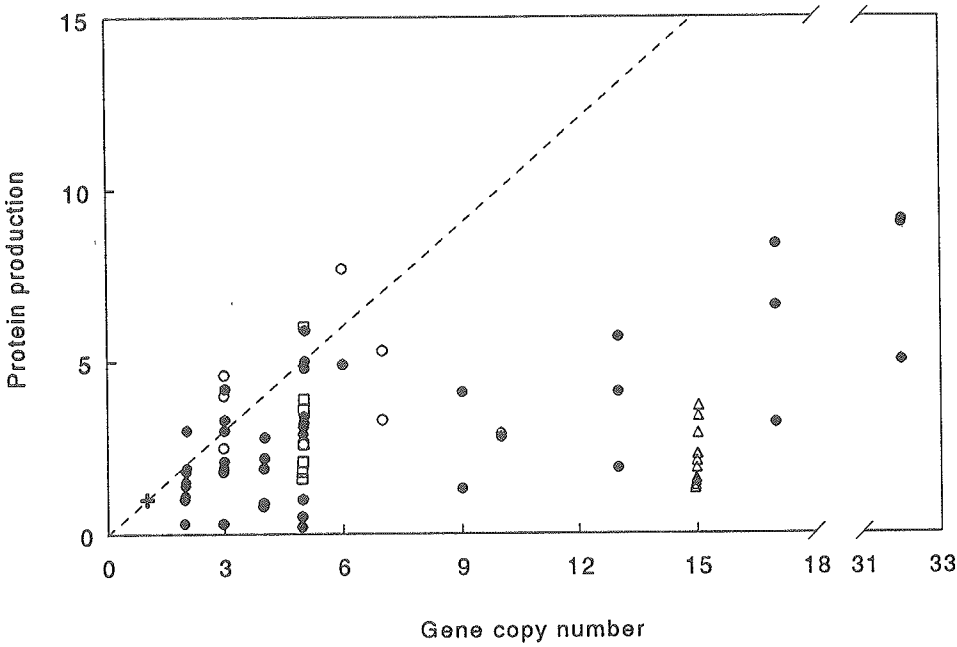

Figure 1. Protein production in multi-copy strains in relation to gene copy number. The protein production is given relative to the level of that of the wild-type strain (=1). The protein production in the wild-type recipient strain (carrying a single gene copy) is indicated by a +. The production levels reported for a number of multi-copy strains are indicated: ●, glucoamylase in *A. niger* (Finkelstein, 1989); □, α-amylase in *A. oryzae* (Tada et al., 1989), ○, glucoamylase in *A. oryzae* (Hata et al., 1991) and; △, cellobiohydrolase II in *T. reesei* (Kubicek-Pranz et al., 1991). In those cases where the copy number for each transformant was not specified; the mean value of the copy number determined was used. The dashed line represents a linear dose-response relation extrapolated from the production level in the wild-type strain.

As already indicated in section IIB, an increase in the level of transcription can be achieved using expression signals of highly expressed fungal genes. The use of heterologous transcription control sequences (*glaA*, *gpdA* and *cbh1*; see Table 1) resulted in a considerably improved production as was shown for the production of phytase in *A. niger* (van Gorcom et al., 1990) and *A. awamori* (Houston et al., 1992), aspartic protease in *A. nidulans* (Ward and Koama, 1991), acid phosphatase (Houston et al., 1992) and glucoamylase P in *T. reesei* (Joutsjoki et al., 1993).

Table 1. (Over)production of extracellular fungal proteins in filamentous fungi

PROTEIN [A]	CLONING STRATEGY [B]	PRODUCTION ORGANISM	EXPRESSION SIGNAL [C]	YIELD [D]	IMPROVEMENT FACTOR [E]	REFERENCE
Alkaline protease (*A. oryzae*)	het	*A. oryzae*		41,500 U/g P	5	Chevadhanarak et al., 1991
α-Amylase (*Aspergillus oryzae*) (TAKA-amylase)	rev	*A. oryzae*		49,000 U/g	5	Tada et al., 1989
				12 g/l F	12	Christensen et al., 1988
		A. awamori		10.3 U/g P	< 1	Ruttkowski et al., 1989
		Trichoderma viride		4 mg/l P	-	Cheng et al., 1990
			cbh1	1 g/l P	-	Cheng and Udaka, 1991
		A. nidulans		2070 U/g	-	Lachmund et al., 1993
α-L-Arabinofuranosidase A (*A. niger*)	imm	*A. niger*		2.48 U/ml	8	Flipphi et al., 1993
		A. nidulans		2.64 U/ml	9	
Aspartic protease (*A. oryzae*)	rev	*A. oryzae*		5236 U/g	6	Gomi et al., 1993
Aspartic protease (Cryphonectria parasitica)	rev	*C. parasitica*		N.R.	7	Choi et al., 1993
Aspartic protease (*Mucor miehei*)	rev	*A. nidulans*		2 mg/l	-	Gray et al., 1986
			glaA	0.15 g/l	-	Ward and Kodama, 1991
		A. awamori	*glaA*	0.15 g/l	-	
			glaA	0.8 g/l F P	-	
			glaA	3.0 g/l M	-	
			glaA	1.97 g/l M	-	Ward et al., 1993
		A. oryzae	*amy*	0.15 g/l	-	Christensen et al., 1988
			amy	3.3 g/l F	-	
			glaA	0.15 g/l	-	Boel et al., 1987
			glaA	0.4 g/l F	-	
		M. circinelloides		1.2 mg/l	-	Dickinson et al., 1987

Table 1 (continued)

PROTEIN [A]	CLONING STRATEGY [B]	PRODUCTION ORGANISM	EXPRESSION SIGNAL [C]	YIELD [D]		IMPROVEMENT FACTOR [E]	REFERENCE
Catalase * (*A. niger*)	rev	*A. niger*		3.7 U/mg		5	Fowler et al., 1993
			glaA	11 U/mg		10	
Cellobiohydrolase II (*T. reesei*)	rev	*T. reesei*		37 μg/l		4	Kubicek-Pranz et al., 1991
Endoglucanase I (*T. reesei*)	imm	*T. reesei*	*cbh1*	N.R.		2	Uusitalo et al., 1991
α-Galactosidase (*A. niger*)	rev	*A. niger*	*glaA*	N.R.		3	Den Herder et al., 1992
Glucoamylase (*A. niger*)	het	*A. niger*		4.0 g/l		8	Finkelstein et al., 1989
				9.0 g/l	F	18	
				7.5 g/l	R	15	
			alcA	1.2 g/l	F	-	Davies et al., 1989
			gpdA	1.1 mg/g		3.7	Punt et al., 1990
		A. awamori		4.6 g/l	P	9	Finkelstein et al., 1989
		A. nidulans	*gpdA*	0.2 mg/g		< 1	Punt et al., 1990
Glucoamylase (*A. oryzae*)	rev	*A. oryzae*		29.4 U/ml		8	Hata et al., 1991
Glucoamylase (*A. shirousami*)	het	*A. oryzae*		4970 U/g		< 1	Shibuya et al., 1990
Glucoamylase P (*Hormoconis resinae*)	rev	*T. reesei*		N.P.		-	Joutsjoki and Torkkeli, 1992
			cbh1	0.7 g/l	P	20	Joutsjoki et al., 1993
Glucoamylase (*Humicola grisea* var. *thermoidea*)	rev	*H. grisea*		2.8 g/l	P	3	Allison et al., 1992
		A. awamori		0.66 g/l	F	-	Berka et al., 1988

Table 1 (continued)

PROTEIN [A]	CLONING STRATEGY [B]	PRODUCTION ORGANISM	EXPRESSION SIGNAL [C]	YIELD [D]	IMPROVEMENT FACTOR [E]	REFERENCE
Glucose oxidase * (*A. niger*)	rev	*A. niger*		13.8 U/mg	6	Whittington et al., 1990
				0.35 U/ml	3	Sharif and Alaeddinoglu, 1992
				1.22 U/ml M	10	
				5.5 U/mg	3.5	Witteveen et al., 1993
		A. nidulans		5.4 U/mg	2.2	Whittington et al., 1990
				2.7 U/mg F	1.8	Witteveen et al., 1993
β-Glucosidase (*T. reesei*)	rev	*T. reesei*		33 U/mg F P	5	Barnett et al., 1991
Invertase (*Neurospora crassa*)	com	*N. crassa*		1.74 U/mg	< 1	Carú et al., 1989
Invertase (*A. niger*)	com	*T. reesei*		0.47 U/mg	< 1	Bergès et al., 1993
Lignin oxidase (*Phlebia radiata*)	imm	*T. reesei*	*cbh1*	20 mg/l F P	-	Saloheimo et al., 1991
Lignin peroxidase (*P. radiata*)	imm	*T. reesei*	*cbh1*	N.P. P	-	Saloheimo et al., 1989
Lipase (*H. lanuginosa*)	rev	*A. niger*	*amy*	N.R.	-	Boel and Huge-Jensen, 1989
		A. oryzae	*amy*	N.R.	-	
Pectin lyase A (*A. niger*)	het	*A. niger*		N.R.	-	Harmsen et al., 1990 and Kusters-van Someren, 1991
		A. nidulans		N.R.	-	
Pectin lyase B (*A. niger*)	het	*A. niger*		N.P.	-	Kusters-van Someren et al., 1992
			pki	N.R.	-	
		A. nidulans		N.R.	-	
Pectin lyase C (*A. niger*	het	*A. niger*		N.P.	-	Kusters-van Someren, 1991
		A. nidulans		N.P.	-	

Table 1 (continued)

PROTEIN [A]	CLONING STRATEGY [B]	PRODUCTION ORGANISM	EXPRESSION SIGNAL [C]	YIELD [D]	IMPROVEMENT FACTOR [E]	REFERENCE
Pectin methyl esterase (*A. niger*)	rev	*A. niger*		45 U/mg	20	Khanh et al., 1991
			amy	20 U/mg	9	Khanh et al., 1992
			gpdA	17 U/mg	8	
Phosphatase (acid) (*A. niger*)	rev	*A. niger*		N.R. P	24	Piddington et al., 1993
			gpdA	N.R. P	75	Houston et al., 1992
			glaA	N.R. P	70	
Phytase (*A. awamori*)	rev	*A. awamori*		N.R. P	330	Piddington et al., 1993
			glaA	N.R. P	1930	Houston et al., 1992
Phytase (*A. niger var. ficuum*)	rev	*A. niger (var.ficuum)*		270 U/ml	450	Van Gorcom et al., 1990
		A. niger		95 U/ml	160	
			glaA	280 U/ml	460	
Polygalacturonase I (*A. niger*)	rev	*A. niger*		N.R.	10	Bussink et al., 1991b
		A. nidulans		510 U/ml	-	Bussink et al., 1992
Polygalacturonase II (*A. niger*)	rev	*A. niger*		88 U/ml	50	Bussink et al. 1990, 1991a and 1992
Polygalacturonase II) (*A. tubigensis*)	het	*A. niger*		N.R.	10	Bussink et al., 1991a
Polygalacturonase C (*A. niger*)	het	*A. niger*		N.R.	-	Bussink et al., 1992
		A. nidulans		33 U/ml	-	Bussink et al., 1992
Triglyceride lipase (*M. miehei*)	rev	*A. oryzae*	*amy*	2 mg/l	-	Huge-Jensen et al., 1989

Table 1 (continued)

PROTEIN [A]	CLONING STRATEGY [B]	PRODUCTION ORGANISM	EXPRESSION SIGNAL [C]	YIELD [D]	IMPROVEMENT FACTOR [E]	REFERENCE
Xylanase (*A. awamori*)	rev	*A. awamori*		78 kU/ml	6	Van Gorcom et al., 1991
			glaA	71 kU/ml	5	
			gpdA	32 kU/ml	2	
		A. niger		140 kU/ml	10	
			glaA	72 kU/ml	5	
			gpdA	12 kU/ml	1	

[A] Between brackets the species from which the gene was isolated is indicated. * both intra-and extracellular production.

[B] The strategy of cloning (for description of the methods see section IIB2): com, complementation; het, heterologous hybridization using sequence data from related genes; imm, immunochemical screening and; rev, reverse genetics.

[C] If not indicated homologous expression signals are used. Heterologous transcription control sequences used are: *alcA*, alcohol dehydrogenase I gene of *A. nidulans*; *amy*, α-amylase gene of *A. oryzae*, *cbh1*, cellobiohydrolase I gene of *T. reesei*; *glaA*, glucoamylase gene of *A. niger* sp; *gpdA*, glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus* sp.; *pki*, pyruvate kinase gene of *A. niger*.

[D] The amount of protein of the best producing transformant is indicated. Due to the different quantification methods to indicate yields, comparison of the yields, expressed as improvement factor, is only possible within one study. Yields are indicated as: amount of protein per volume culture fluid (m)g/(m)l; specific enzyme activity (Units) per gram dry weight mycelia U/gr; Units per volume culture fluid U/(m)l or amount of protein per gram dry weight mycelium mg/g. N.R. indicates that the amount of protein produced is not reported; N.P. indicates that no protein is produced. If not indicated production was determined in shake flask cultures. Special production conditions are indicated by; F, production level determined in a controlled fermentation process; P, production level obtained in production strain (see section IIB5) in which multiple copies were introduced; M, production level obtained after mutagenesis of a multi-copy transformant; and R, production level in a haploid segregant obtained after genetic recombination of multi-copy transformants.

[E] The increase in production in relation to the production level obtained with the strain from which the gene was isolated cultivated under inducing conditions is indicated as improvement factor. In several cases this factor could not be determined as either the production level in the strain from which the gene was isolated is unknown or the production level of the transformant has not been quantified.

However, for the production of pectin methyl esterase (Khanh et al., 1992) and xylanase (van Gorcom et al., 1991) it was shown that the use of heterologous expression signals did not result in a further increase in the level of protein production compared to the homologous transcription control sequences. It should be noted that in these studies strains with different copy numbers, integration sites and different culture conditions have been compared precluding unambiguous conclusions of the expression signals used.

The amount of protein found in the culture medium is not only influenced by the level of gene expression. In several studies the effect of protein degradation has also been reported. In a study aimed at overproduction of pectin lyase B (Kusters-van Someren, 1992) no increase in protein production was found although an increase in *pelB* specific mRNA level was observed in the transformants. Degradation of the protein by proteolytic enzymes in the medium was shown to be the major reason for this result. Changing cultivation conditions eventually resulted in increased production levels (Kusters-van Someren, 1992). Similar situations may have affected the results of studies intended to overproduce lignin peroxidase (Saloheimo et al., 1989) and pectin lyase C (Kusters-van Someren, 1992).

Obviously, not only the production level but also the (specific) activity and / or authentic structure of the recombinant protein produced are important aspects. Concerning enzyme activity, it was shown that limited proteolytic processing of recombinant proteins as e.g. observed for aspartic protease (Ward and Kodoma, 1991) and triglyceride lipase (Huge-Jensen et al., 1989) may result in inactivation of the protein produced (Ward and Kodoma, 1991). Also other types of protein modification such as glycosylation may affect enzymatic activity. In several cases where a protein was produced in a heterologous fungal host overglycosylation was observed compared to the protein produced in its natural host (e.g. Flipphi et al., 1993; Boel and Huge-Jensen, 1989; Joutsjoki et al., 1993 and; Ward and Kodama, 1991). However in none of these cases it was reported that this type of modification affected the (specific) activity of the recombinant protein.

5. Additional Strain Improvement Methods

The results presented in Table 1 indicate that considerable success in fungal protein overproduction in filamentous fungi has already been obtained using laboratory strains and shake flask fermentation. A number of approaches for further improvement of the production levels of multi-copy strains have been employed. These include both traditional methods (see section IIa) of strain improvement (e.g. Barnett et al., 1991; Piddington et al., 1993) or recombinant DNA techniques (e.g. Uusitalo et al., 1991; Ward and Kodama, 1991). In both cases the approaches were aimed at the isolation of an improved recipient strain with either improved secretion capacity (e.g. Finkelstein, 1989 and Piddington et al., 1993) and / or reduced extracellular protease levels (e.g. Cheng et al., 1990 and; Ward and Kodama, 1991), Unfortunately no systematic comparison of the mutant strain (P(roduction) strains in Table 1) and the wild-type strain have generally been made, precluding clear conclusions on the effect of these mutations on protein production. Traditional mutagenesis approaches can also be used to improve enzyme production of multi-copy strains constructed (M(utagenized) strains in Table 1). Significant improvement of protein production by mutagenesis was found for glucose oxidase (Sharif and Alaeddinoglu., 1992) and aspartic protease (Ward and Kodama, 1991) and by genetic recombination for glucoamylase (Finkelstein, 1989).

Another field of improvement of the production level in multi-copy strains is medium development (e.g. Smiley, 1967; Smith and Wood, 1991) and controlled (large scale) fermentation (for reviews Greasham, 1991; Dunn-Coleman, 1992). In a few cases (F strains in Table 1) large scale fermentations were carried out which resulted in a further increase in protein production (Finkelstein, 1989 and Ward and Kodama, 1991). Especially in relation to large scale fermentation the use of well defined (induction) processes will be of considerable importance. For a number of fungal proteins (e.g. glucoamylase) the fermentation conditions have been optimized for the production process and / or production strain. It is in this respect that the use of heterologous expression signals in particular those of the *glaA* gene

may be of great importance. This may allow high level production of proteins under the optimized culture conditions for *glaA* expression as was shown for e.g. phytase (van Gorcom et al., 1990) and aspartic protease (Ward and Kodama, 1991).

The use of heterologous expression signals can also have other advantages. It is possible to produce the protein of interest under (induction) conditions completely different from the wild-type situation which may be favourable e.g. when the protein of interest is sensitive for degradation by proteases produced under the wild-type induction conditions (e.g. PELB; Kusters-van Someren, 1991). The use of heterologous expression signal can also be used to uncouple the production of different proteins. This will especially be useful e.g. when the gene encoding the protein of interest is a member of a gene family of which, in the wild-type strain, all members are expressed under the same cultivation conditions (Den Herder et al., 1992; Kusters-van Someren, 1992).

IV. OUTLINE OF THIS THESIS

As illustrated in this chapter a large number of examples have been described in the literature of overproduction of enzymes in filamentous fungi by genetic engineering techniques. Most of the studies only provide quantitative data concerning the overexpression. In a limited number of studies, the overproducing strains were analyzed further. In those cases the data obtained indicate that there is no clear relation between the number of introduced gene copies and the amount of protein produced (Fig. 1). Detailed studies to identify the limiting factor(s) and to subsequently improve the production by overcoming these limitations have not been carried out. Therefore, a systematic study on the effect(s) and limitation(s) of overproduction of extracellular enzymes in filamentous fungi by genetic engineering was started. As a model study the overproduction of homologous glucoamylase (GLA) in a wild-type *A. niger* strain was initiated. Eventually, the identification of limiting steps should lead to a better understanding of the mechanisms involved in protein overproduction and suggestions for new strategies for the construction of

improved overproducing filamentous fungi.

GLA overproduction in *A. niger* was chosen as a model system for a number of reasons. Firstly, GLA (amyloglucosidase; γ-amylase; 1,4-α-D-glucan glucohydrolase EC 3.2.1.3) is an extracellular glycoprotein, with industrial applications (for review Bigelis, 1991). The protein catalyses the release of β-D-glucose units from the nonreducing-ends of starch and other related oligo-and polysaccharides. GLA attacks α-1,4 as well as α-1,6 and some α-1,3 glycosidic linkages although these latter two with lower affinity. GLA of *A. niger,* which is known to digest raw starch, is used for the production of glucose syrup by the saccharification of starch from various sources (e.g. corn, barley and potato). This syrup is further used for the production of dextrose, fructose syrup, or for the fermentation to ethanol.

Secondly, GLA encoding genes from *A. niger* and a number of other fungal species have been cloned and characterized (for reviews Berka et al., 1991 and Sakaguchi et al., 1992). The amino acid sequence suggests that GLA in *A. niger* has three domains: 1) a large catalytic domain (aa 1 - 440); 2) a C-terminal starch binding domain (aa 513 - 616); and 3) an *O*-glycosylated region (aa 441-512) linking the catalytic and starch binding domain (Fig. 1). GLA can be isolated from the culture medium in two forms; a larger form designated G1, and a smaller form (G2) which lacks the starch binding domain.

Thirdly, the expression of the *glaA* gene is regulated. The level of *glaA* specific mRNA isolated from cultures cultivated on different carbon sources appeared to correlate with the amount of GLA produced, indicating that the regulation of the GLA production occurs at the level of transcription (Fowler et al., 1990), most probably through the action of *trans*-acting regulatory proteins. The highest levels of GLA are produced using maltose, maltodextrin or starch.

After the overview of results and current knowledge concerning various strategies of strain improvement given in this chapter, the construction of *glaA* multi-copy strains is described in Chapter 2. In most of the transformants analyzed, a more than 5-fold increase in GLA production is observed. Analysis of the transformants indicated that the expression of the *glaA* genes in many of these

strains is limited at the level of transcription.

To determine into which chromosome(s) the introduced *glaA* genes had been integrated, genetic analysis of these multiple copy strains is carried out (Chapter 3). In each transformant integration occurred at a single chromosome, presumably at a single site. In combination with the results described in chapter 2, the results of genetic analysis suggest that in the transformants expression of the introduced genes is affected by the site of integration. In this chapter we also descibe approaches to further improve GLA production in multi-copy strains by the introduction of additional copies of the *glaA* gene, either by recombination or retransformation. However, in none of the strains was there a significant increase in GLA production, in contrast, in some strains there was a decrease in GLA production. The results of a detailed analysis of one of these strains confirm that the expression of the *glaA* gene is regulated by *trans*-acting regulatory factor(s).

The results from Southern analysis (Chapter 2) and genetic analysis (Chapter 3) of *glaA* multi-copy strains revealed that the vectors introduced by transformation integrated at a single chromosome in each transformant. The integration of a large number of gene copies as found in some transformants results in chromosomes with an altered electrophoretic mobility. The use of some of these transformants for chromosome assignment of any cloned gene in *A. niger* is described in chapter 4.

In Chapter 5 the regulation of transcription of the *glaA* gene is analyzed. The results from deletion and titration analysis of the *glaA* promoter confirms that, as already suggested from the data presented in Chapter 3, transcription of the *glaA* gene is regulated by *glaA* specific regulatory proteins and reveals which part of the promoter region is involved in transcription regulation. The results suggest also that the GLA overproduction observed in the multi-copy strains (Chapter 2) is most probably limited by the available amount of (one of) these regulatory proteins.

To further improve GLA production in multi-copy strains, it will probably be necessary to increase the expression level of gene(s) encoding the regulatory protein(s). For the isolation of (one of) these genes, a new cloning strategy in *A. niger* based on the use of an autonomously replicating plasmid was developed

(Chapter 6).

In Chapter 7, the results described in this thesis are discussed in relation to the construction of a second generation of overproducing strains.

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CHAPTER 2

Glucoamylase overexpression in *Aspergillus niger*: molecular genetic analysis of strains containing multiple copies of the *glaA* gene

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(transcription regulation; regulatory protein; *amdS* selection marker; cosmid vector; CHEF analysis)

SUMMARY

A strategy, based on the usage of the *amdS* selection marker and a cosmid vector containing 4 copies of the glucoamylase gene (*glaA*), was developed to obtain glucoamylase (GLA) overproducing *A. niger* strains. With this strategy fungal strains could be isolated at a relatively high frequency which carry up to 200 copies of the *glaA* gene. In each transformant analysed integration occurred at a single chromosome. A significant increase in the extracellular GLA production was observed in most of the transformants carrying multiple copies of the *glaA* gene. Further analysis showed that the amount of GLA that is produced was not proportional to the number of *glaA* copies in these transformants. However, the level of GLA production clearly correlated with the amount of *glaA* mRNA produced in these transformants. From these results it is concluded that GLA production is limited at the level of transcription.

INTRODUCTION

Filamentous fungi, especially several *Aspergillus* species, are widely used in the fermentation industry for the production of (extracellular) enzymes. Obviously, there is considerable interest in methods to improve the production of these enzymes. Traditionally, this was achieved by strain improvement based on a time-consuming process of rounds of mutagenesis and recurrent selection (Ward, 1989). With the development of recombinant DNA techniques strain improvement by increasing the efficiency of expression of the gene of interest has become feasible. One way to achieve this is by increasing the number of copies of the gene of interest. In general, an increase of the number of copies results in an increased production of the enzyme encoded by the amplified gene.

For the application of genetically engineered strains in industrial fermentation, which is carried out in long time processes in large fermentation vessels with complex growth media, it is of importance that the multiple gene copies are stably maintained. Furthermore, the newly introduced gene copies should be expressed under conditions provided in the production process. At present, little knowledge is available about the stability of genetically engineered strains of *Aspergillus niger* under the physiological conditions of protein production. As a first step in this line of research, *A. niger* strains have been constructed containing multiple copies of the *A. niger* gene encoding the extracellular protein glucoamylase [GLA; α-(1-4),(1-6)-D-glucan glucohydrolase (EC 3.2.1.3)]. The *glaA* gene was chosen, since under appropriate induction conditions GLA is efficiently produced in the wild-type strain. Furthermore, detection of GLA is facilitated by the availability of polyclonal antibodies and a relatively simple activity assay. Isolation of the *glaA* gene from the *A. niger* strain N402, which has been used in many genetical and molecular genetical studies including this study, was facilitated by the data available from cloned *glaA* genes from other *Aspergillus* strains (Boel et al., 1984; Nunberg et al., 1984 and Hata et al., 1991). In addition, some data have been reported on the regulation of *glaA* expression (Nunberg et al., 1984 and Fowler et al., 1990).

In this paper the development of an efficient procedure is reported to obtain and select *glaA* multi-copy transformants of *A. niger*. In addition, an extensive molecular analysis of these transformants is described. Part of this work has appeared previously in preliminary form (van den Hondel et al., 1992).

