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**Construction And Assessment Of A Vector For
Gene Expression In The Fission Yeast
*Schizosaccharomyces pombe***

by

Eric Robert Stephen, B.Sc.

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science**

National Research Council of Canada

and

**Department of Biology
Carleton University
Ottawa, Ontario**

November 19, 1991

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"Construction And Assessment Of A Vector For
Gene Expression In The Fission Yeast
Schizosaccharomyces pombe"

submitted by Eric Robert Stephen, B.Sc.
in partial fulfilment of the requirements
for the degree of Master of Science.

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Abstract

An expression plasmid (pEX1) for use with the fission yeast has been constructed containing an engineered *Schizosaccharomyces pombe* secretion cassette. A prokaryotic endo- β -(1,4)-glucanase structural gene from *Bacillus subtilis* was chosen as a reporter to assess the utility of this vector. For comparative purposes, the *B. subtilis* structural gene with its native controlling elements contained on an *E. coli*/yeast shuttle vector was employed. The native *Bacillus subtilis* controlling elements were utilized by *Schizosaccharomyces pombe* to direct synthesis of the endoglucanase protein. However, the endoglucanase activity remained essentially within the confines of the cell wall. Results presented reveal that the secretion vector pEX1 was able to effect both the expression and export of the reporter gene product to the cell wall. The endoglucanase protein appears to be bound in that location, with little being found in the extracellular medium.

Acknowledgements

I would like to give special thanks to my supervisor Dr. V. N. Iyer for providing continued support throughout my work. As well, I would like to thank my co-supervisors Dr. H. Yamazaki, and especially Dr. V. Seligy who provided an environment rich in scientific excellence and who gave selflessly of his time and knowledge throughout my studies. I would in addition, like to thank Dr. A. Nasim who introduced me to the world of "the fission yeast".

I would like to thank the following National Research Council of Canada staff for contributing towards this project: Dr. M. Ujiie for help with protein purification; Dr. V. Seligy for the *E. coli*/yeast shuttle vector pBTI 1.6 ; Dr.'s V. Seligy and M. Hefford for the plasmid pMut31.1A containing the endoglucanase structural gene; T. Devesceri for excellent photographic assistance and Dr. J. Phipps and Ms. G. Dubuc for their advice on protein extraction and Western analysis.

I would like to thank Dr. H. Heslot for providing plasmid pBK02 containing the 54/1 *S. pombe* promoter.

I would like to thank my parents Frank and Kathleen Stephen, who encouraged me towards a higher ideal and without whom, I would not be.

Finally, I would like to thank from the bottom, length and breadth of my heart, my wife Theresa and our son Adam who endured my absence both at and away from home. Their constant love, understanding and encouragement sustained me through the darkest hours.

**Great is the truth and mighty above all things;
It endureth and is always strong;
It liveth and conquereth for ever more;
The more thou searchest, the more thou shalt marvel.**

- The Apocryphal Books of Esdras

Table of Contents

Title	i
Acceptance page	ii
Abstract	iii
Acknowledgements	iv
Table of Contents	vi
List of Figures	x
List of Tables	xi
List of Abbreviations	xii
I. Introduction	
A. Objectives of research	1
B. Yeasts as model eukaryotes	1
C. <i>Schizosaccharomyces pombe</i> molecular genetics	
a) Vector mediated transformation and recombination in <i>S.pombe</i>	3
b) Genetic markers for use in <i>S. pombe</i>	7
c) Heterologous expression in <i>S.pombe</i>	8
d) Selection of reporter gene	10
e) Selection of homologous protein export signal	12

II. Materials and Methods

A) Growth of bacteria and yeasts

- a) Bacteria (*Escherichia coli* K12) 15
- b) Yeast (*Schizosaccharomyces pombe*) 15
- c) Culture conditions for protein purification 16
- d) Plasmid stability in *Schizosaccharomyces pombe* 16

B) Isolation and characterization of DNA

a) Isolation of *Escherichia coli* plasmid DNA

- i) Large scale procedure 17
- ii) Mini-prep procedure 18
- iii) Rapid screening for recombinant plasmid 19

b) Isolation of *Schizosaccharomyces pombe* DNA

- i) Isolation of total cellular DNA 20
- ii) Rapid plasmid recovery procedure 21

c) Characterization of DNA by restriction enzyme

- analysis 21

d) Purification of DNA from agarose gels 22

C) DNA mediated transformations

- a) *Escherichia coli* transformation 23
- b) *Schizosaccharomyces pombe* transformation 24