MATERIALS AND METHODS

Strains and transformation procedures

A. niger N402 (*cspA*1 derivative of ATCC9029; Bos, 1986) was used for the isolation of the *glaA* gene and as recipient strain in transformation experiments. A glucoamylase deletion strain of *A. niger* N402 was constructed using a gene replacement strategy (van Hartingsveldt et al., 1990). In the resulting strain, N402ΔGLA, the coding region of the *glaA* gene is replaced by a fragment carrying a phleomycin resistance gene under control of the *gpdA* promoter and the *trpC* terminator region of *A. nidulans* (I. v.d. Veen and J.C. Verdoes, unpublished).

E. coli K12 strain JM109 (Yanisch-Perron et al., 1985) was used for transformation and propagation of plasmids. *E. coli* transformation was carried out as described previously (Hanahan, 1983). *E.coli* 1046 (Cami et al., 1978) was used in transfection experiments. An *in vitro* packaging kit (Gigapack XL, Amersham) was used for transfection experiments. Fungal transformations were carried out as described previously (Goosen et al., 1987).

Molecular methods

Fungal DNA isolations were performed as described by Kolar et al.(1988). Fungal RNA isolations were carried out using the RNAzol™ kit from CINNA/BIOTECX. Preparation of intact chromosomal DNA and CHEF electro-phoresis were carried out as described by Debets et al. (1990c). All other molecular methods were essentially as described in Sambrook et al. (1989).

Construction of *glaA* expression vectors

The *glaA* gene was isolated from a λ EMBL genomic library of *A.niger* N402 using an oligonucleotide, corresponding to the N-terminal part of the *glaA* sequence of *A. awamori* (5' tgctgaggtgtaatgatctctggg 3'; Nunberg et al., 1984), as a probe. From one of the hybridizing λ clones a 8.2-kb *Nco*I (partial) - *Xba*I fragment was isolated and cloned in pMTL25P, yielding pAB6-7.

Considering the results obtained by Kelly and Hynes (1985), the *amdS* gene was used as selection marker for the construction of *A. niger* strains carrying multiple copies of the *glaA* gene. Two glucoamylase expression vectors were constructed, pAB6-8 and pAB6-10 (Fig. 1). Vector pAB6-8, which contains a single copy of the *glaA* gene was obtained by cloning a 8.2-kb *Hin*dIII fragment of pAB6-7 in the *Hin*dIII site of pAN4.1. Transformation vector pAN4.1 was constructed by cloning an *Eco*RI-*Kpn*I fragment derived from pGW325 (Wernars et al., 1986), containing the *amdS* gene, in the corresponding sites of pUC19 (W. van Hartingsveldt and M. Harteveld, unpublished). Vector pAB6-10, which is a cosmid vector, contains 4 copies of the *glaA* gene. This vector was obtained by cloning 8.2-kb *Hin*dIII *glaA* fragments from pAB6-7 in the *Hin*dIII site of pAN4-cos1. Vector pAN4-cos1 was generated by cloning the 5.0-kb *Eco*RI-*Sal*I fragment from p3SR2 (Hynes et al., 1983), containing the *amdS* gene from *A. nidulans*, in pJB8 (Ish-Horowitz et al., 1981) digested with *Hpa*I and *Sal*I (R.F.M. van Gorcom, unpublished). Initially, ligation mixtures for the construction of pAB6-10 were packaged and transfected into *E. coli* JM109. DNA isolated from transfectants obtained in JM109 revealed structural instability of the cosmids. This problem could be largely solved using *E. coli* 1046 as host strain. Interestingly, all stable cosmids contained the copies of the *glaA* gene in a head to tail orientation.

Methods for protein analysis

Western analysis using polyclonal antibodies raised against purified glucoamylase (Punt et al., 1990) was carried out as described previously (Sambrook et al., 1989).

For iso-electric focusing (IEF) analysis SERVALYT^R Precotes^R gels (pH 3-6) were used. Glucoamylase activity was measured as described by Metwally et al. (1991). The concentration of protein was determined with the Biorad protein assay kit using purified glucoamylase (lot 12005320-30; Boehringer-Mannheim, F.R.G.) as standard.

Fungal growth conditions

All liquid cultures were carried out in 300 ml Erlenmeyer flasks containing 50 ml growth medium, inoculated with 5 x 10^7 fungal spores. Minimal growth medium (Bennett and Lasure, 1991) with 5 % maltodextrin as carbon source was used. Flasks were incubated at 30 °C in a rotary shaker revolving at 300 r.p.m.. Unless indicated otherwise, all samples were taken after 48 h of incubation.

RESULTS

Isolation of multi-copy transformants

Vectors pAB6-8 and pAB6-10 were introduced into *A. niger* N402 and several independent AmdS$^+$ transformants were obtained. For selection of amdS$^+$ transformants agar plates containing acetamide or acrylamide as sole nitrogen source were used (Kelly and Hynes, 1985). As overexpression of GLA could have adverse effects on the growth of multi-copy transformants, selection was carried out on osmotically stabilised agar medium containing xylose. Xylose is known to repress *glaA* expression (Nunberg et al., 1984 and Fowler et al., 1990).

To select transformants containing different numbers of vector copies, a number of the AmdS$^+$ transformants were tested for their growth characteristics on agar plates containing acetamide or acrylamide as sole N(itrogen)-source. As already described by Kelly and Hynes (1985), transformants which can grow on agar plates containing either acetamide or acrylamide generally contain more *amdS* copies than those that only grow on agar plates containing acetamide. For a further selection of *A. niger*

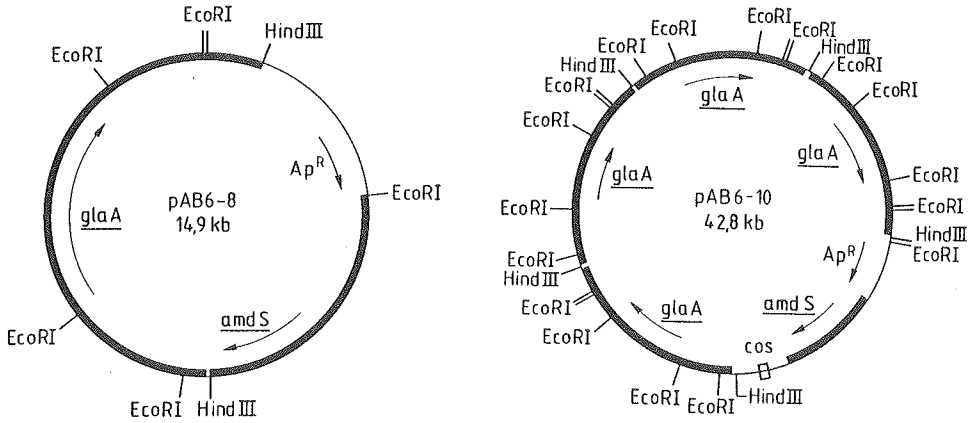


Figure 1. The *glaA* expression vectors pAB6-8 and pAB6-10.

strains with a very high number of *amdS* gene copies advantage was taken of the observation by Kelly and Hynes (1985) that a slight inhibition of growth occurs with *amdS* multiple copy strains in liquid media containing ω-aminoacids (β-alanine or γ-amino butyric acid (GABA)) as sole N-source. As suggested by the authors, this inhibition of growth is probably due to the fact that in these *A. niger* strains a regulatory protein, similar to the *amdR* protein of *A. nidulans* (Andrianopoulos et al., 1990), was titrated out (Kelly and Hynes, 1987). After testing different agar media containing combinations of ω-amino acids it was found that a combination of 50 mM β-alanine and 20 mM GABA as sole N- and C-sources was the best medium composition to identify strains which carry a very high number of *amdS* gene copies (results not shown). Therefore, this composition was used to select very high copy strains.

A number of putative transformants, obtained after transformation of *A. niger* N402 with pAB6-8 or pAB6-10, were purified. No indications for genetic instability were observed during subculturing of single conidiospore colonies. Twenty-three pAB6-8 transformants were tested for their growth characteristics on agar plates. Fourteen transformants showed strong growth on acrylamide and 4 out of these 14 showed

clear inhibition of growth on ω-amino acids. From the 31 pAB6-10 transformants tested, 20 showed strong growth on acrylamide, of which 3 showed inhibition of growth on ω-amino acid medium. A number of pAB6-8 and pAB6-10 transformants with different growth characteristics were chosen for further analysis.

DNA analysis of multi-copy transformants

The number of *glaA* gene copies in the selected AmdS$^+$ multi-copy transformants was determined by Southern analysis (Fig. 2). The copy number of the *glaA* gene in pAB6-8 transformants ranged from 2 to 60. In the pAB6-10 transformants the copy number of the *glaA* gene ranged from 12 to more than 200 copies (Table 1). From these results it is clear that a significantly higher copy number can be obtained with a vector containing multiple copies of the *glaA* gene than with a vector with a single copy of the *glaA* gene. Furthermore, a relationship has been found between the growth characteristics of the transformants and the copy number of the *glaA* gene (Table 1). Southern analysis revealed, besides the expected hybridization pattern (3.3-kb fragment in an *Eco*RI digest), some extra (minor) bands hybridizing with the *glaA* probe in most of the transformants (only seen after longer exposure). These could originate from vector-genome fragments created by the integration event, whereas also rearrangements or deletions can not be excluded. Interestingly, the ethidium bromide stained agarose gel of digested DNA of strains carrying the highest number of copies revealed, besides the rDNA bands, the presence of additional, very intensively fluorescing bands (Fig. 2A, lanes 4 and 6). These additional bands correspond with the position of *Eco*RI fragments of the vector pAB6-10 (lane 8) in the gel. From the intensity of these additional bands and those of the rDNA bands it can be concluded that the number of copies of the introduced *glaA* genes is larger than the number of copies of the rDNA repeat, which based on data for other fungi related to *A. niger* (Timberlake, 1978 and Borsuk et al., 1982), is assumed to be between 60 and 300 per haploid genome. These data correspond well with our data obtained with Southern analysis (Table 1).

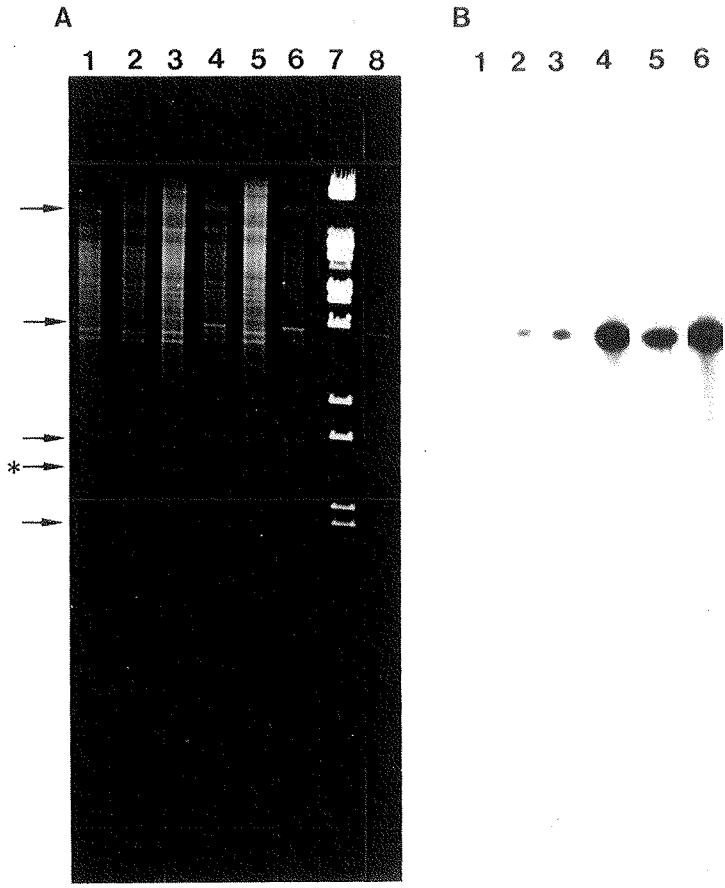


Figure 2. Determination of *glaA* copy number.
A. Ethidium bromide stained agarose gel of *Eco*RI digested chromosomal DNA. *Eco*RI digests of 1 = N402, 2 = N402[pAB6-10]B1, 3 = N402[pAB6-10]B3, 4 = N402[pAB6-10]B13, 5 = N402[pAB6-10]B36, 6 = N402[pAB6-10]B38, 7 = lambda marker *Bst*EII, 8 = pAB6-10. Arrows indicate the position of restriction fragments of pAB6-10 after *Eco*RI digestion (lane 8). An asterisk indicates the *Eco*RI fragment from pAB6-10 running at the same position as the smallest *Eco*RI fragment of the ribosomal repeat of *A. niger* (O'Connel et al., 1990).
B. Southern blot analysis of *Eco*RI digested chromosomal DNA from *A. niger* pAB6-10 transformants probed with the *glaA* gene: 1 = N402, 2 = N402[pAB6-10]B1, 3= N402[pAB6-10]B3, 4 = N402[pAB6-10]B13 5 = N402[pAB6-10]B36, 6 = N402[pAB6-10]B38. DNA was digested with *Eco*RI, separated on an 0.7 % agarose gel, transferred to nitrocellulose, and probed with a ^{32}P-labelled *glaA* fragment (3.3-kb *Eco*RI fragment). To determine the copy number signals obtained were compared with a serial dilution of the 3.3-kb *Eco*RI *glaA* fragment (see Table 1). Standard deviations were up to 20 %. A probe of the A. niger *gpdA* gene (van Gorcom, unpublished results) was used as an internal standard. The signal of the single copy of *glaA* in N402 could only be seen after longer exposure.

Table 1. Analysis of glucoamylase overproducing *A. niger* strains

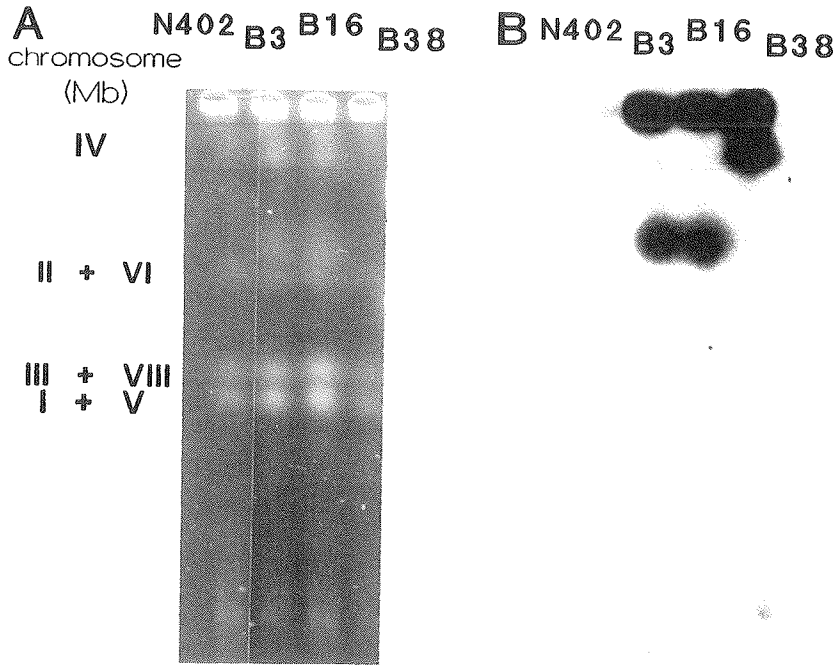
Strain	growth properties [A]	*glaA* copy number [B]	*glaA* mRNA level	excreted glucoamylase in medium (mg/l) [C]
N402	minimal	1	+	50
N402[pAB6-8]A12	acetamide	6		250
N402[pAB6-8]A24	acetamide	6		250
N402[pAB6-8]A9	acrylamide	10		365
N402[pAB6-8]A34	acrylamide	2 *		25
N402[pAB6-8]A35	acrylamide	10		425
N402[pAB6-8]A6	ω-aminoacids	60		425
N402[pAB6-8]A10	ω-aminoacids	60		365
N402[pAB6-8]A31	ω-aminoacids	60		325
N402[pAB6-10]B1	acetamide	20	+++	900
N402[pAB6-10]B8	acetamide	12	++	415
N402[pAB6-10]B3	acrylamide	30	+/-	30
N402[pAB6-10]B16	acrylamide	40	+/-	25
N402[pAB6-10]B36	acrylamide	80	+++	800
N402[pAB6-10]B39	acrylamide	80	+++	900
N402[pAB6-10]B13	ω-aminoacids	160	+	100
N402[pAB6-10]B38	ω-aminoacids	200	++	355

(A) minimal; growth on minimal medium
acetamide; growth on 10mM acetamide as sole N-source
acrylamide; growth on 10mM acrylamide as sole N-source
ω-aminoacids; inhibition of growth on 50mM ß-alanine/20mM GABA as sole N- and C-sources
(B) copy number determined by Southern blots
* rearrangements
(C) cultivation conditions: 1 x 10^6 spores/ml in 50 ml culture medium (5% maltodextrine) in shake flask (300 ml) grown at 48h at 30°C

To determine the site(s) of integration of the *glaA* gene copies in the fungal genome the Contour-clamped Homogeneous Electric Field (CHEF) gel electrophoresis technique was used. The chromosomal CHEF pattern of *A. niger* consist of 4 bands (Fig. 3A). Based on the results of genetic analysis it is assumed that *A. niger* has 8 chromosomes (Debets et al., 1990a). Apparently, some of the *A. niger* chromosomes

have the same size, since only 4 instead of 8 bands can be identified on a CHEF gel (Debets et al., 1990c). The chromosomes of 8 pAB6-10 transformants were separated by CHEF electrophoresis, transferred to nitrocellulose and subjected to Southern analysis. In 4 of these transformants a changed chromosomal CHEF pattern was observed. Besides the 4 wild-type bands a new band was seen (Fig. 3A). Southern analysis showed that in these transformants all integrated *glaA* copies are located on this new band (Fig. 3B and 3C). Our interpretation of this result is that due to the integration of a large number of *glaA* genes the size of one of the chromosomes has been increased, which leads to a lower electrophoretic mobility of that chromosome. For some of these strains the chromosome in which the *glaA* genes have been integrated could be determined. This was performed using Southern blot analysis with different linkage groups specific probes (Debets et al., 1990b). In this way it was shown for transformants B39 and B13 that the *glaA* genes were integrated on chromosomes corresponding to linkage groups III and I, respectively (results not shown). In the other transformants, in which no shift of chromosomal band(s) was observed, the *glaA* genes are located on a band at the position of one of the wild-type bands (Fig. 3C). In these transformants genetic analysis is needed to determine the chromosome of integration.

Figure 3. Separation of chromosomal DNA of *A. niger* transformantson a contour-clamped homogeneous electric field (CHEF) gel.
A. Ethidium bromide stained CHEF gel with a number of pAB6-10 transformants. Agarose plugs containing intact chromosomal DNA were prepared as described by Debets et al 1990c. The chromosomes were electrophoresed in an agarose gel (0.5 % chromosomal grade) for 3 periods of 48 hr, with pulse intervals of 55, 47 and 40 min, respectively.
B. Southern blot of separated chromosomes of some multiple-copy *glaA* strains hybridized with a ^{32}P-labelled 3.3-kb *Eco*RI fragment, containing part of the *glaA* gene. The signal of the wild-type *glaA* gene could only be seen after longer exposure and was located at the positon of chromosomes II or VI (not shown).
C. Artist impression of Southern analysis of chromosomal patterns in selected transformants. Open boxes indicate the position of wild-type chromosome(s). Black figures indicate the position of the chromosome with the introduced *glaA* genes. (The chromosome corresponding to linkage group (LG) VII is not yet identified in CHEF pattern).


A
N402 B3 B16 B38
chromosome
(Mb)
IV
II + VI
III + VIII
I + V
B N402 B3 B16 B38
C
chromosome
(Mb)
N402 B8 B1 B3 B16 B36 B39 B13 B38
IV
(6.6)
II + VI
(5.0)
III + VIII
(4.1)
I + V
(3.5)

Analysis of *glaA* expression in the transformants

In most of the multi-copy transformants a considerable increase of the amount of secreted protein was measured (data not shown). To test whether this increase was specifically caused by an increase of the GLA production, medium samples were analyzed using Iso-Electric-Focusing (IEF) gels (SERVALYT^R Precotes^R, pH 3 - 6). After protein separation and staining with Coomassie Brilliant Blue one major protein band is visible on the IEF gel with an isoelectric point corresponding with the pI of purified GLA (pI= 3.5) (van den Hondel et al., 1992). This band is absent in culture medium from strain N402ΔGLA, in which the *glaA* gene has been deleted (results not shown). Western analysis was used to confirm that this band was GLA (results not shown). From these data it was concluded that the increase of the amount of secreted proteins is indeed due to the increase of GLA production. Under the conditions used, GLA is the major secreted protein. Determination of the total amount of secreted protein produced by the strains N402, N402ΔGLA and the transformants, was used to calculate that N402 produces about 50 mg GLA per liter culture fluid, whereas several of the transformants secreted about 20 fold more GLA (900 mg/l)(Table 1). Glucoamylase activity assays indicated that all GLA secreted is also enzymatically active (results not shown). From the data presented in Table 1 it is clear that no relationship exists between the number of *glaA* copies and the amount of GLA that is found in the culture medium. One explanation for this result could be intracellular accumulation of GLA in multi-copy strains. However, analysis of mycelium extracts of the pAB6-10 transformants gave no support for this explanation (results not shown). Another explanation for the discrepancy between the number of *glaA* copies and the amount of produced GLA could be that in multi-copy transformants GLA production is limited at the level of transcription. To analyze this, the glucoamylase mRNA level was determined using Northern analysis (Table 1). A clear difference in *glaA* mRNA levels was observed between different multi-copy transformants (Fig. 4). In some multi-copy transformants (B3, B16 and B13) hardly any *glaA* mRNA was found, in spite of the high copy number of *glaA* genes. From the data obtained with Northern analysis

it is shown that the amount of GLA produced correlates with the mRNA level of *glaA* in the different transformants. From these data, taken together with the results from Southern analysis, we conclude that in multi-copy transformants the GLA production is limited at the level of transcription.

Limited expression is not caused by methylation

From the results obtained by Northern analysis it is clear that in most of the multi-copy strains not all *glaA* genes copies are transcribed at a wild-type level. It has been

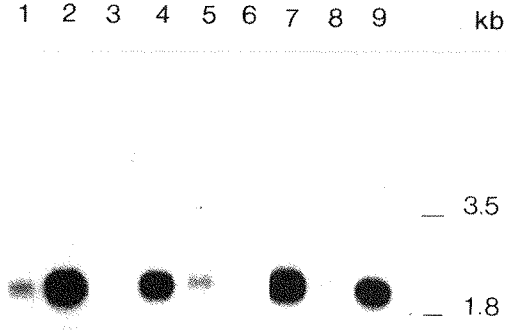


Figure 4. Northern blot of mRNA samples from glucoamylase over-producing *A. niger* strains. Lanes 1 - 9 contain 10 μg RNA from *A. niger* N402 (1) and pAB6-10 transformants (2 - 9).
1= *A. niger* N402,
2= N402[pAB6-10]B1,
3= N402[pAB6-10]B3,
4= N402[pAB6-10]B8,
5= N402[pAB6-10]B13,
6= N402[pAB6-10]B16,
7= N402[pAB6-10]B36,
8= N402[pAB6-10]B38,
9= N402[pAB6-10]B39.
The same *glaA* fragment as described in legend 3 was used as probe.

reported for other filamentous fungi that expression of introduced genes can be inactivated by methylation (Selker and Garett, 1988; Goyon and Faugeron, 1989; Mooibroek et al., 1990 and Durand et al., 1992). To analyze whether inactivation of gene expression of introduced gene copies also occurs in *A. niger* by this mechanism, chromosomal DNA from N402, N402[pAB6-10]B1, B13, and B36, was digested with pairs of isoschizomeric restriction enzymes which show a different sensitivity to methylation (*Hpa*II/*Msp*I and *Sau*3A/*Mbo*I). These digests were subjected to Southern analysis. For both pairs of enzymes identical hybridization patterns were observed in all 4 strains (results not shown), indicating that the limited expression of the *glaA* genes present in transformant B13 and B36 is not caused by methylation of the introduced *glaA* genes.

Transformation of multi-copy transformants with additional copies of pAB6-10

In an attempt to further improve GLA production additional copies of the *glaA* gene were introduced in N402[pAB6-10]B1, B13, and B36 by co-transformation of pAB6-10 with vector pAN7-1, containing the hygromycin resistance marker (Punt et al., 1987). For all three strains a large number of hygromycin resistant transformants (supertransformants) was obtained. The amount of GLA produced was determined in a number of supertransformants. In none of the 54 supertransformants analyzed an increase of the GLA production was found, compared to the recipient strains. Southern analysis of several of these supertransformants revealed that the number of *glaA* copies in most of them was higher than in the recipient multi-copy transformant. These data are consistent with the hypothesis that GLA production in multi-copy strains is limited at the level of transcription. Introduction of new gene copies did not elevate this limitation. Surprisingly, in 6 supertransformants, derived from N402[pAB6-10]B1 and B36, a striking decrease of the GLA production was found. This result indicates that the introduction of new *glaA* copies may even lead to a decreased expression of the resident *glaA* genes.

DISCUSSION

A. niger strains containing multiple copies of the glucoamylase gene of *A. niger* were isolated. A significant increase of the extracelluar GLA production was observed in most of the multi-copy strains. In the *glaA* multi-copy transformants no clear relationship was found between the copy number of the *glaA* gene and the amount of GLA that is produced. Northern analysis showed that in some transformants (N402[pAB6-10]B3, B13, and B16) hardly any *glaA* mRNA was produced, despite the high copy number. From this data we conclude that in these transformants not all the *glaA* genes are transcribed or that all genes are transcribed at a low level. It has been reported that expression of new introduced genes in filamentous fungi can be inactivated by methylation of newly introduced DNA. However, analysis of several transformants revealed no indications that the introduced *glaA* gene copies were methylated, making inactivation of gene expression by this process unlikely. Another possibility to explain the reduced expression of the *glaA* genes in the transformants N402[pAB6-10]B3, B13, B16, and B38 is that the expression is influenced by the site of integration. It has been reported earlier that gene expression in fungi can be controlled by position-dependent mechanisms (Miller et al., 1987). CHEF analysis showed that in all pAB6-10 transformants the *glaA* genes are integrated at different chromosomes but that in each transformant the integration is restricted to one chromosome. Debets et al. (1990b) also found, with multi-copy *amdS* transformants in *A. niger*, that all these copies were integrated together in one site of the chromosome. Although not analyzed yet, it seems very likely that in most cases integration of all introduced vector copies occurs at a single site of one of the chromosomes. Therefore one may assume that the level of *glaA* expression in the different transformants is influenced by the different sites of integration.

A further explanation for the transcription limitation in the multi-copy strains may come from the results obtained by the introduction of additional *glaA* gene copies in some of these strains. Determination of the GLA production in these supertransformants showed that in none of the supertransformants analyzed an

increase in GLA production was observed. In some supertransformants even a decrease of GLA production was observed. Based on these results we hypothesize that in the presence of a large number (≥ 20) of *glaA* gene copies a shortage of specific regulatory proteins may occur. Similar observations were reported in expression studies of the *alcA* (Gwynne et al., 1987) and *amdS* (Hynes et al., 1986) genes of *A. nidulans*. Further research will concentrate on testing this hypothesis by analyzing factors involved in (the regulation of) the expression of the *glaA* genes in these multi-copy transformants. In addition, a study will be carried out on the regulation of the *glaA* expression and GLA production under different fermentation conditions. Special attention will be given to genetic stability of multi-copy transformants under these physiological conditions.

ACKNOWLEDGEMENTS

The authors wish to thank R.F.M. van Gorcom, W. van Hartingsveldt, M. Harteveld and I. van der Veen for their contribution to the vector constructions. Drs. C.J. Bos and A.J.M. Debets from the Department of Genetics of the Agricultural University Wageningen are gratefully acknowledged for their introduction to the CHEF system and further collaboration. This project is supported by the Dutch Programme Commission Industrial Biotechnology. This paper is dedicated with great appreciation to Dr. Frits Berends on the occasion of his retirement as head of the Biochemistry Department of TNO Medical Biological Laboratory.

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Submitted

CHAPTER 3

Evaluation of molecular and genetic approaches to generate glucoamylase overproducing strains of *Aspergillus niger*

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(Genetic stability; Protein overproduction; CHEF analysis; Gene regulation; Filamentous fungi)

SUMMARY

To evaluate the possibility to improve glucoamylase (GLA) production in *A. niger* strains, carrying multiple copies of the GLA encoding gene (*glaA*), additional *glaA* copies were introduced either by genetic recombination or retransformation. For strains to be used in such experiments a genetic analysis was first carried out. The results of this analysis clearly revealed that in each transformant integration had occurred at a chromosome corresponding to a single linkage group. The GLA production per gene copy showed considerable variation in these strains, indicating a clear effect of the site of integration on gene expression. Introduction of additional gene copies by genetic recombination experiments was carried out for different combinations of strains, carrying *glaA* copies in different chromosomes. The introduction of additional *glaA* gene copies by genetic recombination did not result in a considerable increase in GLA production compared to the parental strains. In some strains recombination resulted in genetic instability, observed by the frequent loss of *glaA* copies. Also retransformation of multi-copy *glaA* strains did not result in increase in GLA production. In several strains even a decrease in GLA production was found after retransformation. Southern analysis of these transformants suggested that newly introduced gene copies were heavily rearranged which partly explains why GLA production was not increased. Further analysis of one such transformant provided evidence that the overexpression of the *glaA* gene is limited by the amount of *trans*-acting regulatory protein(s) available.

INTRODUCTION

Filamentous fungi are frequently used as production organisms for industrial enzymes (Berka et al., 1992). In many cases strains selected for having desirable characteristics have been subjected to further strain improvement programs. Traditionally, strain improvement strategies are based on the empirical method of mutagenesis and selection (e.g. reviews Hara et al, 1992; Rowlands, 1992). The use of recombinant DNA techniques, which were developed for fungal species during the last 10 years (for review see Timberlake, 1992; van den Hondel and Punt, 1993) facilitates a controlled strategy of strain improvement, allowing the introduction and expression of additional copies of fungal genes of interest and the expression of non-fungal genes.

Recently, a number of glucoamylase (GLA) overproducing *A. niger* strains have been constructed by introduction of multiple copies of the GLA encoding gene (*glaA*) of *A. niger* (Verdoes et al., 1993). Initial molecular genetic analysis indicated that the *glaA* expression in these strains was limited at the level of transcription. These results suggested that *glaA* expression in multi-copy strains is affected by the site of integration and the available amount of regulatory factor. To evaluate the strategy of strain improvement by introduction of multiple gene copies and to elucidate the molecular basis of the observed transcription limitation in our multi-copy strains, further molecular as well as classical genetic research is needed. Although *A. niger*, like many other fungal species lacks a conventional sexual cycle, the so-called parasexual cycle (Bos, 1988a) can be used to perform genetic analysis of (manipulated) strains. For detailed genetic analysis both a genetic map and an electrophoretic karyotype are important tools. Genetic maps of each of the eight linkage groups (LG) of *A. niger* have been reported (Debets et al., 1993) and a complete set of master strains is available for LG assignment (Bos et al., 1993). Also a partial electrophoretic karyotype of *A. niger* has been elucidated (Debets et al., 1990c).

In this paper the analysis of multi-copy strains is described, in which, to improve GLA production additional copies of the *glaA* gene were introduced using genetic recombination or retransformation. Also a detailed genetic analysis of the original *glaA* multiple copy strains is reported.

MATERIALS AND METHODS

Strains

All *A.niger* strains used, originated from strain N402 (*cspA1*; Bos et al., 1988b). The isolation of the *A. niger glaA* multi-copy strains and supertransformants was described before (Verdoes et al., 1993). Strains N915 (*cspA1, fwnA1*[I], *argH12*[II], *bioA1*[III], *leuA1*[IV], *pheA1*[V], *oliC2*[VII], *crnB12*[VIII]) and N917 (*cspA1, fwnA1*[I], *ntrB3*[IV], *pheA1*[V], *lysD25*[VI], *oliC2*[VIII] (C.J. Bos; unpublished) were used as master strains in linkage group analysis. Strain AB6.4 is a fawn coloured derivative of strain AB4.1ΔGLA (*pyrG1, glaA::ble*^{R}; Verdoes et al., 1994).

Media and cultivation conditions

Media used for genetic analysis have been described by Bos et al. (1988b) and Debets et al. (1989, 1990a). The oligomycin concentration used in the media was 3 μg ml^{-1}, while the hygromycin concentration was 0.2 mg ml^{-1}. Cultivation conditions for GLA production in liquid cultures were as described by Verdoes et al. (1993).

Genetic techniques

Heterokaryons of prototrophic multi-copy transformant strains and master strains N915 or N917 were obtained on minimal medium (MM; Bennett and Lasure, 1991) containing oligomycin. Isolation of heterokaryons and heterozygous diploids and haploidization was carried out as described by Bos et al. (1988b). Approximately 100 haploid segregants out of each diploid combination were purified and tested for their markers as described previously (Bos et al. 1988b; Debets et al., 1989, 1990a). The presence of the *amdS* gene copies, which was used as selection marker in the transformation experiments (Verdoes et al., 1993), was tested in the segregants by analysis of their growth phenotype on 3 different media; MM (Oxoid agar) with 20 mM acetamide as only C- and N-source with 10 mM $CsCl_2$; MM (Oxoid agar) with 10 mM acrylamide as sole N-source and; supplemented MM (SM) with 50 mM ß-alanine. The loss or inactivation of *amdS*

gene copies was determined by testing for resistance to fluoracetamide as described by Debets et al. (1990a).

Suitable segregants obtained from linkage analysis experiments were used to construct strains with *glaA* copies at two different chromosomes. No direct method is available that can be used to select for the presence of both chromosomes with the *glaA/amdS* copies in a haploid segregant. Therefore, indirect selection of *glaA/amdS* was based upon the (auxotrophic) markers present at the homologous chromosomes of the other strain used in the combination. For example, for the combination of B36 and B39, selection of heterokaryons and diploids was carried out on minimal medium supplemented with only leucine, as both strains used in this combination are *leuA1* (see Table 2). In the diploid the two chromosomes carrying the *glaA/amdS* inserts; V (from B36) and III (from B39), are present together with the homologous chromosomes carrying an auxotrophic marker, from the other parent strain; *pheA1* (V) and *bioA1* (III). Isolation of haploid segregants containing both *glaA/amdS* inserts was carried out by selecting Phe^+/Bio^+ segregants. Selected segregants were subsequently tested by CHEF/Southern analysis for the presence of the *glaA/amdS* gene copies. This last step was to ensure that in the selected segregants no recombination, due to mitotic crossing-over, had occurred between the markers used for selection and the original *amdS/glaA* inserts. From the data in Table 1 it is clear that mitotic crossing-over, albeit at low frequency (< 20 %) may occur.

Biochemical and molecular techniques

The total amount of protein was determined essentially as described by Verdoes et al. (1993) using GLA (or BSA; Fig. 1) as standard and can be used as indication for GLA production. Preparation of intact chromosomal DNA was carried out as described by Debets et al. (1990c). Electrophoresis was performed using a CHEF DRII apparatus and a Model 1000 Mini Chiller (Bio-rad). Gels were made with 0.6 % chromosomal-grade agarose (Bio-rad) in 1x TAE (Sambrook et al.,

1989). Electrophoresis was carried out in 1 x TAE at 12.5 °C. Standard DNA isolations from *Aspergillus* were carried out as described by Kolar et al. (1988). All other methods were carried out essentially as described by Sambrook et al. (1989).

RESULTS

Genetic analysis of glaA multi-copy strains

In a previous study GLA overproducing strains carrying multiple copies of the *glaA* gene have been described (Verdoes et al., 1993). To facilitate improvement of GLA production in these strains by genetic recombination the linkage group (LG) at which the *glaA* copies were integrated was determined in a number of these strains by genetic analysis. For such an analysis heterozygous diploids had to be constructed from *glaA* multi-copy strains and tester strains. The isolation of diploids requires selective markers for both parental strains. Generally, auxotrophic markers are used for this purpose. However, the *glaA* multi-copy strains are prototrophic and, moreover, the *amdS* marker in these strains can not efficiently be used as a so-called "forcing" selection marker. Therefore, a new strategy was developed to obtain heterokaryons and heterozygous diploids. This strategy was based on the use of the nuclear oligomycin resistance marker in the tester strains N915 and N917. These master strains together cover all eight LG's. Agar plates containing minimal growth media and oligomycin were used to select heterokaryons and diploids from the tester strain and the prototrophic *glaA* multi-copy strains. Haploid segregants of these constructed diploids were analyzed for their markers. A summary of the results of the genetic analysis is shown in Table 1. These results show that in each transformant for which genetic analysis could successfully be carried out, linkage to a single LG was observed. These results are in agreement with the results of CHEF electrophoresis and Southern analysis, using the *glaA* gene as probe, which indicated that for each transformant integration had taken

place at a single chromosome (Verdoes et al., 1993). The LG of *glaA/amdS* copies could not be determined with genetic analysis of transformant N402[pAB6-10]B38 due to their loss in the diploids obtained with both master strains, as shown by resistance of diploids for fluoracetamide, and by Southern analysis (results not shown). Southern analysis of separated chromosomes of this transformant showed that the *glaA* copies were integrated at the chromosome corresponding to LG VI, which also contains the multiple copies of the rDNA repeat (Verdoes et al., 1994). The presence of two highly repetitive sequences (rDNA and *glaA/amdS*) on one single chromosome may explain the instability observed during the isolation of diploids. Alternatively, the instability may be a result of an increased frequency of intra-chromosomal recombination caused by the endogenous *glaA* gene, which is located at this chromosome (Verdoes et al., 1994).

Comparison of the results of relative GLA production and genetic localization of the *glaA* copies (Table 1) shows that integration at chromosomes corresponding to different LG may result in nearly wild-type level of GLA production per gene copy (e.g. LG I (B1) and LG IV (B8)). Integration at other chromosomes (e.g. LG VI; B38 and LG VIII; B3 and B16) results in very low relative GLA production levels. Comparison of the relative level of GLA production of B1 and B13, both carrying multiple copies of the *glaA* gene at the chromosome corresponding to LG I, shows that also different integrations at one chromosome can have different effects on the level of gene expression per gene copy.

Effect of introduction of additional glaA copies in multi-copy strains

Two approaches have been used to further increase the *glaA* copy number and to analyze the effect on GLA production: 1) combining chromosomes with the *glaA* inserts of different multi-copy strains using genetic techniques and 2) retransforming multi-copy strains. As in various *glaA* multi-copy strains integration had occurred at chromosomes corresponding to different LG's (Table 1), it should be possible to isolate recombinants carrying multiple *glaA* copies at different

Table 1. Linkage group assignment of *glaA* multi-copy strains.

Strain [A]	Linked markers [B]		Segregants				Recombinant (%)	Linkage group *glaA/amdS*	Relative GLA production [C]	
	A	B	AB	A+	+B	++				
N402	--	--	-	-	-	-	--	VI[D]	50	(1)
N402[pAB6-10]B1	*amdS*	*fwnA1*	3	48	48	2	5.0	I	45	(20)
N402[pAB6-10]B3	*amdS*	*crnB12*	3	57	42	2	4.8	VIII	1.0	(30)
N402[pAB6-10]B8	*amdS*	*leuA1*	3	24	58	10	13.7	IV	35	(12)
N402[pAB6-10]B13	*amdS*	*fwnA1*	0	53	42	0	0.0	I	0.6	(160)
N402[pAB6-10]B16	*amdS*	*crnB12*	6	48	42	3	9.1	VIII	0.6	(40)
N402[pAB6-10]B36	*amdS*	*pheA1*	1	54	43	4	4.9	V	10	(80)
N402[pAB6-10]B38	*amdS*		N.D.					VI[D]	1.8	(200)
N402[pAB6-10]B39	*amdS*	*bioA1*	14	40	35	41	19.4	III	11	(80)

[A] *A. niger* strains carrying multiple copies of the *glaA* gene (Verdoes et al., 1993).
[B] Linkage group assignment of the introduced *glaA/amdS* copies was carried out with two master strains (N915 and N917) by following the *amdS* marker. In all cases for each combination a single diploid was used in segregation analysis. Only linked markers with tester strain N915 are given. However, data with N917 completely correspond.
[C] Data from Verdoes et al., 1993. Relative GLA production is expressed in mg GLA produced per litre culture fluid per gene copy. The variation in the level of GLA production in different experiments is up to 50 %. Between brackets the number of *glaA* gene copies is indicated.
[D] Linkage group assignment by CHEF/Southern analysis (Verdoes et al., 1994). Linkage group assignment for N402[pAB6-10]B38 could not determined by genetic analysis due to loss of the *amdS/glaA* copies in both diploids isolated.

chromosomes by genetic recombination of these strains. For these recombination experiments suitable segregants, with introduced auxotrophic markers obtained from the genetic analysis (Table 1), were selected (see Materials and Methods). Analysis of protein production (Table 2) and Southern analysis (results not shown) was used to show that these segregants (indicated V) had characteristics comparable to those found for the parental *glaA* multi-copy strains.

Table 2. Protein production in recombinant strains[a].

Strain [A]	Relevant genotype [B]	Total protein production [C] (mg l^{-1})
PARENTAL STRAINS:		
N402		190 ± 20
N402[pAB6-10]B1	*glaA/amdS*(I)	400 ± 10
V1-V (S)	*glaA/amdS*(I), *argH12, bioA1, pheA1*(V), *oliC2*	360 ± 160
N402[pAB6-10]B36	*glaA/amdS*(V)	520 ± 100
V36-IV (S)	*glaA/amdS*(V), *fwnA1*(I), *bioA1, leuA1*	490 ± 90
V36-V (S)	*glaA/amdS*(V), *fwnA1, bioA1*(III), *leuA1, oliC2*	440 ± 120
N402[pAB6-10]B39	*glaA/amdS*(III)	390 ± 40
V39-IV (S)	*glaA/amdS*(III), *argH12, leuA1, pheA1*(V)	340 ± 50
RECOMBINANTS:		
V1/V36 (R)	*glaA/amdS*(I and V), *argH12, bioA1, leuA1, oliC2, crnB12*	640 ± 60
V36/V39 (R)	*glaA/amdS*(V and III), *fwnA1, leuA1*	370 ± 40

[A] All segregants used originated from the cross with master strain N915. Between brackets is indicated segregant (S) and recombinant (R). The strategy for selection of recombinants is described in the Material and Methods section.

[B] Between brackets the LG of integration of the *amdS/glaA* copies and that of the auxotrophic marker, used for selection of the recombinants is indicated.

[C] The total amount of protein produced was determined as described by Verdoes et al., 1993 using purified GLA as standard. Standard deviations in total protein production are given.

Genetic recombination experiments with derivatives from the multi-copy strains were carried out for four different combinations; (V1 x V36, V13 x V36, V36 x V39 and V1 x V39), including combinations of both high and low GLA production strains (Table 1). Recombinant strains resulting from the recombination experiments were analyzed by CHEF/Southern analysis for the presence of the different sets of parental *glaA/amdS* copies. As expected, progeny from V1 x V36 and V36 x V39 carried *glaA/amdS* copies at both parental chromosomes. However, in the progeny obtained from V13 x V36 the *glaA/amdS* copies from parent B13 were lost and in the progeny of V1 x V39 both sets of *glaA/amdS* copies were lost. In the recombinant strains with the expected combination of *glaA* copies GLA production was determined. The results, as presented in Table 2, show that in the progeny of V1 x V36 a slight increase in protein production was found compared to the parental strains used, whereas for the progeny of V36 x V39 no increase in protein production was found. For V1 x V36 recombinants the resulting GLA production is lower than the sum of the protein production of the two parental strains (Table 2).

Another approach to introduce additional *glaA* gene copies in the multi-copy strains was carried out by retransformation. Three of the multi-copy strains, N402[pAB6-10]B1, B13 and B36, were chosen because of their different levels of relative GLA production (Table 1). A number of so-called supertransformants obtained from these strains were analyzed for GLA production. As shown in figure 1, no significant increase in protein production was found in these strains. Surprisingly, even a clear decrease in protein production of the recipient strain was found in 6 (out of 14) B36 transformants and 3 (out of 14) B1 transformants (Fig. 1). Two of each group of supertransformants with a decreased protein production (N402[pAB6-10/pAN7-1]B1.9, B1.17, B36.15A and B36.24) and one B36 supertransformant with an unchanged level of protein production (N402[pAB6-10] B36.25) were used for further analysis. Southern analysis of digested chromosomal DNA isolated from these supertransformants and the recipient strains indicated a small increase of the number of intact *glaA* gene copies (as indicated by the more intense band corresponding to intact gene copies; Fig. 2). In addition, in the

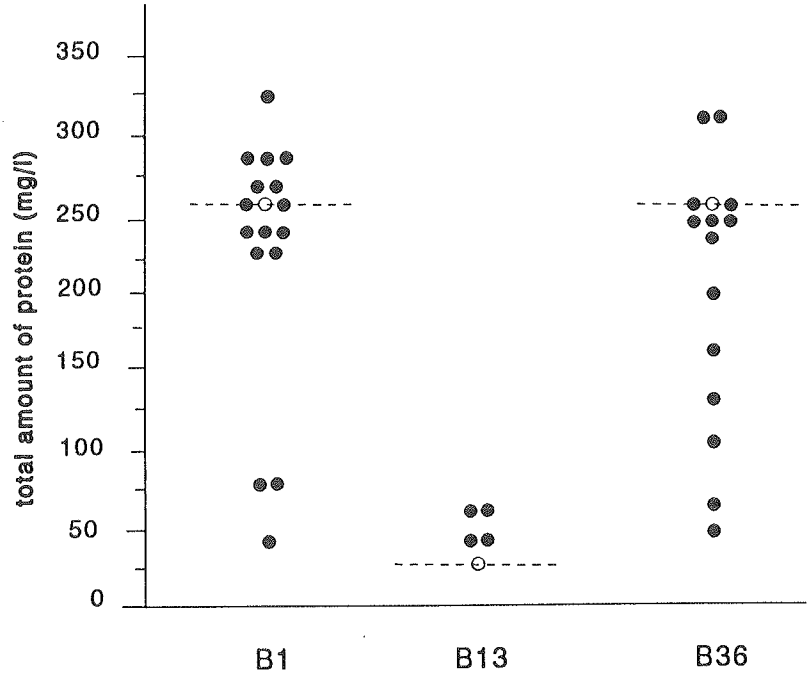

Figure 1. Total amount of protein secreted in the medium of *glaA* super-transformants. Supertransformants were obtained after retransformation of *glaA* multi-copy strains B1, B13 and B36 with pAB6-10 together with pAN7-1 (Verdoes et al., 1993). The amount of protein secreted by the recipient strains are indicated by open circles. Solid circles indicate the amount of protein secreted by different supertransformants. The amount of protein was determined using BSA as standard. (It should be noted that the relative extinction for BSA is about 2 fold higher than that of GLA. Accordingly data presented in Fig. 1 are about 2-fold lower than those in Tables 2 and 3).

transformants with a decreased GLA production level a number of apparently rearranged gene copies was observed, indicated by the presence of additional bands (Fig. 2). To analyze at which chromosome the additional *glaA* copies were integrated CHEF / Southern analysis was carried out for these supertransformants, using the *glaA* gene as probe. In 4 out of 5 transformants analyzed (N402[pAB6-10/pAN7-1]B1.9, B36.15A, B36.24 and B36.25) one specific hybridization signal was found in the autoradiogram (Fig. 3). From this result it was concluded that integration of additional *glaA* copies in these strains has occurred at the same chromosome as the initial integration. In one supertransformant,

N402[pAB6-10/pAN7-1]B1.17 a second specific signal at the position of a chromosomal band was found (Fig. 3) in addition to the hybridization signal at the same position of the recipient strain. The chromosomal band at this position of this additional signal contained also the pAN7-1 vector, as determined by Southern analysis using the HmB resistance gene as probe, which was used as selective marker in the retransformation experiment (results not shown). These results indicate that in this strain the new copies were integrated at a chromosome different from that of the original *glaA* gene copies. Genetic analysis was carried out to determine the LG of integration of these additional copies. LG assignment was carried out by following the HmB^R marker in genetic analysis using both master strains N915 and N917. Linkage (27.4 and 11.5 % recombination,

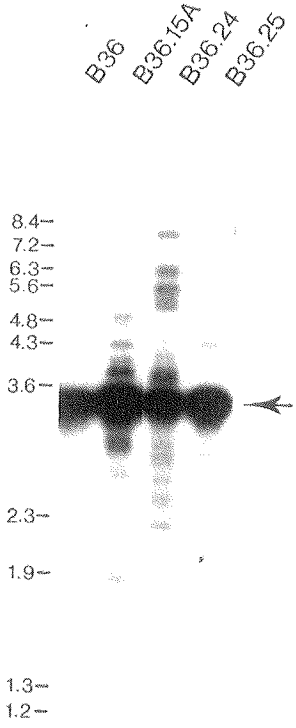

Figure 2. Southern blot analysis of *Eco*RI digested chromosomal DNA isolated from N402[pAB6-10]B36 and derivatives obtained after retransformation. DNA was digested with *Eco*RI, separated on an 0.7 % agarose gel, transferred to nitrocellulose, and probed with a ^{32}P-labelled *glaA* fragment (3.3-kb *Eco*RI fragment). The position of intact *glaA* genes is indicated by an arrow. Numbers on the left give the size (in kilobase pairs) of *Bst*EII-digested bacteriophage lambda DNA markers.

respectively) was found between the HmB^R marker and the *oli*C2 mutation which is assigned to LG VII. From these results we conclude that the new copies have integrated at the chromosome corresponding to LG VII. The original *glaA* gene copies in strains B1 are located at the chromosome corresponding to LG I (Table 1).

GLA production in segregants from supertransformant N402[pAB6-10/pAN7-1]B1.17

To study why in supertransformants a decrease in GLA production was observed despite of an increase in *glaA* copy number, further analysis of transformant N402[pAB6-10/pAN7-1]B1.17 was carried out. As the *glaA* copies are present at two different chromosomes in this transformant it was possible to segregate both LGs with the *glaA* copies by genetic recombination. A spontaneous chlorate resistant mutant strain of B1.17 and strain AB6.4 (*fwnA*1, *pyrG1, glaA*::*ble*^{R}) were used to form and select heterokaryons and diploids on minimal medium supplemented with phleomycin. Haploid segregants obtained from one of the isolated diploids were tested for the *glaA* gene copies, by the relevant markers (*ble*^{R}, HmB^R and *amdS*). The different classes of segregants were tested for the level of protein production (Table 3). From this data it is clear that segregants which only contain the B1 derived *glaA* gene copies (class IIB; Table 3), have a similar level of protein production as the original B1 strain. This result indicates that in transformant B1.17 the expression of the *glaA* copies integrated at chromosome was not affected by rearrangements or irreversible inactivation of the B1 gene copies but apparently by the introduction of additional *glaA* copies at LG VII. The protein production found for segregants carrying only the new copies (Class III) indicates that these *glaA* gene copies are not or hardly expressed. Southern analysis of chromosomal DNA isolated from Class III segregants indicated that these *glaA* gene copies are heavily rearranged (similarly as shown in Fig. 2; results not shown) explaining the expression results in these segregants.