D) Immobilization of DNA and hybridization probe procedures

- a) Immobilization of DNA from agarose gels 25

b) Immobilization of DNA from <i>E. coli</i> recombinants . . .	25
c) Immobilization of DNA from <i>S. pombe</i> recombinants .	26
d) DNA hybridizations	26
E) Protein purification and analysis	
a) Extraction of proteins	28
b) Polyacrylamide gel electrophoresis of proteins	28
c) Isoelectric focusing	29
d) Immunodetection of endoglucanase	30
e) Detection of endoglucanase activity	31
f) Concentration of proteins	32
g) Fractionation of cultures for endoglucanase activity	32
F) Preparation of ³² P-radiolabelled DNA probes	
(a) Nick translation procedure	33
(b) 5'-end labelling procedure	34
G) Preparation and separation of <i>S. pombe</i> chromosomes	35
H) Dideoxynucleotide synthesis and purification	36

III. Results

(A) Construction of the *S. pombe* expression/secretion vector

pEX21E

(a) Modification of plasmid pIRT2	38
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(b) Isolation and modification of the 54/1	
promoter	41
(c) Construction and isolation of the acid phosphatase	
secretion cassette components	47
(d) Isolation and cloning of the <i>B. subtilis</i> endoglucanase	
structural gene in plasmid PEX1	53
(B) Endoglucanase gene cloning, detection and stability	56
(C) Endoglucanase expression studies in <i>S. pombe</i>	57
IV. Discussion	74
V. Conclusions	84
VI. References	85

List of Figures

Construction of plasmid pIRT2Δ	39
Isolation of the 54/1 promoter	42
Synthesis and assembly of the <i>S. pombe</i> secretion cassette components	46
Dideoxy-sequence data from a segment of the synthetic signal sequence in plasmid pUCD1	49
Structure of expression plasmid pEX1	52
Structural features of the <i>Bacillus subtilis</i> endoglucanase gene	54
Southern hybridization analysis of <i>S. pombe</i> pBT11.6 transformants	57
Southern hybridization analysis of <i>S. pombe</i> pEX21E transformants	60
Determination of plasmid stability in <i>S. pombe</i> transformants	62
Congo Red plate assay	64
Cellular location of endoglucanase activity	66
Western analysis of total cellular protein from <i>S. pombe</i> transformants	69
Isoelectric point comparison of endoglucanase produced in pEX21E and pBT11.6 transformed <i>S. pombe</i> cells	71

List of Tables

Selective markers used in <i>Schizosaccharomyces pombe</i>	
transformation	09
Plasmids used in this study	14

List of Abbreviations

A	adenine
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
bp	base pair
BCIP	5-bromo-4-chloro-3-indoyl phosphate
C	cytosine
°C	degrees centigrade
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CMC	carboxymethyl cellulose
cpm	counts per minute
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	N,N-ethylenediaminetetraacetic acid
FCS	fetal calf serum
g	gram
G	guanine
HPLC	high pressure liquid chromatography
IEF	isoelectric focus
kbp	kilobase pair
kDa	kilodalton
M	Molar
Ma	milliampere
MCS	multiple cloning site
mg	milligram
ml	milliliter
mM	millimolar
mmol	millimole
N	Normal
NBT	nitro blue tetrazolium
³² P	radioactive isotope of phosphorus
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pH	-log ₁₀ [H ⁺]
PMSF	phenylmethylsulfonyl fluoride

RNAse	ribonuclease
RP	reverse phase
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	150 mM NaCl, 15 mM sodium citrate (pH 7.0)
T	thymine
TAE	40 mM Tris-acetate Ph 8.0, 2 mM EDTA
TBE	89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.0)
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
μg	micrograms
μl	microliter
UV	ultraviolet
V	volt

I. Introduction

A) Objectives of research

The purpose of this research was to develop a vector for the fission yeast *Schizosaccharomyces pombe* which could be used to examine the expression of heterologous proteins. The plasmid construct contains an engineered secretion cassette consisting of a native *S. pombe* promoter and a chemically synthesized acid phosphatase secretion signal sequence containing a multiple cloning site with unique restriction sites for cloning. The *Bacillus subtilis* endo- β -1,4-glucanase gene was chosen as a reporter gene to assess the utility of the vector. This gene has been well characterized and codes for an activity which is easily detected and quantifiable and which has no analog or homolog in yeast. In *B. subtilis* this gene product is synthesized with a secretion signal which directs the export of the endoglucanase to the cell's exterior. The ability of *S. pombe* to utilize a native *Bacillus* secretion signal to export the endoglucanase protein was also examined.

B) Yeasts as model eukaryotes

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been developed as successful model systems for the study of eukaryotic molecular biology. As microorganisms, they are easily manipulated and are amenable to both biochemical and genetic characterization. As eukaryotes, they share many of the fundamental cell

biology properties of higher eukaryotes. Although *S. pombe* and *S. cerevisiae* both belong to the same taxonomic group, the Ascomycetes, they exhibit a number of dissimilar features. These include: morphology, mode of cell division (binary fission in *S. pombe* vs budding in *S. cerevisiae*), chromosome number (3 in *S. pombe*, 17 in *S. cerevisiae*), and gene arrangement, including the number of introns (Yamamoto et al., 1981).