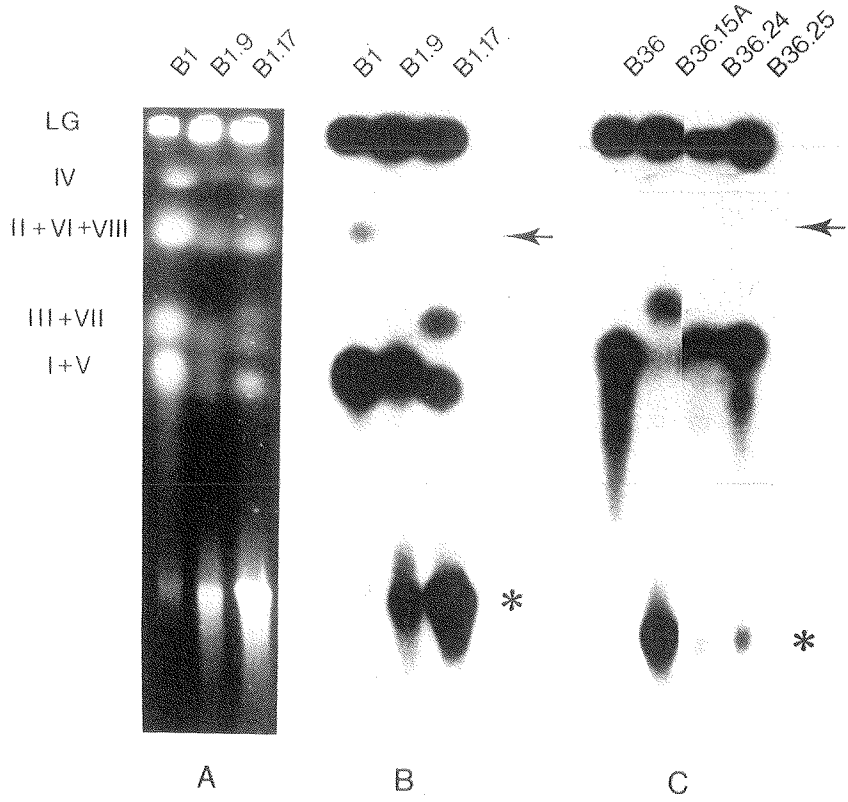

Figure 3. Separation of chromosomal DNA of *A. niger* transformants on a contour-clamped homogeneous electric field (CHEF) gel. (**A**). Ethidium bromide stained CHEF gel from transformant N402[pAB6-10]B1 and derivatives. Electrophoresis was carried with pulse intervals of 3200, 2800 and 2200 s, for 36, 36 and 72 h. respectively. Position and number of chromosomes in the wild-type strain N402 (Verdoes et al., 1994) are indicated on the left. Southern blot of separated chromosomes of multiple copy strain B1 and derivatives (**B**) and B36 and derivatives (**C**) hybridized with a ^{32}P-labelled 3.3-kb *Eco*RI fragment, containing a part of the *glaA* gene. Arrows indicate the position of the wild-type *glaA* gene which could be seen in all lanes only after longer exposure. The position of chromosome degradation products is indicated by asterisks.

Comparison of the GLA production of "deletion" (A) and "wild-type" (B) derivatives indicates that the expression of the endogenous *glaA* gene is reduced in class III and IV segregants, compared to class I and II (Table 3). This suggests that the newly introduced gene copies also affect the expression of the endogenous *glaA*

Table 3. Protein production in segregants of N402[pAB6-10/pAN7-1]B1.17 x AB6.4

		Linkage group of relevant markers			Total amount protein ($mg\ l^{-1}$) [A]
		I	VI	VII	
PARENTAL STRAINS:					
N402		--	*glaA*	--	190 ± 10
N402[pAB6-10]B1		*glaA/amdS*	*glaA*	--	510 ± 20
N402[pAB6-10/pAN7-1]B1.17		*glaA/amdS*	*glaA*	*glaA*/HmB	210 ± 10
DIPLOID: [B]					
N402[pAB6-10/pAN7-1]B1.17 X AB6.4		*glaA/amdS* --	*glaA* *glaA::ble*^{R}	*glaA*/HmB --	ND
SEGREGANTS: [C]					
Class I: ("N402")	A	--	*glaA::ble*^{R}	--	70 ± 5
	B	--	*glaA*	--	190 ± 10
Class II: ("B1")	A	*glaA/amdS*	*glaA::ble*^{R}	--	360 ± 20
	B	*glaA/amdS*	*glaA*	--	520 ± 20
Class III: ("B17")	A	--	*glaA::ble*^{R}	*glaA*/HmB	70 ± 5
	B	--	*glaA*	*glaA*/HmB	90 ± 10
Class IV: ("B1.17")	A	*glaA/amdS*	*glaA::ble*^{R}	*glaA*/HmB	150 ± 10
	B	*glaA/amdS*	*glaA*	*glaA*/HmB	200 ± 10

A For details see legend Table 2.
B For the isolation of heterokaryons/diploids a spontaneous, chlorate resistance, nitrate non-utilising mutant of B1.17 was used.
C Two independent segregants of each class were used to determine protein production.

gene. In conclusion, from comparison of the protein levels produced in the different classes of B1.17 segregants it is clear that introduction of additional *glaA* copies results in a decrease of the expression of resident, active *glaA* gene(s).

DISCUSSION

In this paper two approaches to improve GLA production in *A. niger* strains carrying multiple *glaA* copies are presented and evaluated. As one of the approaches involved genetic recombination, genetic analysis of the original multi-copy strains was carried out. For this analysis of prototrophic *glaA* multi-copy strains a new strategy was used based on the usage of a nuclear oligomycin resistance marker to select for heterokaryons and heterozygous diploids. The advantage of this strategy is that it obviates the need for introduction of additional markers in the strains to be analyzed. Therefore the strategy can be used for the genetic analysis of strains without suitable selection markers (e.g. production strains and wild-type strains).

The data of the LG assignment of 8 *glaA* multi-copy transformants shows that integration occurred at chromosomes corresponding to six different LG's (Table 1). The results summarized in Table 1, also show that the GLA production per *glaA* gene copy (relative GLA production) greatly differs between the different transformants. The relative GLA production of transformants B8 and B1 is comparable to that of the wild-type strain, whereas all other strains have a significant lower relative GLA production. From previous results, it was suggested that GLA production reaches a maximum level of about 20 times the level observed in the wild-type strain (Verdoes et al., 1993). Although this maximum level of production may partly explain the reduced relative production level in strains producing this maximum level and carrying more than 20 gene copies (B36 and B39), the extremely low relative production levels observed in strains not producing this maximum level (B3, B13, B16 and B38) can not be explained in the same way. Apparently, in these strains the site of integration affects the expression of the introduced gene copies.

Although homologous *glaA* sequences are present in the transformation vector pAB6-10, the integration occurred in only one of the eight transformants analyzed (N402[pAB6-10]B38) at the chromosome carrying the endogenous *glaA* gene

(Verdoes et al., 1994). This result indicates that in the transformation experiments with the wild-type strains the integration is not targeted to homologous sequences. On the contrary, in 4 out of 5 supertransformants analyzed the integration of the additional *glaA* copies occurred at the chromosome containing the original *glaA/amdS* copies. These results suggest that in retransformation targeting occurs more frequently to a chromosome with homologous sequences. Similar results have been described for other fungi as well (Farman and Oliver, 1992). An alternative explanation for these results could be that the presence homologous sequences introduced by integration at different chromosomes may cause genetic instability, resulting in (the generation) of abortive (inviable) transformants. This latter explanation is supported by the results obtained in the genetic recombination of multi-copy strains, as in 2 out of 4 combinations, one or both sets of *glaA/amdS* copies were lost in the progeny. Also during purification of supertransformants, indications for genetic instability of complex multi-copy strains were obtained in some cases, such as segregation of a primary transformant into phenotypically different progeny. However, it should be noted that instability was never observed in purified segregants.

To improve GLA production levels in multi-copy strains, we introduced additional *glaA* copies in some of the original multi-copy strains by either genetic recombination or retransformation. Our observations, as mentioned in the previous paragraph, concerning genetic recombination with multiple copy strains, indicated serious problems of genetic stability during heterokaryon and/or diploid formation, which makes the isolation of this type of complex multi-copy strains not straightforward. In addition, the analysis of the GLA production level in genetically recombined transformants (with additional *glaA* gene copies) showed that the GLA production was less than the sum of that found in the recipient strains (Table 2). The results obtained with retransformants show that also with this approach we were unable to obtain strains with improved protein production (Fig. 1). Moreover, in 9 out of 37 supertransformants analyzed, even a significant decrease in protein production was found compared to the recipient strains (Fig. 1). Southern analysis

indicated that most of the newly introduced gene copies were rearranged which may explain why no increase in protein production was observed. However, this does not explain why in some transformants a decrease in protein production was found. Therefore, one of these latter type of transformants was analyzed further. In this supertransformant (B1.17) the additional copies were integrated at a chromosome corresponding to a linkage group (LG VII) different from that of the original *glaA/amdS* copies (LG I). Analysis of the protein production in segregants derived from B1.17, in which both sets of *glaA* copies were separated, showed that the copies in the original *glaA/amdS* inserts (at LG I) were still active, whereas the additional gene copies (at LG VII), of which most are rearranged, were inactive. Apparently, the active copies were affected in *trans* by the integration of the additional inactive *glaA* copies in supertransformant B1.17. The protein production levels in classes of segregants carrying these inactive gene copies indicated that also the endogenous *glaA* was affected by this *trans* effect. A similar explanation may hold for the decrease in protein production in other low producing supertransformants as in these strains also indications for rearrangements of *glaA* gene copies was observed (Fig. 2). However, as in these strains the new copies integrated at the same chromosome as the original ones, easy analysis of the different gene copies by genetic recombination is precluded.

Taken together, the results presented give additional support for the hypothesis that in the multi-copy strains the overexpression of the *glaA* gene may be limited by a *trans*-acting regulatory factor (Verdoes et al., 1993). A limiting amount of regulatory factor would explain the observed maximum production level and the inability to significantly increase GLA production by the introduction of additional gene copies. Furthermore, sequestering of this factor by (promoter) sequences of inactive *glaA* gene copies, as observed in transformant B1.17, may explain the decrease in GLA production in supertransformants.

From the data presented in this paper it is clear that new strategies should be developed to further increase GLA production using multi-copy strains. The introduction of additional gene copies encoding the putative *trans*-acting regulatory

factor seems to be the most direct approach. Such an approach has shown to be successful with the *alcA/alcR* expression system in *A. nidulans* (Gwynne et al., 1987). Once the gene encoding the regulatory factor is cloned, the strains with the additional *glaA* gene copies as described in this paper will be very useful to further improve the GLA production by the introduction of additional copies of this gene.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. C. J. Bos and Ms. M. Slakhorst (Agricultural University Wageningen, The Netherlands) for providing various master strains. Dr. K. Swart and Prof. Dr. P. H. Pouwels are gratefully acknowledged for their helpful discussions and critical reading of the manuscript. This project is supported by the Dutch Programme Commission for Industrial Biotechnology.

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CHAPTER 4

The complete karyotype of *Aspergillus niger*: the use of introduced electrophoretic mobility variation of chromosomes for gene assignment studies

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(gene replacement; CHEF analysis; filamentous fungi; linkage group analysis)

SUMMARY

A method is described for unambiguous assignment of cloned genes to *Aspergillus niger* chromosomes by CHEF-Southern analysis. All of the 8 linkage groups (LGs) with the exception of LG VII have previously been assigned to specific chromosomal bands in the electrophoretic karyotype of *A. niger* (Debets et al. (1990) MGG 224: 264-268). Using a LG VII specific probe (*nicB* gene of *A. niger*) we have shown that LG VII corresponds to a chromosome of about 4.1 Mb. Furthermore, genetic localization of three unassigned genes (*glaA, aglA* and *pepA*) in strains in which these genes were disrupted using a selectable marker gene led to a revised karyotype with respect for the chromosomes corresponding LG VIII and VI. The size of the chromosome corresponding to LG VIII was shown to be 5.0 Mb instead of 4.1 Mb, whereas the chromosome corresponding to LG VI was shown to be slightly larger than previously reported (5.2 Mb instead of 5.0 Mb). The revised electrophoretic karyotype reveals only 5 distinct bands. The presence of 3 pairs of equally sized chromosomes precluded assignment of genes to one specific chromosome in the wild-type strain. However, unambiguous chromosome assignment of cloned genes using CHEF-Southern analysis was demonstrated using a set of *A. niger* strains with introduced chromosomal size variation. The availability of these tester strains obviates the need of isolating or constructing mutant strains for the purpose of chromosome assignment.

INTRODUCTION

For the localization of genes in the genome of *Aspergillus niger* to individual chromosomes two approaches can be used. The first method is based on genetic analyses and can be used when mutants with a detectable phenotype are available. In case of non essential genes such mutants may also be generated by gene disruption experiments (for review see Timberlake, 1991). Genetic linkage group analysis may not only lead to the chromosomal localization but also provides some information about the genetic distance of the specific mutation to other linked markers. Recently, a complete set of tester strains for genetic analysis has been described by Bos et al. (1993), containing a full set of auxotrophic, morphological and resistance markers distributed over all eight linkage groups (LGs). A more detailed genetic analysis of mitotic crossover recombinations may be subsequently used to determine the position relative to the centromere and other markers on the chromosome. A genetic map of *A. niger* based on such mitotic mapping strategies has been published recently (Debets et al., 1993).

In many cases mutants resulting in a detectable phenotype may not be available and/or may not be generated by gene disruption experiments (e.g. in case of essential genes). Although, any gene may be marked with a selectable marker by site specific integration (e.g. Jones and Sealy-Lewis, 1989; Punt et al., 1990), the isolation of appropriate strains may be laborious as site specific integration in some cases was shown to be very infrequent (Goosen et al., 1989; Binninger et al., 1991; Akileswaran et al., 1993).

With the development of pulsed-field gel electrophoresis techniques (Schwartz and Cantor, 1984; Skinner et al., 1991) a second approach for gene mapping became available. All pulsed-field gel electrophoresis systems are essentially based on the same principle: intact chromosomes migrate through an agarose gel matrix, driven by a pulsed electric field, with a velocity dependent on their size and three-dimensional structure. As a consequence of this, the genome is resolved into chromosomal bands, the so-called electrophoretic karyotype. Any cloned DNA

segment may be assigned to one of the chromosomal bands of the electrophoretic karyotype by Southern hybridization studies.

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Chu et al., 1986) was used to obtain a partial electrophoretic karyotype of *A. niger* in which seven of the eight known LG's were assigned to one of the 4 chromosomal bands (Debets et al., 1990c). The position of the chromosome corresponding to LG VII was not resolved, as neither a LG specific gene or any transformant in which integration of introduced DNA had occurred in LG VII was available (Debets et al., 1990b/c).

The assignment of the other seven LG's to a chromosomal band in the CHEF pattern showed that in *A. niger* at least three pairs of chromosomes of almost the same size are present (Debets et al., 1990c). Therefore, unambiguous chromosome assignment of newly cloned sequences was not possible for *A. niger* without employing genetic analysis of the corresponding mutant strains. In *A. nidulans* (Brody and Cantor, 1989), *Podospora anserina* (Javerzat et al., 1993) and *S. cerevisiae* (Schwartz and Cantor, 1984) assignment of genes to chromosomes localized at a similar position in electrophoresis patterns of wild-type strains was accomplished using the altered electrophoretic karyotype of well-characterized translocation strains. Unfortunately, for *A. niger* to date translocation strains are not available. However, recently we succeeded in generating a number of strains with altered electrophoretic karyotypes by the introduction of multiple copies of the *glaA* gene (Verdoes et al., 1993a). In several of these strains chromosomes, which are of equal size in wild-type strains were separated in the electrophoretic karyotype.

In this paper the application of a set of these *A. niger* strains with introduced variation in electrophoretic mobility of chromosomes for chromosome assignment is presented. The complete electrophoretic karyotype of *A. niger* is described by localizing LG VII, using the recently cloned *nicB* gene of *A. niger* (Verdoes et al., 1993b). The use of this set of tester strains for chromosome assignment is illustrated for several genes recently cloned in our laboratory.

MATERIALS AND METHODS

Strains and plasmids

Intact chromosomal DNA molecules of *A. niger* were prepared from *A. niger* N402, N402[pAB6-10]B3, B13, B17, B38 and B39 transformants (Verdoes et al., 1993a; unpublished results), carrying multiple copies of the *A. niger glaA* gene . The *A. niger* strains AB4.1ΔGLA (this paper), ΔΔ20 (den Herder et al., 1992) and AB1.1 (Mattern et al., 1992) were used for the LG assignment by genetic analysis. Strain AB4.1ΔGLA is a derivative of AB4.1 (van Hartingsveldt et al., 1987) in which the *glaA* gene is replaced by the phleomycin-resistance gene. In strain ΔΔ20 the *aglA* gene and in strains AB1.1 the *pepA* gene is replaced by the *pyrG* gene of *A. oryzae* and the *pyrG* gene of *A. nidulans*, respectively. Master strains N890 and N849 were used (Bos et al., 1993), for the genotypes see Table 2. It should be noted that *pyrG1* and *pyrA5* are allelic and can both be complemented with the *A. oryzae pyrG* gene. The plasmids and DNA fragments used as LG specific probes, are described in Table 1.

Genetic analysis of deletion strains

Genetic analysis was essentially carried out as described by Bos et al. (1988) and Debets et al. (1990a). Benomyl was used at a final concentration of approximately 0.25 μg/ml. For the isolation of heterozygous diploids minimal medium supplemented with oligomycin (strain combinations N890//AB1.1 and N890//ΔΔ20) or phleomycin (strain combination N592//AB4.1ΔGLA) at concentrations of 1 μg/ml was used.

CHEF electrophoresis

Preparation of intact chromosomal DNA was carried out essentially as described by Debets et al. (1990c). Electrophoresis was performed using CHEF DRII apparatus and Model 1000 Mini Chiller (Bio-rad). The conditions were as described

Table 1. Plasmids and LG specific probes used in this study.

GENE:	PLASMID AND PROBE FRAGMENT:	REFERENCE:	LG ASSIGNMENT:
bphA	2.7-kb *Bgl*II fragment isolated from pAB8-2	van Gorcom et al. 1990	I Boschloo et al. 1991
pepA	1.4-kb PCR amplified fragment of *A. niger* containing complete coding region of *pepA*	Berka et al. 1990	I this paper
pyrG	3.7-kb *Hin*dIII fragment isolated from pAB4-1	van Hartingsveldt et al. 1987	III Bos et al. 1989
gpdA	1.4-kb *Hin*dIII fragment from pAB5-2	van Gorcom, unpubl. results	V this paper
glaA	1.4-kb *Not*I- *Nco*I fragment from pAN52-6	van den Hondel et al. 1991	VI this paper
rDNA	3.6-kb *Eco*RI fragment from pMN1 (*A. nidulans*)	Borsuk et al. 1982	VI this paper
nicB	2.8-kb *Hin*dIII fragment from pNIC1	Verdoes et al. submitted	VII this paper
aglA	9.5-kb *Sal*I fragment from pAB1-1A	den Herder et al. 1992	VIII this paper
cprA	1.2-kb *Eco*RI-*Bam*HI fragment from pCPR1	van den Brink et al. in prep.	VIII this paper

by Debets at al. (1990c) with some minor modifications. Gels were made with 0.6 % chromosomal-grade agarose (Bio-rad) in 1x TAE (Sambrook et al., 1989). Pulse duration of 24 h, 48 h and 72h with a pulse switch of 55, 47 and 37 min respectively, in 1 x TAE buffer chilled at 12.5 °C was used to separate the smaller chromosomes (3.5 - 4.3 Mb) (Condition A). For the separation of the larger chromosomes (5.0 - 6.6 Mb) electrophoresis was carried out for 3 periods of 48 hr, with pulse intervals of 55, 47 and 40 min, respectively. The temperature of the running buffer was 11 °C (Condition B).

Molecular genetic DNA techniques

General molecular biological techniques were essentially as described by Sambrook et al. (1989).

RESULTS AND DISCUSSION

Localization of LG VII

To localize the position of LG VII, which remained unresolved in the previously established karyotype (Debets et al., 1990a, c), a recently cloned LG VII specific gene, *nicB* (Verdoes et al., 1993b), was used. By Southern hybridization analysis the position of chromosome VII in the electrophoretic karyotype could be identified (Fig. 1, panel *nicB*). From the result obtained we conclude that LG VII is associated with a band of 4.1 Mb in the karyotype of *A. niger,* thus completing the electrophoretic karyotype of *A. niger*.

Genetic localization of genes using mutant strains obtained by gene disruption

In order to carry out chromosome localization studies of cloned genes using CHEF-Southern analysis, chromosome specific probes for all LG's are required. For *A. niger* only for a few cloned genes linkage data are available (Debets et al., 1990c). To obtain some additional LG specific probes, three recently cloned genes; *glaA, aglA* and *pepA* of *A. niger* were mapped using genetic analysis. For this purpose, strains were used in which these genes were deleted by gene replacement (AB4.1ΔGLA; this paper, ΔΔ20; den Herder et al., 1992 and AB1.1; Mattern et al., 1992, respectively). In two of these strains the gene was disrupted by the *pyrG* marker gene of *A. oryzae* (*aglA*) and the *pyrG* marker gene of *A. nidulans* (*pepA*). Diploids were constructed from these transformant strains and a *pyrA5* tester strain so that segregation of the Pyr^+ marker could be analyzed and thus the location of the disrupted genes could be inferred. The *glaA* gene was localized similarly by analysing segregation of the phleomycin-resistance marker used for replacing the *glaA* gene in a haploidization analysis. The results of these experiments are summarised in Table 2. From these data the three genes could be mapped unambiguously; *pepA* on LG I, *glaA* on LG VI and *aglA* on LG VIII. The

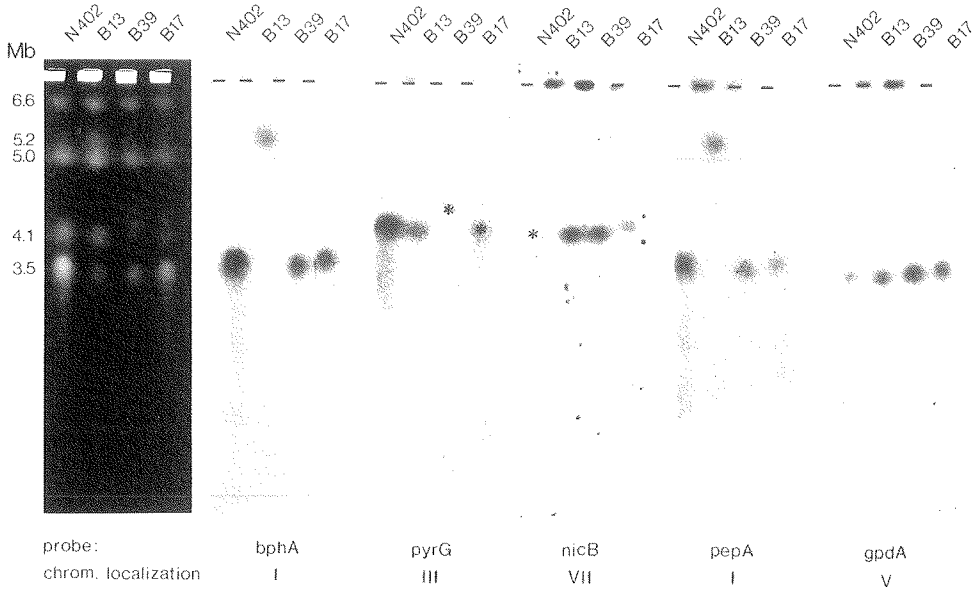

Figure 1. Chromosome assignment by hybridization analysis. Chromosomal DNA was separated under condition A (see M&M). The left panel shows an ethidium bromide stained CHEF gel with separated chromosomes of wild-type *Aspergillus niger* strain N402 and three *A. niger* strains with introduced electrophoretic mobility variation of one of the chromosomes (B13, B39 and B17). The sizes (in Mb) of the chromosomal bands in the wild-type strain are indicated on the left. Probes used for hybridization analysis and chromosome assignment are indicated under each panel. The quality of some samples of isolated chromosomal DNA results in fainter hybridization signals. The position of these signals (which could be seen clearly after longer exposure, results not shown) is marked by an asterisk.

result of *pepA* localization is in agreement with the results obtained by Mattern et al. (1992) who reported the genetic localization of a protease mutation, allelic to *pepA,* on chromosome I.

Revised localization of LG's VI and VIII

Subsequently, the three genes were used as LG specific probes in hybridization analysis of the karyotype of *A. niger* N402. Due to a slight modification in electrophoretic conditions compared to Debets et al. (1990c), a band of approximately 5.2 Mb was revealed separate from the previously identified 5.0 Mb

Table 2. Genetic analysis of deletion strains

		Linkage group:							
Strain:	?	I	II	III	IV	V	VI	VII	VIII
N849		*fwnA1*	*argH12*	*lysA7*	*hisC3*	*pheA1*	*pdxA2*	*nicB5*	*nirA3*
AB4.1ΔGLA	Δ*glaA::ble*			*pyrG1*					
%		44	53	55	49	50	8	65	57
N890		*fwnA1*	*argH12*	*pyrA5*	*leuA1*	*pheA1*	*lysD25*	*oliC2*	*crnB12*
AB1.1	Δ*pepA::pyrG*$_{AN}$			*pyrG1*					
%		0	58	--	60	53	67	75	47
ΔΔ20	Δ*aglA::pyrG*$_{AO}$			*pyrG1*					
%		48	39	--	37	48	44	63	14

fwnA1, fawn-coloured conidiospores; *argH12, lysA6/lysD25, hisC3, pheA1, pdxA2, nicB5, pyrA5/pyrG1, leuA1,* requirements for arginine, lysine, histidine, phenylalanine, pyridoxine, nicotinamide, uridine,leucine, respectively; *nirA3/crnB12, oliC2*, resistance to chlorate, oligomycin. All strains carry *cspA1* (short conidiospores) in addition to the markers shown. The percentage of recombinants between the disruption alleles and the genetic markers in the tester strain used are given. In strains AB1.1 and ΔΔ20 the genes are replaced by the *pyrG* gene of *A. nidulans* (*pyrG*$_{AN}$) and *A. oryzae* (*pyrG*$_{AO}$) respectively. For each analysis 140 - 260 haploid segregants were analyzed.

band (Fig. 1 and 2, first panel). The *glaA* probe hybridized to this newly observed band (Fig. 2, panel glaA). Based on these results LG VI was assigned to a 5.2 Mb chromosome. Unexpectedly, hybridization analysis with the *aglA* gene showed hybridization to a band of 5.0 Mb (Fig. 2, panel aglA) which, based on the previously published karyotype (Debets et al., 1990c) and the new position of the chromosome corresponding to LG VI, would indicate localization of *aglA* on LG II. This contradicts the data from the genetic analysis of strain ΔΔ20 which showed that the *aglA* gene is located on LG VIII (Table 2). However, re-examination of previous hybridization experiments using a (heterologous) LG VIII specific probe (*A. nidulans niaD)* which have lead to the initial assignment of LG VIII to a chromosome of 4.1 Mb (Debets et al., 1990c), indicated that LG VIII indeed

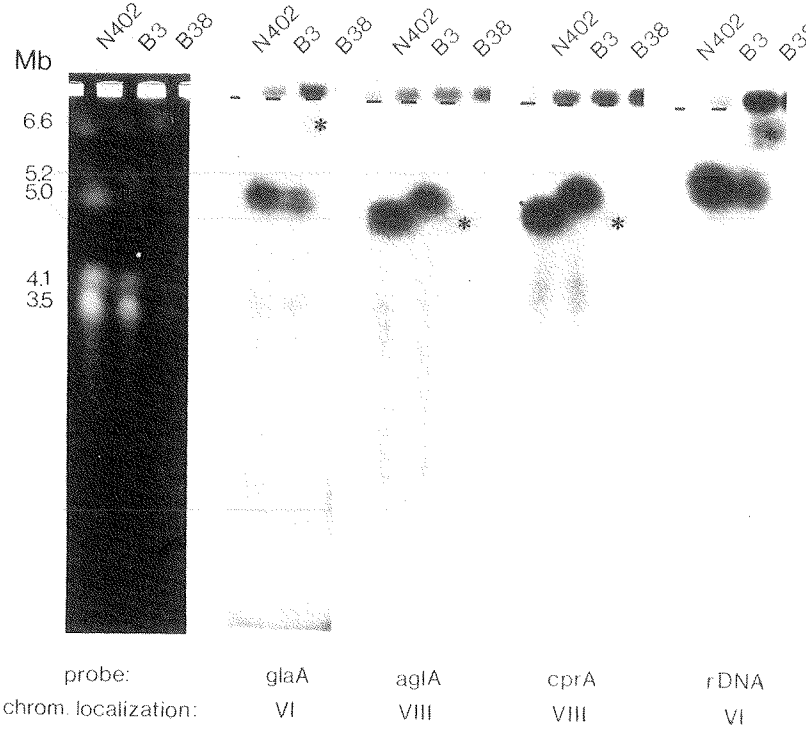

Figure 2. Chromosome assignment by hybridization analysis. Chromosomal DNA was separated under condition B (see M&M). The left panel shows an ethidium bromide stained CHEF gel with separated chromosomes of wild-type *Aspergillus niger* strain N402 and two transformant strains with introduced electrophoretic mobility variation of one of the chromosomes (B3 and B38). For further details see legend Fig. 1.

corresponds to a chromosome of 5.0 Mb. Hybridization with the *pepA* gene indicated that LG I corresponds to a chromosome of 3.5 Mb which is in agreement with previous experiments (Debets et al., 1990c). The complete, revised electrophoretic karyotype of *A. niger* N402 is schematically shown in Fig. 3.

Chromosome assignment using strains with introduced variation in chromosomal electrophoretic mobility

Although the electrophoretic karyotype of *A. niger* is complete by elucidating the position of LG VII and correcting the position of LG's VI and VIII, it still can not be

used for unambiguous chromosome assignment of cloned genes. This is due to the fact that of the eight chromosomes, 3 pairs are of almost identical size, which prevents that discrimination within these pairs can be made by hybridization analysis.

Recently, however, we obtained a number of strains with altered electrophoretic karyotypes generated by the introduction of multiple copies of the *glaA* gene (Verdoes et al., 1993a). These new copies are integrated at a single chromosome in each transformant. Out of these strains, a set of 5 strains was selected in which pairs of chromosomes, originally of almost identical size, are separated as a consequence of the increase in size of one of these chromosomes. Genetic analysis (Verdoes et al., in preparation) was carried out to determine in which LG the integration of the *glaA* gene copies had taken place. In addition, the position of the chromosomes with increased size in the electrophoretic karyotype was determined by Southern analysis using a fragment of the coding region of the *glaA* gene as probe (Verdoes et al., 1993a). Finally, the identification of these altered chromosomes with respect to the LG they belong to was determined using all available LG specific probes (panels *bphA, pyrG, nicB, pepA* in Fig. 1; *aglA, glaA* in Fig. 2). In all cases these results correspond to that of the genetic analysis. The electrophoretic karyotype of the strains with the introduced changes in electrophoretic mobility of the chromosomes is schematically shown in Fig. 3.

This set of tester strains has been used to localize three *A. niger* genes for which corresponding mutant strains were not available. Hybridization analysis of *A. niger* N402 with a probe from the glyceraldehyde-3-phosphate dehydrogenase gene (*gpdA*) showed that the *gpdA* gene is located on either LG I or V. (Fig. 1, panel *gpdA*). From the result that in strain B13 (with an altered mobility of chromosome I) the signal is found at the same position as in N402 we conclude that *gpdA* gene is located on LG V (Fig. 1, panel *gpdA*). Hybridization analysis of *A. niger* N402 and B38 with a probe from the *A. nidulans* rDNA which is expexted to be highly similar in sequence with *A. niger* rDNA (O'Connel et al., 1990) showed that the rDNA repeats are located on LG VI (Fig. 2, panel rDNA). Similar analysis

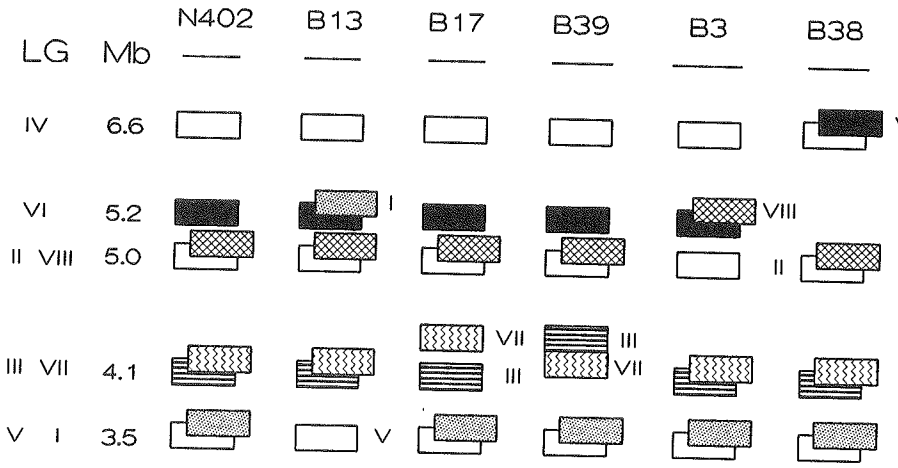

Figure 3. Schematic representation of the electrophoretic karyotype of the wild-type *Aspergillus niger* strain (N402) and the set of 5 *A. niger* strains with introduced variation in electrophoretic mobility of chromosomes. On the left the LG's corresponding to the different chromosomes in the wild-type strain N402 are indicated by roman numbers. The size of these chromosomes is indicated in Mb. The position of the chromosomes in the (tester) strains is indicated by differently marked boxes. Chromosomes specifically separated in the tester strains are indicated by LG numbers.

with a probe from the *A. niger* cytochrome-P450 reductase gene (*cprA)* resulted in an altered hybridization signal (compared to N402) in B3 (Fig. 2, panel *cprA*), indicating that *cprA* is located on LG VIII.

CONCLUSIONS

The complete, revised electrophoretic karyotype of *A. niger* was determined based on CHEF/Southern analysis with LG specific probes. The electrophoretic karyotype together with a set of strains with introduced electrophoretic mobility variation of chromosomes allows the assignment of any cloned *A. niger* gene to one of the eight chromosomes as illustrated by the localization of the rDNA, *gpdA* and *cprA* genes. Based on these results the number of genes useful as LG specific probes is extended and now enables detection by hybridization of the position of

chromosomes corresponding to six of the eight LG's in *A. niger* N402: *bphA/pepA* (LG I), *pyrG* (LG III), *gpdA* (LG V), *glaA*/rDNA (LG VI), *nicB* (LG VII) and *aglA/cprA* (LG VIII).

The availability of strains carrying chromosomes with an introduced variation in electrophoretic mobility may be used for chromosomal localization of genes in those species which lack useful sexual or parasexual genetic cycles.

Strains with such an altered karyotype may also be used to elucidate complex karyotypes, in which many chromosomes of equal size are present.

ACKNOWLEDGEMENTS

Prof. P.H. Pouwels is thanked for critical reading of the manuscript. M.R. Calil was supported by a scholarship of the CNPq of the Secretary for Science and Technology of Brazil. This project is supported by the Dutch Programme Commission for Industrial Biotechnology.

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Gene, in press

CHAPTER 5

The effect of multiple copies of the upstream region of the *Aspergillus niger* glucoamylase-encoding gene on expression

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(β-Glucuronidase; activator; expression; catabolite repression; deletion analysis; transcription regulation)

SUMMARY

The regulation of transcription of the glucoamylase-encoding gene (*glaA*) of *Aspergillus niger* was studied. To facilitate this study a reporter strain containing a fusion of the *glaA* promoter (p_{glaA}) of *A. niger* to the β-glucuronidase-encoding gene (*uidA*) of *Escherichia coli* was constructed. To analyze whether regulatory proteins are involved in the regulation of *glaA*, multiple copies of p_{glaA} were introduced into this reporter strain. Analysis of the resulting strains revealed that introduction of an increasing number of p_{glaA} copies resulted in decreasing expression levels of the *uidA* reporter gene and the endogenous *glaA* gene. These results indicate that the expression of genes under control of p_{glaA} are regulated by specific *trans*-acting regulatory protein(s). Similar so-called titration effects were observed in cultures cultivated in the presence of different inducing carbon sources, indicating that in these conditions the expression is controlled by the same regulatory protein. However, repression by xylose is not influenced by the copy number of p_{glaA}. Deletion analysis of p_{glaA} indicated that regulatory proteins interact with DNA sequences within 0.5 kb upstream from the ATG, whereas sequences between about 0.8 and 0.5 kb upstream from the ATG are identified for high-level expression of *glaA*.

INTRODUCTION

For many genes data have been obtained concerning (regulated) gene expression in filamentous fungi. In most cases these data mainly indicate growth conditions which are favourable or unfavourable for the expression of the gene of interest. Especially for genes encoding proteins involved in carbon catabolism, such as amylases, pectinases, cellulases and xylanases, induction and repression conditions are well established (Berka et al., 1992). However, for a relatively small number of genes and gene clusters regulation of gene expression has been studied at a molecular level in more detail. In most cases, primarily in *Aspergillus nidulans*, regulation was found to be mediated by pathway specific and/or wide domain regulatory proteins. During the last few years several genes encoding such regulatory proteins have been identified and cloned using regulatory mutants (see reviews: Davis and Hynes, 1992 and Scazzocchio, 1992). Generally, these regulatory proteins have similarity with DNA binding proteins as identified from studies in *S. cerevisiae* and higher eukaryotes (Johnson and McKnight, 1989), indicating a general mechanism for molecular gene regulation.

One of the approaches to study regulation of gene expression involves an *in vivo* deletion analysis of the promoter of interest. Serial deletions within the promoter region are made to study the effect of the deletion on gene expression. In this way promoter regions important for gene expression have been identified (for review see Punt and van den Hondel, 1992). An other method, titration analysis, may be used to identify promoter sequences which interact with *trans*-acting proteins. To do this, multiple copies of a promoter fragment containing putative target site(s) for transcription activators/ repressors are introduced and the effect is studied on the expression level of the gene of interest (Kelly and Hynes, 1987; for further review see Davis and Hynes, 1992).

In both deletion analysis and titration analysis the use of a reporter gene fused to the expression signals of interest will facilitate qualitative and quantitative analysis of the effects of deletion and titration. Two reporter gene systems, based on the β-galactosidase (*lacZ*) or the β-glucuronidase gene (*uidA*) of *E. coli*, are available

for *A. niger* (van Gorcom and van den Hondel, 1988; Roberts et al., 1989).

In our research we have focused our attention on the regulation of expression of the glucoamylase gene (*glaA*) of *A. niger*. The expression signals of *glaA* are frequently used for the overexpression of homologous and heterologous genes in various filamentous fungi (for review see van den Hondel et al., 1991). Gene expression controlled by these signals is induced by growth on starch or maltose, whereas no expression is observed in the presence of xylose as carbon source (Nunberg et al., 1984; Fowler et al., 1990). For *A. niger* it was shown that in the presence of glucose *glaA* expression reaches an intermediate level (Fowler et al., 1990). The molecular mechanisms underlying regulated expression of the *glaA* gene have not been elucidated yet. However, it is suggested that regulation of *glaA* expression is controlled primarily at the level of transcription (Fowler et al., 1990). Furthermore, it is as yet unclear whether *glaA* expression in *A. niger* is effected by carbon catabolite repression as was shown in a related species (Ghosh et al., 1990).

The lack of knowledge of the mechanism(s) of regulated expression has been shown to be a (serious) drawback in research aimed at selective overexpression of genes which are controlled by the *glaA* expression signals. Although considerable increase of the glucoamylase (GLA) production level could be obtained by introducing multiple gene copies (Finkelstein et al., 1989; Verdoes et al., 1993), the level of GLA production level seems to reach a maximum. This level corresponds with the expression of only a relatively small number of active gene copies. In our own experience, the GLA production levels could be increased 10 - 20 fold at maximum by increasing the gene copy number 20 up to 200-fold (Verdoes et al., 1993).

Further analysis indicated that the expression level in strains carrying many more copies of the *glaA* gene was limited at the level of transcription (Verdoes et al., 1993). To investigate the reason for this limitation a detailed analysis of the regulation of the *glaA* expression was started.

In the present paper the analysis of the transcription regulation of the *glaA* promoter is described using both titration and deletion analysis. For this study the glucoamylase promoter (p_{glaA}) was fused to the β-glucuronidase gene.

RESULTS AND DISCUSSION

(a) Construction of reporter strain

For the construction of the reporter strain, a vector (pGUS64; Fig. 1) was made in which the glucoamylase promoter (p_{glaA}) of *A. niger* was fused to the (reading frame of the) β-glucuronidase (GUS) encoding gene (*uidA*) of *E. coli*. A mutant *A. niger pyrG* allele (van Gorcom and van den Hondel, 1988) was used to facilitate

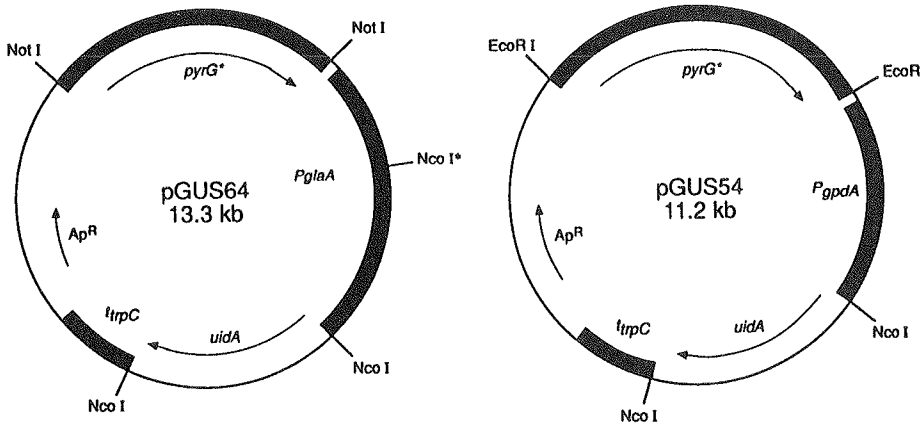


Figure 1. Plasmids used for the construction of the reporter strains.
The vector pGUS64 was constructed by introducing a 2.1-kb *Nco*I fragment from pNOM102 (Roberts et al., 1989), containing the gene coding for β-glucuronidase of *E. coli*, into *Nco*I digested pAN52-7NotI. Vector pAN52-7NotI is a derivative of pAN52-1NotI (Van den Hondel et al., 1991) in which the *A. nidulans gpdA* promoter is replaced by a 4.1-kb *Hin*dIII - *Nco*I fragment containing the *glaA* promoter of *A. niger* N402. For this construction, the *Nco*I site in p_{glaA} located 2.7-kb upstream of the ATG, was removed by filling in with Klenow polymerase (*Nco*I *). A new *Nco*I site was introduced at the ATG startcodon using a synthetic 76 bp *Xmn*I - *Nco*I fragment with a single base pair substitution compared to the wild-type *glaA* sequence, creating a *Nco*I site at the ATG (see Figure 3). A mutant *pyrG* gene (*pyrG* *) was introduced into the resulting vector as a *Not*I fragment isolated from pAB*pyrG*Not, in which the 3.7-kb *Xba*I fragment of pAB94-11 (van Gorcom and van den Hondel, 1988), carrying *pyrG* *, was cloned into *Not*I digested pBluescript using *Xba*I-*Not*I adaptors. The vector pGUS54 was constructed by cloning *pyrG* * as an *Eco*RI fragment into the unique *Eco*RI site of pNOM102 (Roberts et al., 1989). In both vectors the *trpC* terminator (t_{trpC}) of *A. nidulans* was used.

selection for integration of the reporter construct at the *pyrG* locus. As control for *glaA* specific transcription regulation, a similar reporter construct was made using the promoter region of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene (p_{gpdA}) of *A. niger* fused to the *uidA* gene, resulting in vector pGUS54 (Fig. 1). Both vectors were introduced into *A. niger* strain AB4.1 (*pyrG*$^-$; van Hartingsveldt et al., 1987) and a number of Pyr$^+$ transformants were obtained. To analyze *uidA* expression, transformants were tested in a GUS filter assay (legend Table 1). Transformants resulting in blue colonies in this assay were further studied by Southern analysis (results not shown) to identify strains with one copy of either pGUS64 or pGUS54 at the *pyrG* locus, designated GUS64 and GUS54 respectively. The expression of the reporter gene in GUS64 and GUS54 was analyzed after growth in the presence of different carbon sources. Highest expression levels are observed with GUS64 grown in the presence of maltose or maltodextrin, whereas levels on glucose are roughly 50-80 % of this level. Hardly any expression is observed in the presence of xylose (see Table 2). The expression levels found in GUS64 are in agreement with previously published data for *glaA* expression (Nunburg et al., 1984; Fowler et al., 1990). Therefore the level of *uidA* expression in this reporter strain may be used as measure for *glaA* expression in *glaA* regulation studies. Expression levels of the p_{gpdA} - *uidA* fusion gene in GUS54 is similar for all different carbon sources used, further confirming *glaA* specificity of the different expression levels observed for the GUS64 strain (results not shown).

(b) Construction of p_{glaA} multi-copy strains

Introduction of multiple copies of a *cis*-acting sequence that interact with the regulatory protein(s) can give valuable information about gene regulation (Kelly and Hynes, 1987; Davis and Hynes, 1992). Based on the results obtained with this type of research, similar experiments were designed to demonstrate the involvement of *trans*-acting regulatory proteins involved in the expression of the *A. niger glaA* gene. For these experiments two different vectors were constructed (Table 1),

containing either one copy of p_{glaA} (pAB6-15) or approximately 10 copies of p_{glaA} (pAB6-18). Both vectors contain the *amdS* gene of *A. nidulans* as selection marker. As it is not known where the bindingsite(s) for the proposed regulatory protein(s) are located within p_{glaA}, a 2.7-kb *Nco*I - *Bss*HII fragment was used for these constructions. This fragment contains in addition to the *glaA* promoter region and upstream sequences, the 5' part of the transcribed region of the *glaA* gene up to codon 24.

Strain GUS64 was transformed with pAB6-15 or pAB6-18. For the isolation of strains carrying different numbers of integrated vectors, selection was carried out on plates containing either acetamide or acrylamide as sole nitrogen source. In the latter case higher numbers of integrated vectors can be expected (Kelly and Hynes, 1985; Verdoes et al., 1993).

(c) Expression analysis of p_{glaA} multiple copy strains

A large number of GUS64 transformants were assayed for glucuronidase activity (GUS) using a qualitative filter assay. Approximately 17 % of transformants (21 out of 124) obtained after transformation with pAB6-15 and 30 % (3 out of 10) of transformants obtained after transformation with pAB6-18 (acetamide selection) displayed a reduced blue coloration in the filterassay compared to the untransformed GUS64, indicating decreased *uidA* expression in these transformants. After selection on acrylamide 37 % of the GUS64 transformants (6 out of 16) with pAB6-15 revealed a reduced *uidA* expression. Analysis of GUS64 transformants obtained after transformation with an *amdS* vector without the *glaA* promoter region (pAN4.1) did not show any effect on the level of *uidA* expression. Furthermore, no indication for a decreased *uidA* expression was observed in pAB6-15 transformants of strain GUS54 (p_{gpdA}-*uidA*) selected on acetamide or acrylamide (24 tested). These data suggest that in some transformants introduction of multiple copies of p_{glaA} leads to a decreased expression of genes under control of p_{glaA}.

For a number of GUS64 transformants the *uidA* expression was determined by a quantitative GUS-assay and the number of p_{glaA} copies present in the genome was estimated by Southern blot analysis (Table 1). As shown in Table 1, the results of

Table 1. Analysis of *uidA* expression in multiple copies p_{glaA} reporter strains

Strain [A]		Plate GUS assay [B]	*uidA* expression [C]		number of p_{glaA} gene copies [D]
GUS64		+	1960	(100)	2
[pAB6-15]	#24	+/-	910	(47)	5
	#48	+	1910	(97)	4-10
	#53	+/-	1400	(71)	10
	#32	+	2300	(117)	20
	#30	+/-	780	(40)	50
	#52	+/-	720	(37)	100-200
[pAB6-18]	# 9	+	1990	(101)	20
	# 8	+/-	410	(21)	200
	# 3	+/-	190	(10)	>200
	#10	+/-	130	(7)	>500

[A] For the construction of both p_{glaA} vectors, a 2.7-kb NcoI - HindIII fragment, containing the promoter sequences and the first 24 codons of *glaA*, was isolated from pAN52-6 NotI (van den Hondel et al., 1991) and cloned into the corresponding sites of pMTL25P (Chambers et al., 1988). From the resulting vector the p_{glaA} sequences were isolated on a *Hin*dIII fragment. The vectors pAB6-15 and pAB6-18 were constructed by cloning this *Hin*dIII fragment in the unique *Hin*dIII site of pAN4-1 and pAN4-cos1 (Verdoes et al., 1993), respectivily. For the construction of pAB6-18, ligation mixtures with a 10 times molar excess of the p_{glaA} fragment were packaged and transfected, using an in vitro packaging kit (Gigapack XL, Amersham), into *E. coli* 1046 as host strain (Verdoes et al., 1993).

[B] Transformants were grown on agar plates containing 1 % maltodextrin as sole carbon source covered with nylon filters (Hybond). As soon as the mycelium has grown through the filter (about 36 h at 30 °C), the filter was lifted from the agar plates and the intracellular GUS protein was released by a freeze-thaw step in liquid nitrogen and *uidA* expression was assayed in a 0.04 % X-gluc solution essentially as described for *lacZ* expression (Kolar et al., 1990). +, blue colonies; +/-, light blue colonies compared to GUS64.

[C] About 5.10^7 spores were inoculated in 50 ml culture medium (Bennett and Lasure, 1991; 5 % maltodextrin as carbon source) in shake flasks (300 ml) at 30 °C for 48 hours. Quantitative GUS assay were carried out on mycelial extracts, essentially as described by Roberts et al., 1989. *UidA* expression is indicated in Units GUS/mg protein. Values in parentheses indicate relative *uidA* expression level (level of GUS64 is set to 100 %). The *uidA* expression levels presented are from a single experiment. Between experiments considerable differences in expression level may be observed. The mean values and standard deviations of *uidA* expression over 9 experiments for GUS64 and GUS64[pAB6-18]#3 are 1500 ± 500 and 270 ± 130, respectively. The relative *uidA* expression for GUS64[pAB6-18]#3 was always between 10 - 20 %.

[D] The copy number of p_{glaA} was determined by Southern blot analysis. Hybridisation signals for the transformants were compared with serial dilution of the p_{glaA} fragment. The signal from the endogenous *glaA* gene was used as internal standard.

the quantitative and qualitative GUS assays are in good agreement with each other. Furthermore, these results clearly show that an increase in the number of p_{glaA} containing fragments leads to a concomitant decrease of *uidA* expression, in case the expression of the reporter gene is under control of the p_{glaA}. From these results we conclude that introduction of multiple copies of the upstream region of the *glaA* gene can titrate one or more activator proteins, which leads to decreased expression of the (reporter) gene. This titration effect is similar to what was observed for the *amdS* gene and the genes driven by the *alcA* promoter in *A. nidulans* (Kelly and Hynes, 1987; Felenbok et al., 1992).

Additional evidence for this titration was obtained by the analysis of the expression of the endogenous *glaA* gene in GUS64 and the transformants with multiple copies of p_{glaA}. If the observed effect of multiple copies of the p_{glaA} on *uidA* expression is specific for $p_{glaA,}$ then the presence of these copies should also effect *glaA* expression. Indeed, in those transformants which showed a decreased *uidA* expression also the amount of secreted GLA protein was decreased compared to the untransformed GUS64 strain as shown by iso-electric focusing and Western analysis using α-GLA antibodies (Verdoes et al., 1993, results not shown). To determine at what level expression of the *glaA* gene was limited Northern analysis was carried out (Fig. 2). From the result it is clear that in strains carrying multiple copies of p_{glaA} (Fig. 2; lanes 2, 3, and 5) the *glaA* mRNA level is strongly reduced while no effect is seen for the *gpdA* mRNA (Fig. 2). From these data we conclude that the reduced expression of p_{glaA} regulated genes is caused by a limitation at the level of transcription.

Interestingly, this limitation of expression at the level of available transcription factors may also explain why in a previous study aimed at the overexpression of glaA by introducing multiple copies of the gene in *A. niger*, a maximum level of GLA production was found, which could not be overcome by introducing additional copies (Verdoes et al., 1993). Apparently the available amount of regulatory protein is only sufficient to activate a limited number of *glaA* gene copies per nucleus.

A further improvement of GLA production in multi-copy strains may therefore only be achieved by introducing additional copies of the gene(s) encoding these regulatory factor(s) in these strains. This type of strategy has been successfully

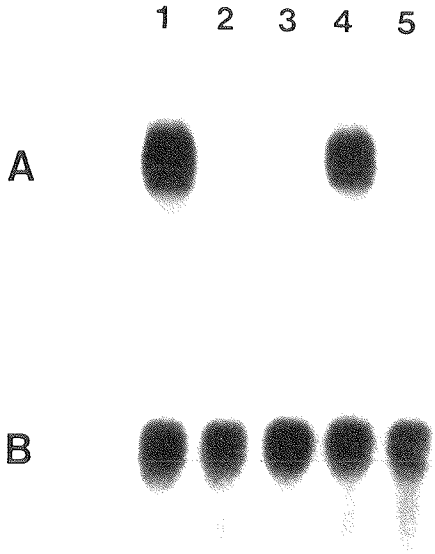


Figure 2. Northern analysis of mRNA samples from reporter strain GUS64 and derivatives carrying multiple copies of p_{glaA}. Lanes 1-5 contain 7.5 ug total RNA from GUS64 (1); GUS64[pAB6-18]#3 (2); GUS64[pAB6-18]#8 (3); GUS64[pAB6-18]#9 (4); GUS64[pAB6-18]#10 (5) (see also Table I). RNA isolations, using the RNAzol™ kit from CINNA/BIOTECX, were carried out as described by the suppliers. RNA was separated on a formaldehyde/agarose gel (Sambrook et al.,1989) and transferred to Hybond N⁺ membrane. RNA was hybridised simultanously with ^{32}P-labelled probes (multiprime labelling kit, Amersham) obtained from the coding region of the *glaA* gene (3.3-kb *Eco*RI fragment, Verdoes et al., 1993) (**A**) and the coding region of the *gpdA* gene of *A. niger* (1.4-kb *Hin*dIII fragment, R.F.M. van Gorcom, unpublished) (**B**)

applied in *A. nidulans*. In a strain carrying multiple copies of the *alcA* expression signals the expression was shown to be limited by the level of the transacting regulatory protein, *alcR* (Gwynne et al., 1987; Davies, 1991). Introduction of multiple copies of the *alcR* gene in this type of strains significantly increased gene expression controlled by the *alcA* promoter region (Gwynne et al., 1987; Davies, 1991). The use of this approach for improving gene expression driven by p_{glaA} awaits isolation and characterisation of gene(s) coding for the regulatory proteins. Furthermore, the study of these genes will be of great help for the understanding of the regulation of *glaA* expression.

(d) Titration effect in multi-copy p_{glaA} strain cultivated on different carbon sources

To further elucidate the mechanism of transcriptional regulation of the *glaA* gene we analyzed whether the observed titration effect was depending on the carbon

source used for cultivation. Therefore, strain GUS64 and the titration strain GUS64[pAB6-18]#3 (Table 1) were cultivated in the presence of different carbon sources and the level of *uidA* expression was determined. The data presented in Table 2 show that under all conditions tested the expression level in GUS64 is clearly higher than in GUS64[pAB6-18]#3. Furthermore, it is shown that titration occurs under all conditions where significant expression is observed. Therefore we conclude that gene expression in the presence of either glucose, maltose or maltodextrin as carbon sources may be regulated by the same activator.

Interestingly, the effect of titration is higher on glucose cultivated cultures than in cultures grown in the presence of maltodextrin or maltose, which may be taken as an indication that the amount of functional activator present under these conditions may be higher than in the presence of glucose.

In the presence of xylose no significant expression was observed. To study whether the lack of expression in the presence of xylose is caused by either the absence (or inactivation) of the activator or another mechanism both strains mentioned above were also grown in media containing xylose and one of the other carbon sources. As shown in Table II the combination of xylose and a second carbon source results in both strains in a level of expression in between that of cultures grown in xylose or the other carbon sources alone. As the relative levels of repression are similar, we conclude that the effect of repression by xylose is not influenced by the introduction of multiple copies of p_{glaA}, implying that xylose repression is mediated by a distinct, not yet saturated, repressor mechanism which is not mediated by the above mentioned regulatory protein(s).

The results of cultivation on mixed carbon sources do not completely correspond with those reported by Fowler et al. (1990) who showed that xylose had no effect on the level of GLA production in maltose and glucose grown cultures. However strains and growth conditions are not completely comparable between both studies.

(e) Localization of target site(s) for the regulatory factor(s)

Deletion analysis of p_{glaA} was carried out to identify the region(s) in the upstream

Table 2. Analysis of *uidA* expression in reporter and titration strain grown in the presence of different carbon sources.

STRAIN	CARBON SOURCE [A]	*uidA* EXPRESSION [B]	% TITRATION [C]
GUS64	XYL	1 ± 1	
GUS64[pAB6-18]#3	XYL	3 ± 3	
GUS64	GLC	1080 ± 460	
GUS64	GLC/XYL	370 ± 170	
GUS64[pAB6-18]#3	GLC	50 ± 15	4.5
GUS64[pAB6-18]#3	GLC/XYL	20 ± 10	5.5
GUS64	MAL	1360 ± 230	
GUS64	MAL/XYL	700 ± 370	
GUS64[pAB6-18]#3	MAL	250 ± 120	18.2
GUS64[pAB6-18]#3	MAL/XYL	80 ± 40	11.3
GUS64	MDX	1320 ± 330	
GUS64	MDX/XYL	140 ± 10	
GUS64[pAB6-18]#3	MDX	230 ± 90	17.6
GUS64[pAB6-18]#3	MDX/XYL	40 ± 10	29.6

[A] Cultivation conditions similar as indicated in Table 1. In combinations 5 % of each carbon source was used. XYL, xylose; GLC, glucose; MAL, maltose; MDX, maltodextrin.
[B] For further experimental details see legend Table 1.
[C] Relative *uidA* expression calculated from the mean value *uidA* expression in GUS64[pAB6-18]#3 / mean value *uidA* expression in GUS64.

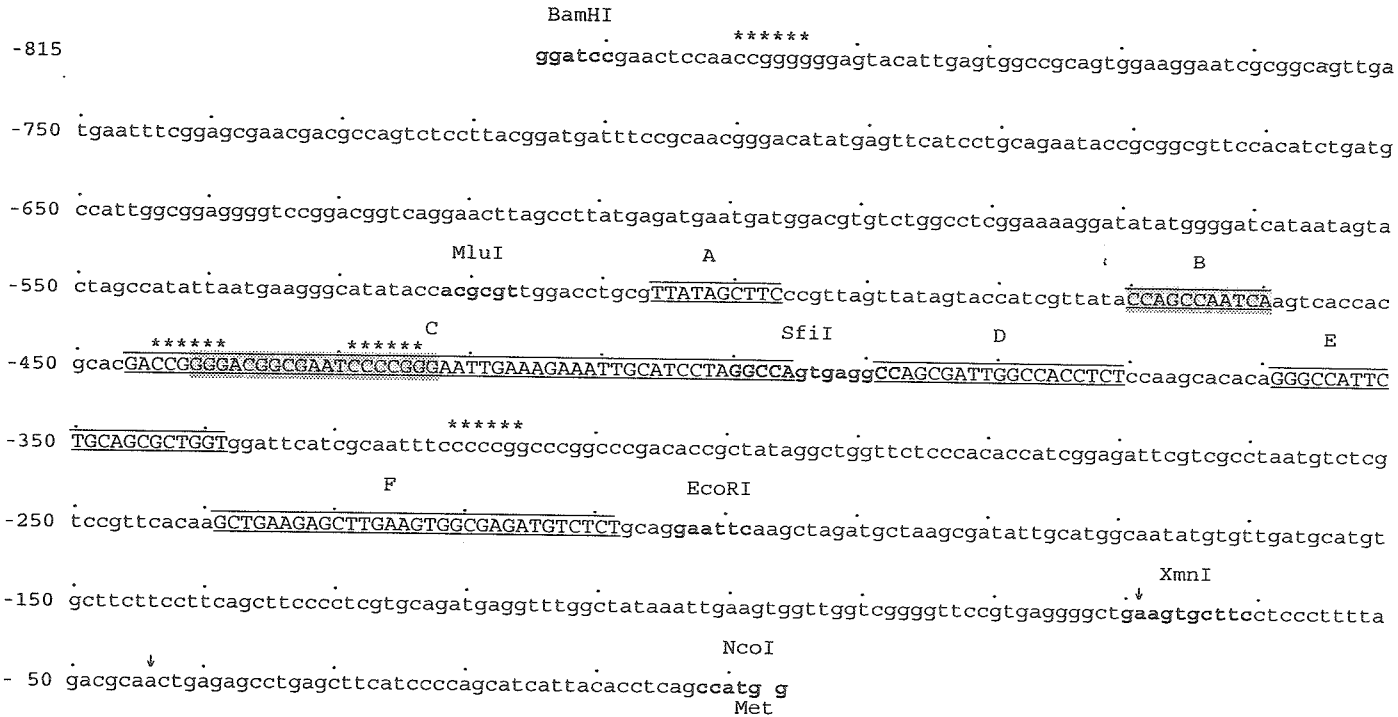
region of the *glaA* gene that contains the target site(s) for the regulatory factor(s). Initially, the complete nucleotide sequence of about 800 bp of the 5' region of the *glaA* gene was determined (Fig. 3). Subsequently, a number of deletion derivatives of p_{glaA} were generated using appropriate restriction sites (Fig. 4). These mutant promoter fragments were fused to the *uidA* gene, generating vectors similar to pGUS64 and introduced into *A. niger* AB4.1 and an appropriate *pyrG*$^-$/*uidA*$^-$ titration strain (AB6-18). The latter strain was obtained by selection of *pyrG*$^-$

mutants of GUS64[pAB6-18]#3 on minimal medium agar plates containing 10 mM uridine and 1 mg/ml 5-fluoro-orotic acid (van Hartingsveldt et al., 1987). Subsequent analysis of some of the resulting *pyrG*$^-$ strains showed that 90% of them lacked *uidA* expression whereas the other 10 % were still *uidA*$^+$. Southern analysis of the *pyrG*$^-$/*uidA*$^-$ mutants showed that these strains had lost the *uidA* gene due to a recombination event between the two *pyrG* genes flanking the *uidA* gene. One of the *uidA*$^-$/*pyrG*$^-$ mutants, designated AB6-18, containing the same number of p_{glaA} copies as GUS64[pAB6-18]#3 (as determined by Southern analysis; results not shown) was used for further experiments.

Transformants from AB4.1 and AB6-18, each containing a single copy of the various deletion vectors (identified by Southern analysis) were subsequently analyzed for *uidA* expression (Fig. 4). As shown in Fig. 4 deletion of the promoter region up to the *Bam*HI site has only a moderate effect on the *uidA* expression level in both strains. However deletion up to the *Mlu*I site significantly reduced the *uidA* expression level to about 5 %, indicating that sequences within the *Bam*HI - *Mlu*I fragment are important for the level of expression. The deletion of sequences up to the *Sfi*I site and further downstream resulted in a complete loss of expression. Comparison of the expression levels in the wild-type and titration strain indicate that all derivatives with significant *uidA* expression are subject to a decrease in expression level due to titration. From these data we conclude that target site(s) for the putative regulatory protein(s) are located downstream of the

Figure 3. Nucleotide sequence of the 5' upstream region of the *glaA* gene of *Aspergillus niger* N402. Numbering is from the translation start codon. Restriction sites used for the construction of deletion derivatives of the glucoamylase promoter are indicated in bold. Elements of similar sequences within the promoter region of the *glaA* gene (Hata et al., 1992) and *amyB* gene (Nagata et al., 1993) of *A. oryzae* (region A to F; see also Fig. 5), obtained by sequence comparison with the GCG analysis program (Devereux et al., 1984), are indicated in over and underlined capital letters. Two of these regions (B and C) overlap with regions previously indicated to be involved in expression of the *A. oryzae amy* genes (region B; Tsuchiya et al., 1992; Nagata et al., 1993) and the *A. oryzae glaA* gene (region C; Hata et al., 1992) are indicated by redlining. Putative *creA* binding sites (Kulmburg et al., 1993) are indicated by *. The transcription start points, as reported by Boel et al., (1984), are indicated by ↓.

Figure 3.


BamHI
******
-815 ggatccgaactccaaccgggggagtacattgagtggccgcagtggaaggaatcgcggcagttga
-750 tgaatttcggagcgaacgacgccagtctccttacggatgatttccgcaacgggacatatgagttcatcctgcagaataccgcggcgttccacatctgatg
-650 ccattggcggaggggtccggacggtcaggaacttagccttatgagatgaatgatggacgtgtctggcctcggaaaaggatatatggggatcataatagta
MluI
A
B
-550 ctagccatattaatgaagggcatataccacgcgttggacctgcgTTATAGCTTCccgttagttatagtaccatcgttataCCAGCCAATCAagtcaccac
C
SfiI
D
E
****** ******
-450 gcacGACCGGGGACGGCGAATCCCCCGGGAATTGAAAGAAATTGCATCCTAGGCCAgtgaggCCAGCGATTGGCCACCTCTccaagcacacaGGGCCATTC
******
-350 TGCAGCGCTGGTggattcatcgcaatttcccccggcccggcccgacaccgctataggctggttctcccacaccatcggagattcgtcgcctaatgtctcg
F
EcoRI
-250 tccgttcacaaGCTGAAGAGCTTGAAGTGGCGAGATGTCTCTgcaggaattcaagctagatgctaagcgatattgcatggcaatatgtgttgatgcatgt
XmnI
-150 gcttcttccttcagcttcccctcgtgcagatgaggtttggctataaattgaagtggttggtcggggttccgtgaggggctgaagtgcttcctccctttta
NcoI
- 50 gacgcaactgagagcctgagcttcatccccagcatcattacacctcagccatg g
Met

*Mlu*I site. As in deletion derivatives downstream of the *Sfi*I no expression was found, we can not localize the region involved in titration more precisely. However, based on our results we can conclude that the target site for the putative regulatory protein(s) is located upstream of the ATG. Comparison of the promoter sequence of the *A. niger glaA* gene with that of the promoter region of similarly regulated *A. oryzae* glucoamylase and amylase genes revealed several regions of similar sequence, which may be involved in gene expression and / or regulation (Fig. 5). Comparison of both *glaA* genes revealed two regions of similar sequence both located in the *Mlu*I - *Sfi*I fragment of the *A. niger* (Fig. 3; Fig. 5A/C). One of the regions (Fig. 5C) overlaps with region I, indicated by Hata et al. (1992). The sequence of region I was shown to be important for the induction of the *glaA* gen in *A. oryzae* by in vivo deletion analysis (Hata et al., 1992). Interestingly, this region (Fig. 5C) overlaps with 2 copies of the CreA/MIG1 target sequences (G/CPyGGGG; Kulmburg et al., 1993). More copies of these putative CreA/MIG1 target sequences were found at position -810 and -321 (Fig. 3). In *A. nidulans* these sequences are shown to be involved in interaction with the CreA regulatory protein (Sophianopoulou et al., 1992; Kulmburg et al., 1993), which mediates carbon catabolite repression (Dowzer and Kelly, 1991). In the *glaA* promoter region these CreA sites overlap with a putative target site for a specific activator protein. This corresponds remarkable well with the situation for the *alcR/alcA* genes (Kulmburg et al., 1993). In the promoter region of these genes the AlcR and CreA target sites are overlapping or in close proximity, which led these authors to suggest a functional role for overlapping target sites of activator and repressor proteins (Kulmburg et al., 1993). Recently the gene coding for the CreA protein of *A. niger* was cloned (Drysdale et al., 1993), suggesting that also in *A. niger* gene regulation by carbon catabolite repression will be CreA mediated. Although similarity was found with functional sequences in other promoter regions we can not conclude anything about the functionality of these regions in our promoter. Obviously, further in vivo analysis is needed to verify the role of the CreA protein and the indicated target sites in the regulation of *A. niger glaA* expression.

Comparison of the *A. niger glaA* upstream region with that of α-amylase genes (TAAG2 and *amyB)* from *A. oryzae* (Tsuchiya et al., 1992; Nagata et al., 1993)

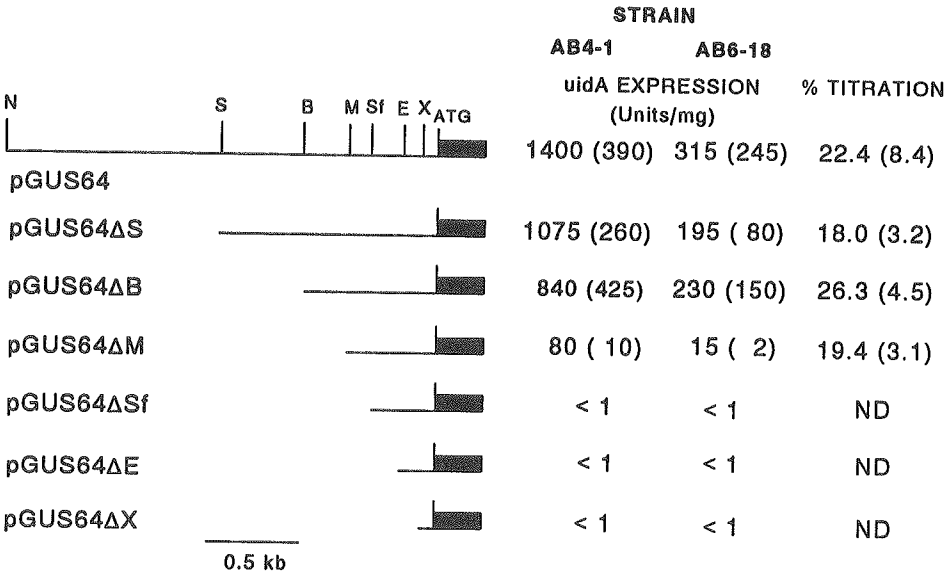


Figure 4. Analysis of the *uidA* expression of transformants of the wild-type strain (AB4.1) and strain carrying multiple copies of p_{glaA} (AB6-18; see text for description) each containing a single copy of the indicated deletion vectors at the *pyrG* locus. Deletion derivatives of pGUS64 were constructed using restriction sites as indicated. pGUS64ΔB indicates that all sequences upstream from this site have been removed using standard cloning techniques (Sambrook et al., 1989). Single copy transformants were selected by Southern analysis. Values in parentheses indicate standard deviation.

revealed 4 other elements of similar sequence (Fig. 5B, D, E and F), one located upstream and three located downstream of the *Sfi*I site. One of these regions (Fig. 5B) was shown to be involved in the level of expression of the *amy* gene using in vivo deletion analysis (Tada et al., 1991; Nagata et al., 1993). Furthermore, Nagata et al. (1993) showed that the core CCAAT sequence present in this region interacts with nuclear proteins from starch and glucose grown *A. nidulans* cultures.

Interestingly, this region is identical to a region involved in the level of expression of the *amdS* gene of *A. nidulans* (Fig. 5B)(Littlejohn and Hynes, 1992). DNA/protein binding experiments with this sequence have shown that this element interacts with nuclear protein from *A. nidulans* (van Heeswijck and Hynes, 1991).

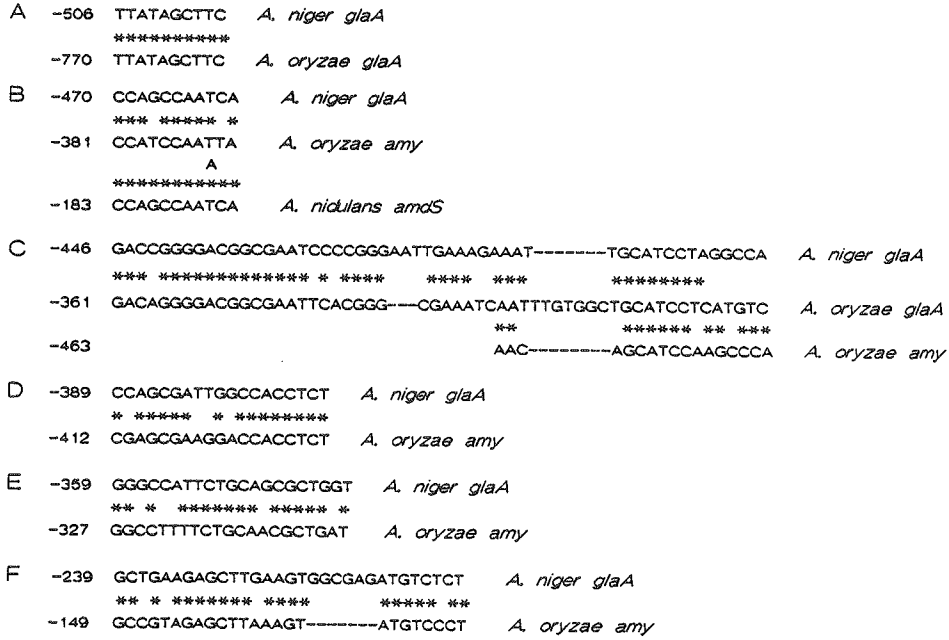


Figure 5. Regions of similar sequences in the upstream regions of different maltose/maltodextrin induced *Aspergillus* genes. (**A**) Nucleotide sequence of a region of similar sequence in the upstream region of the *glaA* genes of *A. niger* and *A. oryzae* (Hata et al., 1992). (**B**) Sequence element similar to a nuclear protein binding site in the *amy* genes of *A. oryzae* (Tsuchiya et al., 1992; Nagata et al., 1993) and the *amdS* gene of *A. nidulans* (van Heeswijck and Hynes, 1991). (**C**) Sequence element similar to the *glaA* gene and *amy* genes of *A. oryzae*. (**D**, **E**, **F**) Sequence elements similar to the *amy* genes of *A. oryzae*. Identical nucleotides to the *A. niger glaA* are indicated by *. Gaps are indicated by -. The number indicate the distance from the first base to the translation start point. The regions of similar sequences are also indicated in Fig. 3. (A to F).

Taken together these data suggest that the sequence in Fig. 5B may be a target site for a general transcriptional activator.

In conclusion, we suggest that sequences in between the *Bam*HI - *Sfi*I sites play an important role in the expression of the *glaA* gene by providing targetsites for both general and specific *trans*-acting regulatory proteins. The presence of three additional regions of similarity between the *A. niger glaA* and *A. oryzae amy* genes downstream of the *Sfi*I site (Fig. 5D, E and F) may indicate that also downstream of *Sfi*I target sequence(s) for *trans*-acting (regulatory) proteins are present. Currently,

further research is being carried out to localize target sites for specific regulatory proteins involved in regulated expression of the *A. niger glaA* gene.

(g) Conclusions

We have studied the regulation of transcription of the glucoamylase gene of *Aspergillus niger* using a combined method of titration and deletion analysis.
(1) Introduction of multiple copies of *glaA* promoter fragments causes titration of *trans*-acting regulatory protein(s). From these data we conclude that the expression of the *glaA* gene is regulated by at least one positive regulatory factor.
(2) Similar titration effects are observed under different inducing conditions (glucose, maltose and maltodextrin) suggesting that the expression in these conditions is controlled by the same activator. The effect of titration is higher in cultures grown in maltose or maltodextrin than in glucose suggesting that the amount of active regulatory protein is lower in glucose grown cultures. Consequently, the regulation of the amount of regulatory protein may be a major component for the regulation of *glaA* expression.
(3) Xylose results in a decreased expression level when it is combined with other, inducing carbon sources. The repression effect of xylose is not influenced by introduction of multiple copies of the *glaA* promoter fragment. These data imply that repression by xylose is not the result of the regulation of expression or inactivation of the regulatory protein shown by titration analysis.
(4) Deletion analysis shows that *glaA* expression and regulation can be assigned to different sequences within the promoter region. At least one of the target sites for regulatory proteins is located within 517 bp upstream of the translation start. Sequences between - 815 and - 517 bp are indicated to be involved in the level of expression.

ACKNOWLEDGEMENTS

The authors wish to thank C. van Rotterdam for her contribution to the vector constructions. Dr. W. van Hartingsveldt and Prof. P. H. Pouwels are thanked for

critical reading of the manuscript. This project is supported by the Dutch Programme Commission for Industrial Biotechnology.

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CHAPTER 6

Characterization of an efficient gene cloning strategy for *Aspergillus niger* based on an autonomously replicating plasmid: cloning of the *nic*B gene of *A. niger*

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(mitotic instability; cotransformation; complementation; *pyr*G; recombination; filamentous fungi; linkage group VII specific gene)

SUMMARY

The development of an improved gene cloning strategy by complementation of mutant alleles in *Aspergillus niger* is described. The strategy is based on the use of a fungal autonomously replicating vector, pAB4-ARp1. This vector was constructed by the introduction of a previously described sequence (AMA1), involved in autonomous replication, into a *pyr*G integrative vector, pAB4-1. With vector pAB4-ARp1, a 10-100 fold increase in transformation frequency was obtained compared to pAB4-1. Furthermore, the transformation frequency of a cotransformed plasmid is also increased using pAB4-ARp1. *A. niger* transformants containing pAB4-ARp1 are mitotically unstable. Cotransformed plasmids strictly cosegregated with the autonomously replicating vector, as a result of recombination between both vectors. The use of pAB4-ARp1 in gene cloning was demonstrated by the complementation of two linkage group VII specific *A. niger* mutants. Complementation of a *lys*F mutant was achieved by cotransformation of pAB4-ARp1 with total genomic *A. niger* DNA ("instant bank"). A *nic*B deficient *A. niger* was complemented by cotransformation with pAB4-ARp1 and an *A. niger* cosmid library. The complementing DNA was reisolated from a *Nic*$^+$ transformant by transforming *E. coli* with total genomic DNA of this transformant. Gene disruption and genetic analysis was carried out to prove that the previous unknown *A. niger* *nic*B gene has been cloned.

INTRODUCTION

A number of strategies have successfully been applied for the cloning of genes of filamentous fungi (for review see Turner, 1991 and references therein). One of these strategies is based on direct complementation of mutant strains. This complementation may be carried out by gene expression in *E. coli* (e.g. Yelton et al., 1983; Kos et al., 1985), *S. cerevisiae* (Berse et al., 1983), or a fungal host (e.g. Johnstone et al., 1985; Yelton et al., 1985). The first gene isolation by direct complementation in a fungal host is described by Johnstone et al. (1985) who used a chromosomal DNA bank constructed in an integrative fungal vector. The complementing DNA was recovered by rescue of the DNA fragment by restriction enzyme digestion, recircularization, and transformation of *E. coli*. An improvement of this method was found in the construction of gene libraries in cosmid vectors (Yelton et al., 1985). As longer DNA fragments (35-40 kb) can be inserted into cosmids compared to plasmids ($\approx$ 10 kb), fewer transformants are needed to represent the complete fungal genome. Furthermore, complementing DNA can be recovered by *in vitro* packaging of λ-phage particles using undigested fungal DNA and subsequent transfection of *E. coli* cells (Yelton et al., 1985; O'Hara and Timberlake, 1989).

The application of direct complementation for gene cloning has been hampered by the relatively low transformation frequency of fungal transformation vectors (in general lower than 1000 transformants/μg). Furthermore the fact that, in general, several vectors integrate into the fungal genome makes re-isolation of complementing sequences far from easy. Even for the reisolation of cosmid vectors two linked λ-*cos* sites separated by 35-50 kb of DNA are needed for packaging. This requirement may have prohibited successful reisolation in some cases (e.g. Adrianopoulos and Hynes, 1988).

The recent isolation of a sequence (AMA1) involved in autonomous replication in *A. nidulans* (Gems et al., 1991) provided a new improvement to the direct complementation strategy. As in yeast, the autonomously replicating plasmid has

proven to increase the transformation frequency and greatly facilitated reisolation of the transforming sequences (Gems et al., 1991). A vector based on the AMA1 sequence was used for cloning of selectable fungal genes by direct complementation in *A. nidulans* using cotransformation of this vector with fragmented chromosomal fungal DNA (Gems et al., 1993).

In this paper we describe the construction and characteristics of an autonomously replicating vector for specific use in gene cloning strategies in *A. niger*. The characteristics of this vector in relation to the cloning of both selectable and nonselectable genes are presented. The usefulness of this vector is illustrated by the isolation of the *nic*B gene from *A. niger*.

RESULTS AND DISCUSSION

(a) Characteristics of pAB4-ARp1

(1) Transformation frequency

The AMA1 sequence, involved in autonomous replication of plasmid DNA in *Aspergillus* species (Gems et al., 1991) was cloned into the *pyr*G integrative vector pAB4.1, yielding pAB4-ARp1 (Fig. 1). The transformation frequency of plasmid pAB4-ARp1 was compared with that of plasmid pAB4.1. After transformation of various *A. niger pyr*G/A (the *pyr*G1 mutation present in the recipient strain AB4.1 and its derivatives, (van Hartingsveldt et al., 1987) is allelic to *pyrA5* in the strains of C.J. Bos, Wageningen) strains with pAB4-ARp1, 10^3 - 10^4 transformants/μg DNA were obtained. This was a 10 - 100 fold increase compared with the number of pAB4.1 transformants obtained in the same experiments. This increase in transformation frequency corresponds to the results obtained by Gems et al. (1991) with vector ARp1 (ArgB selection) when introduced into *A. niger* and indicates that the increase in transformation frequency, caused by the AMA1 sequence, is independent of the selection marker used.

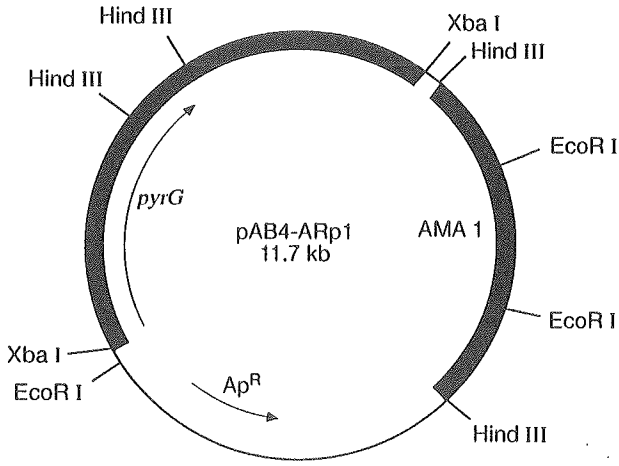

Figure 1. The autonomously replicating plasmid pAB4-ARp1. The AMA1 sequence was isolated as a 5.2-kb *Hin*dIII fragment from pHELP (Gems et al., 1993a) and cloned in the partially *Hin*dIII-digested vector pAB4.1 (van Hartingsveldt et al., 1987). *E. coli* JM109 was used for propagation of plasmid DNA and trans-formation as described by Hanahan et al. (1983). All standard recombinant DNA techniques were essentially carried out as described by Sambrook et al., (1989). Solid boxes represent *Aspergillus* DNA and the thin line represents bacterial DNA.

(2) Stability of pAB4-ARp1 transformants

A. niger [pAB4-ARp1] transformants showed irregular growth on selective agar plates containing amounts of uridine which are unable to support sporulation of Pyr$^-$ segregants. These results suggest instability of the selective marker (Fig. 2). To analyze the stability of the Pyr$^+$ phenotype spores, isolated from transformants purified under selective conditions, were plated on minimal medium agar plates with and without uridine. About 80-90 % of the isolated spores appeared to be Pyr$^-$. From this result we conclude that the selective marker is not stably maintained in pAB4-ARp1 transformants. This instability may result from the loss or modification of pAB4-ARp1. The increased transformation frequency observed with pAB4-ARp1 and the instability of pAB4-ARp1 transformants closely resemble results obtained with the autonomously replicating vector, ARp1 in *A. nidulans* transformants (Gems et al., 1991) suggesting that also pAB4-ARp1 is autonomously replicating.

Figure 2. Stability of the *pyr*G marker in N814 transformants. A N814[pAB4-1] (TOP) and two N814[pAB4-ARp1] transformants (BOTTOM) are shown grown in minimal medium supplemented with nicotinamide and limiting amounts of uridine (2 mM). *A. niger* transformation was carried out essentially as described by Punt and van den Hondel (1993).

(3) Cotransformation with pAB4-ARp1

Cotransformation experiments were carried out with pAB4-ARp1 to analyze the characteristics of pAB4-ARp1 cotransformants. Protoplasts from *A. niger* strain N814 (*csp*A1, *fwn*A1, *nic*B5, *pyr*A5) were transformed with pAB4-ARp1 together with different amounts of pAN7-1, containing the hygromycin B (HmB) resistance

marker (Punt et al., 1987). Transformants were selected on uridine free medium without HmB. Subsequently, these Pyr^+ transformants were tested for HmB resistance. The frequency of cotransformation varied from 11 %, when a similar amount of both vectors was used (0.5 μg each) to 35 % when 10-fold more pAN7-1 (0.5/5 μg) was used. This result indicates that pAB4-ARp1 also greatly increases the transformation frequency of a cotransformed vector, as the transformation frequency of pAN7-1 itself is relatively low (< 50 per μg; Punt and van den Hondel, 1993). This increase in transformation frequency was illustrated by selection of cotransformants directly on uridine free medium with HmB (double selection). HmB^R/Pyr^+ cotransformants were obtained even with very low amounts (1 - 10ng) of pAN7-1, yielding 6-8 transformants per ng pAN7-1.

(4) Stability of cotransformants

Cotransformants obtained under either Pyr or Pyr/HmB selection conditions showed an irregular growth phenotype. The stability of the transformed phenotype (Pyr^+/HmB^R) was analyzed by isolating spores from 3 different N814[pAB4-ARp1/pAN7-1] transformants cultivated on uridine-free medium. Isolated spores were diluted and plated on different agar media (Table 1). The Pyr^+ phenotype was retained in 6 % (transf. #1) to 17 % (transf. #2 and #3) of the conidiospores. Conidiospores isolated from the same transformants cultivated on medium selective for both markers (uridine-free media supplemented with HmB) resulted in a 2-fold higher stability of the Pyr^+ phenotype, in respect to Pyr selection alone (results not shown). An explanation for the increased stability observed for transformants grown under double selection may be that the plasmid is retained at a higher copy number when transformants have been cultivated on this plates.

From the data in Table 1 it is clear that the phenotype of the cotransformant is also unstable for the HmB marker. The HmB marker was retained in 8 % (transf. #1) to 16 % (transform. #3) of the conidiospores. This result indicates that the HmB marker behaves similarly as the *pyr*G marker located on the autonomously

Table 1. Stability of the N814[pAB4-ARp1/pAN7.1] transformants

Growth conditions [B]	Phenotype(s)	Transformant [A]: #1		#2		#3	
		number of growing colonies					
MMA + uridine	Pyr^+ and Pyr^-	462	(100)	281	(100)	226	(100)
MMA	Pyr^+	29	(6)	47	(17)	38	(17)
MMA + hygromycin + uridine	Pyr^+/HmB^R and Pyr^-/HmB^R	37	(8)	45	(16)	25	(11)
MMA + hygromycin	Pyr^+/HmB^R	26	(6)	48	(17)	34	(15)
MMA + uridine + 5-fluoro-orotic acid	Pyr^-	307	(66)	213	(76)	304	(135)
MMA + uridine + 5-fluoro-orotic acid + hygromycin	Pyr^-/HmB^R	0		0		0	

[A] Spores were isolated from a Pyr^+/ HmB^R transformants purified on uridine-free medium.

[B] Agar media containing minimal culture medium (MMA; Bennett and Lasure, 1991). [pAB4-ARp1/pAN7-1] cotransformants were tested for mitotic stability of the *pyr*G marker by plating conidiospores of purified transformants on plates with and with out uridine (10 mM). The stability of the HmB^R marker was tested on plates with HmB (200 μg/ml) with and without uridine. Plates containing 5-FOA (1 mg/ml) with and without HmB were used to determine the frequency of cosegregation of both markers. In brackets the percentage of growing colonies is indicated related to the total number of conidiospores used (Pyr^+ and Pyr^-).

replicating vector. In addition, our results show that Pyr selection, as was used for purification of the transformants, was sufficient to preserve the phenotype of the cotransformed plasmid as well. Furthermore, as shown in Table 1, plating spores on media selective for both markers (plates without uridine and with HmB) resulted in similar numbers of colonies, as with spores plated on media selective for only one marker (plates without uridine or plates with uridine and HmB). These results indicate a cosegregation of both markers. A further confirmation of this cosegregation comes from the results obtained with plates containing 5-fluoro-orotic acid (5-FOA) with and without hygromycin (Table 1). Using these plates a direct determination of the number of Pyr^-/HmB^R conidiospores can be made,

which may result from loss of the *pyr*G marker alone. As shown, no Pyr $^-$/HmB^R conidiospores were detected for any of the analyzed transformants. These results may indicate a physical linkage by recombination of cotransformed vectors during or after transformation process. Recombination of plasmids in fungal transformation is probably not restricted to AMA1 vectors, as the formation of dimers and multimers by recombination is one of the explanations of the integration of multiple copies at a single locus (Timberlake, 1991).

To analyze whether in our case such a recombination had occurred in genomic DNA was extracted from a N814[pAN7-1/pAB4-ARp1] transformant. Southern blot analysis of CHEF separated genomic DNA of this transformant indicated the presence of a plasmid of about 25-kb in *A. niger*. Subsequently, total genomic DNA was used to transform *E. coli* by electroporation. As expected for autonomously replicating plasmids (Gems et al., 1993), a number of ampicillin resistant *E. coli* colonies was obtained. In all cases a plasmid of about 25-kb could be isolated from these cells. Restriction enzyme analysis revealed that this plasmid consisted of DNA sequences originating from both pAB4-ARp1 and pAN7-1 and that recombination had occurred in the pUC sequences of both vectors (results not shown). These results confirm autonomous replication of the AMA1 derived vectors in *A. niger* and indicate that recombination of cotransformed vectors had occurred.

(5) Copy number of pAB4-ARp1 in A. niger

As described by Gems et al. (1991) AMA1 vectors are present in multiple copies per transformed nucleus in *A. nidulans*. We have tested whether free replicating plasmids were also present in multiple copies in *A. niger* using expression analysis of a reporter gene. For this purpose, *A. niger* strain AB4.1 (*pyr*G1; van Hartingsveldt et al., 1987) was cotransformed with pAB4-ARp1 and the vector pGUS64ΔB, containing the *uidA* gene of *E. coli* under control of the expression signals of the glucoamylase gene of *A. niger* (Verdoes et al., 1993). As expected, mitotically unstable Pyr$^+$/*uidA*$^+$ transformants were obtained. The *uidA* expression

level in one of the transformants obtained was compared with that in strain AB4.1[pGUS64ΔB] which contains one copy of pGUS64ΔB at the *pyr*G locus. Both transformants were cultivated in liquid medium containing maltodextrin as inducing carbon source and the *uidA* expression was determined as described by Verdoes et al. (1993b). The *uidA* expression level in the AB4.1[pAB4-ARp1/pGUS64ΔB] transformants was approximately 4 times higher than in the strains AB4.1[pGUS64ΔB] (2700 compared to 670 Units GUS/mg protein). This result suggests an average of 4 copies of the *uidA* gene per nucleus. Taking into account the instability in the pAB4-ARp1 transformants (80 - 90 % marker loss in conidiospores under selective conditions), an average copy number of 20 - 40 per transformed nucleus is indicated, corroborating the results obtained for ARp1 in *A. nidulans* (Gems et al., 1991).

(b) Complementation of *A. niger* mutants

To demonstrate the potential usefulness of pAB4-ARp1 for gene cloning, experiments were carried out to isolate the *nic*B and *lys*F genes of *A. niger*. These genes were chosen because these are located on linkage group (LG) VII. Debets et al. (1990a) showed by genetic analysis that *A. niger* has eight LG's of which all, except LG VII, were assigned to specific chromosomal bands in the electrophoretic karyotype of *A. niger* (Debets et al., 1990b). Isolation of one (or both) of these genes would allow us to assign LG VII to a band in the electrophoretic karyotype of *A. niger*.

Gems et al. (1993) successfully used AMA1 containing vectors in cotransformation experiments for the cloning of genes by complementation in *A. nidulans*. For the cloning of the *nic*B gene a similar approach was used. An *A. niger nic*B mutant strain (N814; *fwn*A1, *nic*B5, *pyr*A5) was cotransformed with pAB4-ARp1 together with an *A. niger* cosmid library in pKBY2 (van Gorcom et al., 1990). One *nic*B complementing transformant was obtained using a total of 22.5 μg (in aliquots of 2.5 and 5.0 μg) of cosmid library DNA. As expected, this

transformant showed an irregular growth phenotype characteristic for transformants carrying pAB4-ARp1. To demonstrate that the Nic$^+$ phenotype is caused by introduced sequences and not by reversion of the *nic*B5 mutation, cosegregation (section a3) of cotransformed vector sequences was analyzed. Pyr $^-$ segregants were selected using plates containing 5-FOA and the Nic$^+$ phenotype of the segregants was tested. In contrast to what is expected for a *nic*B reversion none of the resulting PyrG $^-$ segregants was Nic$^+$. Therefore, it was concluded that *nic*B complemention was caused by cotransformed sequences which recombined to pAB4-ARp1. Southern blot analysis of CHEF separated genomic DNA of the N814[Nic$^+$/pAB4-ARp1] transformant indicated the presence of a plasmid of about 50-kb. To rescue *nic*B complementing sequences, which were thought to be present on this 50-kb plasmid, total genomic DNA was isolated from N814[Nic+/pAB4-ARp1] and used for transformation of *E.coli* DH5α. A number of ampicillin resistant colonies was obtained. Plasmid DNA varying from 15 - 50 kb was isolated from these *E. coli* colonies. The variation in size of the isolated plasmids indicates that deletion of sequences from the 50-kb plasmid present in the Nic$^+$ strain had occurred after the introduction into *E. coli*. To analyze whether the isolated plasmids contained the *nic*B complementing sequences, *A. niger* N814 was transformed with six of these plasmids. With three of the isolated plasmids (pNIC-III, IV and V) *A. niger* Nic$^+$ transformants were obtained, indicating that a *nic*B complementing sequence was cloned on these plasmids. In all three cases the complementing plasmid had a similar size as the plasmid present in N814[Nic$^+$/pAB4-ARp1].

For the isolation of the *lys*F gene of *A. niger* the "instant gene bank" strategy Gems et al. (1993) was used in combination with pAB4-ARp1. This strategy, based on cotranformation of fragmented chromosomal fungal DNA with an AMA1 derived vector, was used for the cloning of genes by complementation in *A. nidulans*. *A. niger* N889 (*csp*A1, *fwn*A1, *bio*A1, *phe*A1, *pyr*A5, *lys*F29) was transformed (Punt and van den Hondel., 1993) using 0.5 μg pAB4-ARp1 together with a 10 to 500 times excess of chromosomal DNA of *A. niger*. One Pyr$^+$/Lys$^+$ transformant was

obtained. From the cosegregation of the Pyr^+ and Lys^+ phenotype it was concluded that complemention was cause by cotransformed sequences. Although the presence of unintegrated plasmid (> 75 kb) was demonstrated by CHEF/Southern analysis (results not shown) plasmid rescue into *E. coli* by electroporation was unsuccessful in a number of different attempts.

(c) Analysis of the *nic*B complementing sequences

Although often not considered, cloning by direct complementation may also lead to the isolation of suppressor genes as was shown in several cases in *S. cerevisiae* (e.g. Nakano and Muramatsu 1989). Therefore, it was necessary to prove that the cloned *nic*B complementing sequence indeed contains the *nic*B gene and not a fortuitous *nic*B suppressor.

To localize the *nic*B complementing gene, several *Eco*RI and *Hin*dIII fragments from pNIC-V, ranging in size from 2 - 10 kb, were subcloned in pUC19. A number of the resulting subclones were used in cotransformation experiments of *A. niger* N814 together with pAB4-ARp1. Efficient *nic*B complementation was expected to occur only in the presence of a complete copy of the *nic*B complementing gene. The presence of a part of the gene would only result in complementation after homologous integration of the vector, which occurs at highly reduced frequencies (van Hartingsveldt et al., 1987). Of all the plasmids tested, only pNIC1 (Fig. 3), containing a 2.8-kb *Hin*dIII fragment, efficiently complemented the *nic*B5 mutation, indicating that a complete copy of the *nic*B5 complementing gene had been cloned in this plasmid. Further subcloning of pNIC1 using *Sma*I yielded the vectors pNIC1.1 to pNIC1.4 (Fig. 3A). Introduction of each of these subclones together with pAB4-ARp1 in *A. niger* N814 did not result in complementation of the *A. niger* *nic*B5 mutant. This result indicated that a part of the complementing gene is located within the internal 1.0-kb *Sma*I fragment.

Based on this result a gene disruption vector was constructed to generate an *A. niger* strain, in which the *nic*B complementing gene was disrupted. Therefore,

the internal *Sma*I fragment from pNIC1 was replaced by a 3.4-kb DNA fragment containing the *pyr*G gene of *A. oryzae* (Fig. 3B). The resulting vector, pNIC::pyrG was linearized with *Hin*dIII and the isolated 5.2-kb *Hin*dIII fragment was used for

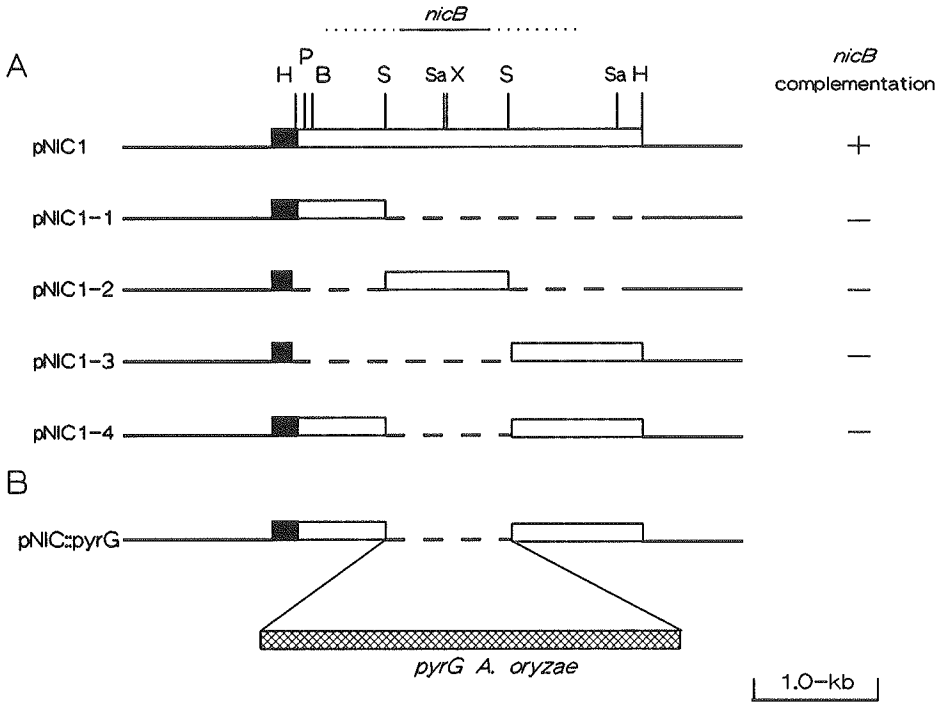

Figure 3. A. linearized map of the *nic*B complementing sequence in pNIC1. *Nic*B complementing plasmids from N814[Nic$^+$/pAB4-ARp1] were isolated by transforming *E.coli* DH5α by electroporation using the Bio-rad Gene Pulser^{TM} as described by the suppliers with total genomic DNA from this strain, isolated as described by Kolar et al., (1988). DNA from one of these plasmids (pNIC-V) was purified by CsCl-ethidium bromide gradient centrifugation. *Hin*dIII and *Eco*RI fragments ranging from 2 to 10 kb were isolated from this plasmid and cloned in pUC19 (Yanisch-Perron et al., 1985). A subclone designated pNIC1 complements the *nic*B mutation in *A. niger* N814. Subclones from pNIC1 (pNIC1-1 to 1-4) were tested for *nic*B complementation by transformation of strain N814; + = *nic*B complementation, - = no *nic*B complementation.
B. Vector pNIC::pyrG used for gene replacement. A 3.4-kb fragment, containing the *pyr*G gene of *A. oryzae*, was isolated as a *Hin*dIII fragment from pAO*pyr*G-Not (unpublished results) and treated with Klenow polymerase to generate blunt-ends. Subsequently, this fragment was cloned in the *Sma*I site of pNIC1-4. Pyr$^+$ transformants of N889 were isolated on osmotically stabilized agar plates with minimal medium supplemented with nicotinamide and other required markers. Restriction enzymes indicated; H, *Hin*dIII; P, *Pst*I; B, *Bam*HI; S, *Sma*I; Sa, *Sal*I; X, *Xmn*I. Black box indicates polylinker of pUC19.

transformation of *A. niger* N889 (*csp*A1, *fwn*A1, *bio*A1, *phe*A1, *pyr*A5, *lys*F29). One out of twenty-five Pyr^+ transformants (N889[*nic::pyr*G]#24) analyzed was nicotinamide deficient. Southern blot analysis of this transformant indicated that a part of the sequence in the chromosome which corresponds to the *nic*B complementing sequence was replaced by the disrupted allele from pNIC::pyrG, illustrated by the loss of the 1.0 kb *Sma*I fragment and the replacement of the 2.8-kb *Hin*dIII fragment by a 5.2-kb fragment in this strain (results not shown). From this result we conclude that disruption of a gene localized on the cloned *Hin*dIII fragment from pNIC1 results in nicotinamide deficiency.

Genetic analysis was carried out to formally prove that this gene is the *nic*B gene. For this analysis heterokaryon complementation, as described by Bos et al. (1988), of strain combinations N889[*nic::pyr*G]#24 x N814 (*fwn*A1, *nic*B5, *pyr*A5) and N889[*nic::pyr*G]#24 x N857 (*nic*B5, *trp*B2) was carried out. Heterokaryons formed in both combinations were unable to grow on media without nicotinamide, indicating that the mutation leading to deficiency for nicotinamide in strain N889[*nic::pyr*G]#24 is allelic to the *nic*B5 mutation. Therefore, we conclude that we successfully cloned the *nic*B gene of *A. niger.*

The cloning of this gene enabled us to localize the chromosome corresponding to LG VII in the electrophoretic karyotype of *A. niger.* Based on Southern analyses of CHEF separated chromosomes we have shown that LG VII corresponds to a chromosome of about 4.1 Mb leading to a total genome size of *A. niger* of about 37 Mb (Verdoes et al., in press).

(d) CONCLUSIONS

In this report the use of an autonomously replicating vector, pAB4-ARp1, is described for a gene isolation method by direct complementation of mutations in *A. niger.*

(1) Vector pAB4-ARp1, carrying the *A. niger pyr*G gene as selection marker, shows a significantly higher transformation frequency (10 - 100 fold) than the corresponding integrative vector, pAB4.1. Also the transformation frequency of cotransformed DNA is (greatly) enhanced by the use of pAB4-ARp1, as shown by

cotransformation with a vector conferring HmB^R.

(2) Transformants obtained after transformation with pAB4-ARp1 are mitotically unstable. However, 10 - 20 % of the conidiospores obtained from [pAB4-ARp1] transformants cultivated under selective conditions retained the *pyr*G marker. Cotransformants, obtained after transformation with pAB4-ARp1 together with a vector carrying another marker, were mitotically unstable for both markers. However, a strict cosegregation of the *Pyr*G marker and the other marker was observed. Cosegregation was due to recombination between pAB4-ARp1 and the cotransforming DNA.

(3) A pAB4-ARp1 derived plasmid is maintained at an average of 4 gene copies per nucleus. Reisolation of pAB4-ARp1 derived plasmid molecules is possible via transformation of *E.coli* with total genomic DNA from (instable) *A. niger* transformants.

(4) Based on the above mentioned characteristics of pAB4-ARp1, this vector was used for gene isolation by direct complementation. Complementation of two *A. niger* mutants was achieved using pAB4-ARp1 either by cotransformation with a cosmid library (*nic*B) or with total genomic DNA (*lys*F). Genetic analysis was carried out to prove that the LG VII specific *nic*B gene of *A. niger* was cloned.

(5) The multi-copy maintenance of pAB4-ARp1 (and other AMA1 derived vectors) may also facilitate the cloning of poorly expressed complementing genes from heterologous (fungal) species or the cloning of multi-copy suppressor genes, of which in *S. cerevisiae* many examples have been described (e.g. Nakano and Muramatsu, 1989).

(6) The recombination of cotransforming DNA with pAB4-ARp1 and the retainment of this plasmid may provide a possibility to use pAB4-ARp1 and other AMA derived vectors with a selection marker for the cloning of non-selectable genes. As *pyr*G selection is sufficient to maintain cotransformed sequences, also efficient isolation of genes which can not be selected by direct complementation, may be feasible. Furthermore pAB4-ARp1 may also be used for gene cloning strategies in other fungal hosts, as *pyr*G mutants may be easily isolated using 5-FOA (van den Hondel and Punt, 1991 and references therein) and AMA derived vectors have shown to be functional in fungal species other than *Aspergillus* (Brüeckner et al., 1992).

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. Clutterbuck for kindly providing the vector pHELP and Dr. C.J. Bos for providing the mutant strains used. Prof. P. H. Pouwels is thanked for critical reading of the manuscript. This project is supported by the Dutch Programme Commission for Industrial Biotechnology.

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CHAPTER 7

GENERAL DISCUSSION

I INTRODUCTION

In this thesis research is described that was aimed at an evaluation of the use of filamentous fungi, modified by genetic engineering, for the overproduction of proteins with the potential to be commercially applied. In chapter 1 an overview is given of the results published on this type of research. Basically, the strategy to overproduce a protein consists of introduction of additional copies of the gene encoding the protein of interest into a fungal host. From the results obtained it is clear that, although considerable overexpression can be achieved, there is no clear correlation between the number of gene copies introduced and the amount of protein produced. In most cases the amount of protein produced is lower than what would be expected from the number of gene copies introduced. As in all cases further detailed analysis is lacking, the molecular basis of this observation remained unresolved. To study, in more detail, the possibilities of protein overproduction by genetic engineering and to identify limiting step(s) in this process, a systematic study, starting with the construction and detailed analysis of GLA overproducing *A. niger* strains, was initiated. In chapters 2, 3 and 5 of this thesis the results of this study are described. In this last chapter the implications of these results for the construction of "second generation" overproducing strains are discussed.

Comparison of the number of *glaA* gene copies and the amount of GLA that is produced showed that also in our study no clear correlation between these two parameters was found (chapter 2). From the results of molecular genetic studies it was concluded that the GLA production in multi-copy strains is limited at the level of transcription. As shown in chapters 2 and 3, the expression of the introduced gene copies can be (negatively) influenced by the site of integration. Furthermore, as analysed in detail in chapter 5, the available amount of *trans*-acting regulatory factor(s) poses an important limitation to protein production in multi-copy strains. Obviously, the construction of "second generation" overproducing strains should be aimed at solving the identified limitations.

II STRATEGIES FOR THE CONSTRUCTION OF SECOND GENERATION OVERPRODUCING STRAINS

A. Site of Integration

The results of the analysis of our GLA overproducing strains showed that the expression of the introduced *glaA* gene copies can be negatively affected by the site of integration of the gene copies (chapters 2 and 3). Based on data presented in chapter 1, it is suggested that this effect is not restricted to GLA overproduction, but is a general phenomenon observed in recombinant fungal strains. Two approaches may be followed towards overcoming the (negative) effect on expression of the site of integration: (1) accomplishing position-independent expression or (2) expression of the genes of interest at the locus of a highly expressed gene.

Position-independent expression

Effects of the site of integration on gene expression are not limited to filamentous fungi but are also suggested to take place in other eukaryotic transgenic systems like mice, tobacco and *Drosophila* (e.g. Al-shawi et al., 1990; Allen et al., 1993; Chung et al., 1993). Analysis of the upstream regions of genes in these eukaryotes led to the identification of DNA sequences involved in the organization of the chromatin structure. These DNA sequences are located at the boundaries of DNAse sensitive regions and interact with the nuclear "scaffold". This interaction results in the arrangement of DNA in topological loops (Cook and Brazell, 1976; Gasser and Laemmli, 1987). Within these loops it is suggested that the chromatin structure is changed, making the DNA sequences in the loops more accessible for transcription activator proteins, resulting in transcriptionally active genes (Workman and Buchman, 1993; Adams and Workman, 1993). Introduction of these elements in transgenic expression cassettes resulted in more or less site-independent and / or high level gene expression in transgenic organisms (Stief et al., 1989; Bonifer et al., 1990; Greer et al., 1990; Whitelaw et al., 1992; Allen et al.,

1993).

Introduction of similar elements of fungal origin in fungal expression vectors may lead to improvement of the protein production in recombinant filamentous fungi obtained with this type of vector. However, at this moment very little is known about chromatin structure and its organization in filamentous fungi and scaffold attachment sequences have not yet been identified in these organisms.

Expression at a locus of a highly expressed gene

An second approach to overcome the negative effect of the site of integration in filamentous fungi may be site-specific integration of gene copies encoding the protein of interest at the locus of a highly expressed gene. In fungi efficient methods for site-specific integration have been described (e.g. Timberlake 1991; Finkelstein, 1991). This approach has already been applied for the production of heterologous proteins (e.g. Van Hartingsveldt et al., 1990). Although site specific integration has primarily been used to target a single gene copy to a specific locus, also tandem integration of a number of gene copies has been found (e.g. Van den Hondel and Punt, 1991).

The number of gene copies may be further increased by using site specific integrative vectors containing multiple copies of the gene of interest, similarly as the cosmid-vectors described in chapter 2. An even further increase in the number of gene copies per vector molecule, compared to the cosmid strategy (chapter 2), may be obtained by the use of cloning vectors based on bacteriophage P1 (insert site 75 - 95 kbp; Sternberg, 1992) or on the F factor episome (insert site in excess of 100 kbp; Leonardo and Sedivy, 1990).

B. Regulatory Factor Limitation

As shown in chapters 2, 3 and 5, in the case of introduction of a large number of gene copies, the expression of the introduced *glaA* genes is limited by the available amount of regulatory factor(s). The negative effect on gene expression of a limiting amount of trans-acting regulatory factor(s) is not restricted to *glaA*

expression. Also for the (over)expression of several other fungal genes this type of limitation has been observed. Overexpression of the *qut*E *A.nidulans* gene could only be achieved by the introduction of multiple copies of the *qut*E gen and the activator gene (*qut*A) (Beri et al., 1990). From data obtained by Burger et al. (1993) it is clear that expression of the *A. nidulans niaD* gene (even in a single copy situation) is limited by the amount of the regulatory protein NirA, as introduction of additional *nirA* gene copies results in increased *niaD* expression. Another example of a limiting amount of a regulatory factor was observed in strains carrying a large number (> 70) of copies of the acetamidase encoding gene (*amdS*) in *A. nidulans*. In these strains expression of genes involved in lactam and omega amino acid catabolism was reduced, as a consequence of limiting amount of the regulatory protein, *AmdR* (Kelly and Hynes, 1987; Andrianopoulos and Hynes, 1988). A similar situation was observed for the genes of involved in the alcohol metabolism of *A. nidulans*. Introduction of multiple copies of a heterologous gene under control of the alcohol dehydrogenase I (*alcA)* promoter resulted in a decrease in expression of the endogenous *alcA* and *aldA* genes (Gwynne, 1987). Introduction of multiple copies of the gene encoding the specific regulatory protein (*alcR*) in these multi-copy transformants resulted in restoration of the *alcA* and *aldA* expression and increased expression of the heterologous gene (Davies, 1991; Gwynne, 1987).

From these examples it is clear that gene expression could be improved by increasing the amount of specific regulatory protein. We assume that the introduction of additional copies of the gene(s) encoding the limiting regulatory factor(s) involved in *glaA* expression can be used to improve the level of GLA production in the *glaA* multi-copy strains. As genes involved in regulation of the expression of the *glaA* gene have not been cloned or even identified, a prerequisite for the suggested approach to work will be the isolation of this gene or these genes.

III OUTLOOK

There are several reasons to expect that the results of the study on GLA overproduction and the proposed approaches to overcome the identified limitations

may have a wider impact than the improvement of GLA overproducing strains only. Firstly, the expression signals of *glaA* are frequently used for the expression of fungal and non-fungal genes (see chapter 1; and van den Hondel et al., 1991). Therefore, the isolation of the gene encoding the regulatory factor is not only important for the improvement of GLA production but also for the (over)production of other fungal and non-fungal proteins. Secondly, the transcription limitation as observed in GLA overproducing strains, as a consequence of the negative effect of the site of integration on expression and the limiting amount of trans-acting regulatory factor(s), may also occur for overexpression of other fungal genes, in particular for those where distinct regulation of gene expression is evident (chapter 1). Therefore, a continuation of the study of GLA overproduction may provide additional information which will be of importance for the improvement of protein (over)production in filamentous fungi by the genetic engineering approach in general.

Although the data on *glaA* expression experiments described in this thesis are carried out in shake flask cultures, the application of genetically engineered strains in production processes will involve controlled (large scale) fermentation conditions. It is obvious that the physiological conditions in shake flask and controlled fermentation are quite different. Thus, it is not unlikely that under the latter conditions the production performance of multi-copy strains will be quite different. For this reason it seems obvious that a detailed study of protein production under controlled fermentation experiments will be needed to analyze whether results found for shake flask conditions can be extrapolated to controlled fermentation conditions. To initiate a study on this subject one of the multi-copy strains (N402[pAB6-10]B1; chapter 2) was used to analyze growth and product formation in controlled fermentation conditions (Schrickx et al., 1993). The initial results show that, compared to the wild-type strain (N402), only a 2-fold increase in GLA activity in the multi-copy strain was found in a glucose-limited continuous culture. In contrast with these data no increase in the level of expression was found in the multi-copy compared to the wild-type strain, in a maltodextrin-limitation continuous culture. For the multi-copy strain cultivated under maltodextrin-limitation in a recycling culture, in which the total weight of the culture vessel is kept constant, a

5-fold increase in GLA activity was found compared to the wild-type strain (Schrickx et al., 1993). This increase is similar to that found for these strains cultivated in the presence of maltodextrin in shake flasks (Schrickx et al., 1993). The observation that the production level of the multi-copy strain cultivated in continuous culture shows a lower relative increase in GLA production compared to the wild-type strain under shake flask and recycling conditions is similar to the data presented by Finkelstein (1989) and van Gorcom et al. (1990). Together the data of Schrickx et al. (1993) suggest clear differences in gene expression under different fermentation conditions, which may be caused by altered gene regulation under these conditions. One of the explanations can be that the available amount of regulatory factor(s) is different under different fermentation conditions.

Another aspect important for successful industrial application of recombinant strains is the genetic stability of the introduced gene copies. In general, the recombinant strains obtained carry multiple, tandemly integrated copies of the gene of interest (e.g. Kelly and Hynes, 1985; Wernars et al., 1985). Although the GLA overproducing strains described in this thesis were genetically stable, when cultivated on solid media or in liquid media in shake flasks, genetic instability was found during genetic analysis of multi-copy strains and the construction of strains carrying multiple copies of the *glaA* gene at different chromosomes (chapter 3). As very little is known about genetic stability in genetically engineered strains cultivated under controlled fermentation conditions further study on this subject has to be carried out. In this respect the *glaA* multi-copy strains may be very useful by providing the opportunity to study genetic stability in relation to gene expression, as the expression of the *glaA* gene can be modified by cultivating cells in the presence of differenr inducing carbon sources.

Although considerable progress has been made concerning the understanding of GLA overproduction, it should be considered that overcoming limitations on the level of transcription, as identified in this thesis, will probably reveal new limitations at other levels e.g concerning cell-physiology and protein secretion. Protein secretion in filamentous fungi has shown to be very expensive in relation to energy consumption (Metwally et al., 1991; Van Verseveld et al., 1991). Therefore, extensive protein overproduction may affect cell physiology considerably, which in

turn may affect gene expression and genetic stability. It may thus be worthwhile to continue the analysis of protein overproduction and cell-physiology with the *glaA* multi-copy strains as started with the work of Schrickx et al. (1993). This will provide valuable information about gene expression, gene regulation, protein production and protein secretion in protein overproducing filamentous fungi under controlled fermentation conditions.

In conclusion, the results presented in this thesis indicate that the genetic engineering approach has been successful for the construction of protein overproducing fungal strains. The approaches to overcome the identified limitation at the level of transcription, as suggested in this chapter, will most likely lead to a further improvement of the level of protein production in these genetically engineered fungal strains.

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SAMENVATTING

Filamenteuze schimmels komen voor op de meest uiteenlopende plaatsen en onder soms extreme omstandigheden. Om daar te kunnen overleven beschikken deze organismen over een grote variëteit aan routes voor biochemische omzettingen. Dit stelt hen in staat een grote diversiteit van koolstof en stikstof verbindingen te gebruiken als substraat. Schimmels zijn verder in staat een groot aantal verschillende hydrolytische eiwitten, betrokken bij de afbraak van polymeren (b.v. koolhydraten en eiwitten),op efficiënte wijze in het medium uit te scheiden. Gezien de vele (biotechnologische) toepassingen van dergelijke enzymen worden filamenteuze schimmels veel gebruikt als produktie organisme van enzymen.

Slechts voor een klein aantal enzymen geldt dat het nivo van produktie "van nature" hoog genoeg is voor commerciële toepassingen. Daarom worden stammen onderworpen aan verschillende methoden van stamverbetering. Klassieke methoden voor stamverbetering bestaan uit mutagenisatie en genetisch recombinatie. Beide methoden, soms in kombinatie, hebben geleid tot verbetering van produktie eigenschappen van schimmels in verschillende produktie processen. Na jarenlange toepassing van de klassieke stamverbeterings methoden is door de ontwikkeling van de moleculaire biologie van schimmels een nieuwe, meer gerichte methode voor stamverbetering mogelijk geworden (genetische modifikatie). In hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van het gebruik en van de resultaten van deze nieuwe methode voor het verhogen van het produktie nivo van schimmeleiwitten in filamenteuze schimmels. Bij deze methode wordt er van uitgegaan dat het introduceren van extra kopieën van het gen coderend voor het eiwit van interesse in een stam zal leiden tot verhoging van het nivo van produktie van het betreffende eiwit. De gevolgde strategie om te komen tot een efficiënt producerende stam bestaat uit een aantal stappen:

1) Selektie van een schimmelstam (op grond van de produktie van een bepaalde enzymatische aktiviteit met b.v. specifieke eigenschappen van het enzym van interesse) waarvan het DNA als uitgangsmateriaal moet dienen voor de isolatie van het gen coderend voor het enzym van interesse;
2) klonering en karakterisering van dit gen;

3) konstruktie van recombinant stammen door de introduktie van extra kopieën van het gekloneerde gen. Hierbij kan de expressie van het gen indien wenselijk verhoogd worden door het vervangen van eigen expressiesignalen voor die van een gen met een hoog (constitutief of reguleerbaar) expressie nivo;
4) analyse van de (gen-expressie en) eiwitproduktie in de verkregen transformanten;
5) verdere vergroting van produktie in de verkregen stam(men) door verbetering van de fermentatie omstandigheden (opschalen, medium-samenstelling enz.) en/of klassieke stamverbeteringsmethoden.

De toepassing van deze methode van stamverbetering heeft in de afgelopen jaren geleid tot een groot aantal voorbeelden van stammen met een vergrote enzym produktie. In een aantal gevallen werd het nivo van produktie zo hoog dat het enzym inmiddels op commerciële basis wordt geproduceerd. Een groot aantal van de in hoofdstuk 1 genoemde studies is overwegend gericht op overproduktie van het relevante enzym.

Om meer inzicht te verkrijgen in mechanismen van enzym overproductie door middel van genetische modificatie van schimmels is een meer systematisch onderzoek gestart. Dit onderzoek zoals beschreven in dit proefschrift heeft als primaire doelstelling het evalueren van de mogelijkheden (en beperkingen) van genetische modificatie voor de konstruktie van eiwit overproducerende filamenteuze schimmels. Als model is gekozen voor de overproduktie van glucoamylase (GLA) in *Aspergillus niger*. Glucoamylase is een commercieel interessant enzym dat betrokken is bij de afsplitsing van glucose van zetmeel en andere di- en polysacchariden en zijn toepassing vindt in b.v. de voedingsindustrie. Daarnaast is veel biochemisch onderzoek uitgevoerd met betrekking tot de karakterisering van GLA, als ook moleculair genetisch onderzoek van de regulatie van expressie van het coderende gen (*glaA*) .

In Hoofdstuk 2 is beschreven hoe met behulp van moleculair genetische technieken stammen zijn gemaakt met een zeer groot aantal kopieën (tot 200) van het *glaA* gen. Deze kopieën zijn stabiel geïntegreerd in het genoom. In een aantal van deze stammen is een 15-20 voudige toename van de GLA produktie gemeten

t.o.v de wild-type stam. Uit nadere analyse van deze stammen bleek dat er, evenals in de in hoofdstuk 1 genoemde voorbeelden, geen duidelijke relatie bestaat tussen het aantal *glaA* kopieën en de geproduceerde hoeveelheid GLA. Uit nader onderzoek blijkt dat de expressie van de geïntroduceerde *glaA* kopieën gelimiteerd is op het nivo van transcriptie.

Op basis van genetische analyse is bepaald in welk chromosoom de geïntroduceerde kopieën geïntegreerd zijn. In kombinatie met de produktie gegevens, zoals beschreven in hoofdstuk 2, blijkt uit deze analyse, dat in een aantal van de multi-copy stammen de expressie van de geïntroduceerde genen beïnvloed wordt door de plaats van integratie. In een aantal van de verkregen stammen is getracht het aantal *glaA* kopieën verder te vergroten teneinde de GLA produktie te verbeteren. Hiertoe zijn door middel van genetische recombinatie of hertransformatie extra *glaA* kopieën geïntroduceerd (Hoofdstuk 3). In geen van de verkregen stammen leidde dit echter tot een duidelijke verhoging van het nivo van produktie ten opzichte van de gebruikte uitgangsstammen. In een aantal stammen leidde de introduktie van extra *glaA* gen kopieën zelfs tot een verlaging van het nivo van GLA produktie. De gegevens, verkregen door nadere analyse van een dergelijke stam, geven aan dat aan de verlaging van de expressie van de *glaA* genen een *in trans*-werkende regulatie factor(en) ten grondslag ligt.

In de geanalyseerde multi-copy transformanten blijkt de integratie te hebben plaats gevonden in één chromosoom (Hoofdstuk 2/3). In die gevallen waarbij een groot aantal kopieën geïntegreerd is, resulteert dit in een duidelijk veranderd electroforetisch gedrag van dit chromosoom. Van een aantal stammen met een dergelijk veranderd karyotype is gebruik gemaakt om een methode te ontwikkelen om langs moleculair genetische weg vast te stellen in welk chromosoom een willekeurig gekloneerd *A. niger* gen is gelokaliseerd (Hoofdstuk 4).

De betrokkenheid van transcriptie factoren bij regulatie van de expressie van het *glaA* gen, zoals gesuggereerd door de resultaten in hoofdstuk 3, is nader onderzocht door het uitvoeren van een functionele analyse van de *glaA* promotor (Hoofdstuk 5). Hiertoe is een promotor deletie-analyse uitgevoerd, waarbij ook de effekten van de introduktie van een groot aantal kopieën van de *glaA* promoter op de gen-expressie zijn geanalyseerd. De verkregen resultaten bevestigen dat de

transcriptie van het *glaA* gen gereguleerd wordt door een (*glaA*) specifiek regulatie eiwit. De resultaten van de deletie analyse leidden tot de identificatie van het promotor gebied dat betrokken is bij de interaktie met de regulatie faktor(en).

Op basis van de in hoofdstuk 5 verkregen resultaten is geconcludeerd dat naast de integratie plaats de beschikbare hoeveelheid (van een) van deze faktoren bepalend is voor het in hoofdstuk 2 en 3 gevonden GLA produktie nivo. Naar verwachting is het vergroten van de hoeveelheid transcriptie factor(en) door bijvoorbeeld het introduceren van extra kopieën van het (de) gen(en) coderend voor deze factor(en) de meest gerichte methode om te komen tot een verdere verbetering van de GLA produktie in multi-copy stammen. Met het oog op de isolatie van dit tot nu toe ongeindentificeerde gen is een voor *A. niger* nieuwe kloneringsmethode ontwikkeld, gebaseerd op het gebruik van een autonoom replicerende vector (Hoofdstuk 6).

In Hoofdstuk 7, tenslotte, worden de resultaten van het in dit proefschrift beschreven onderzoek, in relatie tot de konstruktie van een tweede generatie overproducerende stammen, besproken.

ABBREVIATIONS

A.	*Aspergillus*
amdS	acetamidase encoding gene
amy	α-amylase-encoding gene(s)
bp	base pair(s)
CHEF	contour-clamped homogeneous electric field
E.	*Escherichia*
5-FOA	5-fluoro-orotic acid
GLA	glucoamylase
glaA	GLA encoding gene
gpdA	glyceraldehyde-3-phosphate dehydrogenase encoding gene
GUS	β-glucuronidase
HmB	hygromycin B
IEF	iso-electric focusing
kb	kilobase(s) or 1000 bp
LG	linkage group
*lys*F	gene/mutation involved in lysine biosynthesis
*nic*B	gene/mutation involved in nicotinamide biosynthesis
nt	nucleotide(s)
p	promoter region
*pyr*G	oritidine-5'-phosphate decarboxylase encoding gene
^{R}	resistance
uidA	GUS-encoding gene
X-Gluc	5-bromo-4-chloro-3-indolyl-β-d-glucuronide
[ ]	denotes plasmid-carrier state.

CURRICULUM VITAE

Jan Cornelis Verdoes werd op 5 december 1965 geboren te Katwijk aan Zee. In mei 1984 behaalde hij het V.W.O. diploma aan het Pieter Groen College te Katwijk en begon in hetzelfde jaar aan de studie biologie aan de Rijks Universiteit Leiden. Het doctoraal examen (specialisatie moleculaire biologie; B4) werd op 29 augustus 1989 behaald.

Vanaf september 1989 tot september 1993 was hij, verbonden aan de vakgroep Microbiologie (Prof. Dr. A.H. Stouthamer), in dienst van de Vrije Universiteit van Amsterdam. Het onderzoek, onder leiding van Dr. C.A.M.J.J. van den Hondel en Dr. P.J. Punt, werd uitgevoerd binnen de afdeling Moleculaire Genetica en Gen Technologie (Prof. Dr. P.H. Pouwels) bij het Medisch Biologisch Laboratorium TNO te Rijswijk. De resultaten van dit onderzoek zijn weergegeven in dit proefschrift.

DANKWOORD

Tot slot wil ik graag alle mensen bedanken die op enigerlei wijze hebben bijgedragen aan de totstandkoming van dit proefschrift.

Ik ben er van overtuigd dat de unieke sfeer binnen de afdeling MGG en het (voormalig) MBL een positieve invloed heeft gehad op het onderzoek en het plezier dat ik aan dit "werk" beleefd heb. Zonder namen te noemen wil ik iedereen bedanken die voor het creëren van deze sfeer hebben gezorgd. Ik heb mij er enorm thuis gevoeld.

Daarnaast gaat mijn speciale dank uit naar een aantal personen die nauw betrokken zijn geweest bij de inhoudelijke totstandkoming van dit proefschrift.
Mijn promotor, Prof. Stouthamer, wil ik bedanken voor zijn getoonde interesse voor mijn onderzoek en zijn begeleiding.

Kees van den Hondel, mijn co-promotor, dank ik voor de enthousiaste manier waarop je mij in het onderzoek begeleid hebt. Je was altijd belangstellend naar de laatste resultaten en bereid tijd vrij te maken om resultaten en voortgang te bespreken. Daarnaast waardeer ik je inspanningen om te komen tot een invulling van mijn post-AIO tijdperk.

Beste Peter Punt, jouw invloed op dit proefschrift is veel omvattend. Je leerde mij in het begin de fijne kneepjes van de *Aspergillus* technieken en aan het eind was je nauw betrokken bij de totstandkoming van de publicaties. Je was voor mij een altijd aanwezige vraagbaak, een bron van literatuurreferenties en nieuwe ideeën. Peter, ik waardeer het dat je steeds weer tijd had om "een klein vraagje" te beantwoorden. Ik ben blij dat je tijdens mijn promotie een plaats hebt die je toekomt.

Pieter van den Berg, Anne van Diepeningen en Regine Calil wil ik bedanken voor hun experimentele bijdragen aan dit proefschrift. Bovendien wil ik Fons Debets en Klaas Swart, van de vakgroep Erfelijkheidsleer van de Landbouw Universiteit Wageningen, bedanken voor hun interesse in mijn onderzoek en hun inspanningen om studenten te interesseren voor, en te begeleiden tijdens, dit onderzoek.

Henk van Verseveld en Jaap Schrickx wil ik danken voor de plezierige samenwerking. Jaap succes met de afronding van jouw proefschrift.

Van al mijn MGG kollega's wil ik Jan Jore in het bijzonder bedanken. Beste Jan,

ik heb vier jaar met jou een kamer mogen delen, waarvan misschien het laatste jaar voor ons beiden gold als een tropenjaar. Tijdens deze tijd hebben we elkaar zodanig leren kennen, dat we met elkaar kunnen "lezen en schrijven". Ik hoop je nog veel te ontmoeten en ben blij dat jij mijn paranimf wilde zijn.

Beste Gerrit, ik was verheugd dat je ondanks al die spullen die ik bij je "geleend" heb en al die keren dat ik gezegd heb; " Gerrit, ik ben toch....." toch mijn paranimf wilde zijn.

Ik wil mijn ouders bedanken voor de vrijheid die ze mij geschonken hebben om mijn eigen keuzes te maken en de ongedwongen wijze waarmee ze mij tijdens mijn studie gesteund hebben.

Tenslotte, maar zeker niet in de laatste plaats wil ik op deze manier een onbetaalbare rekening vereffenen. Lieve Coby, slechts weinigen zullen begrijpen hoe belangrijk jouw aandeel is geweest. Ik wil je bedanken voor je getoonde steun, begrip en vertrouwen.