From a genetic point of view, *S. pombe* has diverged significantly from *S. cerevisiae*. Available evidence based on studies in *S. pombe* on cell division (Michison, 1970), purine metabolism (Heslot, 1972), inositol requirement (Megnet, 1964), ribosomal DNA repeat structure (Mao et al., 1982), intron splicing (Tollervy and Mattaj, 1987; Russell, 1989) and codon usage bias (Russell and Hall, 1982) suggests that *S. pombe* is more closely related to the filamentous fungi (Russell and Hall, 1983) and higher eukaryotes than *S. cerevisiae* (Nurse, 1985).

The most highly studied of the two organisms is *S. cerevisiae* (Strathern, 1981; 1982). However, *S. pombe* shares many of the experimental advantages offered by *S. cerevisiae* and the development of gene cloning methods in *S. pombe* has progressed considerably over the last five years. This has encouraged many investigators to begin studying new problems in the fission yeast. (Russell and Nurse, 1986; Russell, 1989). There are indications that for certain applications, such as the expression of some higher eukaryotic genes, *S. pombe* may prove to be more useful than *S. cerevisiae* (Yamamoto, 1989).

Development of expression vectors for use in *S. pombe* has lagged behind that of *S. cerevisiae*. To date, the only reported examples are based on the *S. pombe* alcohol dehydrogenase (ADH) gene (Russell and Nurse, 1986; McLeod et al., 1987), the 54/1 promoter (Kudla et al., 1988) and the viral SV40 early (Kaufman et al., 1985) promoter. The *adh* promoter is highly expressed in *S. pombe* and it is estimated that from 0.5 to 2% of the total soluble protein is alcohol dehydrogenase in glucose grown cells (Russell and Hall, 1983). In contrast, the SV40 early promoter is not as strongly expressed as the *adh* promoter. However, transcripts of this SV-40 promoter initiate at the same nucleotide position in *S. pombe* as they do in mammalian cells (Jones et al., 1988) and intron processing is only partly conserved. The strongest promoter is the *S. pombe* 54/1 which has been shown to drive up to 5% of *S. pombe* total protein synthesis as β -galactosidase when the 54/1 promoter is fused to the promoterless *lacZ* gene (Kudla et al., 1988).

C) *Schizosaccharomyces pombe* molecular genetics

a) Vector mediated transformation and recombination in *S. pombe*

The first reports on DNA mediated transformation of the budding yeast *Saccharomyces cerevisiae* (Hinnen et al., 1978; Beggs, 1978) were followed by the development of a transformation procedure for the fission yeast *Schizosaccharomyces pombe* (Beach and Nurse, 1981). Later improvements involved the use of Novozyme SP234 (Beach et al., 1982; Sipiczki et al., 1985). *S. pombe* cell walls unlike those

of *S. cerevisiae*, contain α -(1,3)-glucans and protoplasting is best achieved with Novozyme SP234 (Stephen and Nasim, 1981). Unlike *S. cerevisiae*, regeneration of *S. pombe* protoplasts does not require an agar matrix to support the resynthesis of the new cell wall. Spheroplast-mediated transformation methods for *S. pombe*, utilizing the cell wall degrading enzymes Glusulase (Endo Laboratories) and Zymolyase (Seikagaku Kogyo) have been published (Sakaguchi and Yamamoto, 1982; Chevallier and Lacroute, 1982; Russell and Hall, 1982) as well as a non-protoplasting method utilizing lithium chloride. These methods are identical to those used for *S. cerevisiae* (Ito et al., 1983; Heyer et al., 1986). The protoplasting methods provide the highest, most efficient levels of transformation, but the lithium chloride method is considerably faster and easier to use. Plasmids containing the *S. cerevisiae* 2 μ autonomously replicating sequence typically give transformation frequencies of 1-4 x 10⁴ transformants/ μ g (Russell, 1989). Transformation by the whole cell method is required when transforming certain mutant cells which tend to lyse if the protoplast method is used, as in the case of some *cdc* mutants (Russell and Nurse, 1986).

Molecular genetic procedures for integration, gene disruption and gene replacement are all based on homologous recombination (Russell, 1989). Recombination events between transforming DNA and the *S. pombe* genome are well documented (Grimm and Kohli, 1988; Wright et al., 1986). Homology-independent integration has been shown to occur at a relatively high frequency in *S. pombe* but has not been described in *S. cerevisiae* (Grimm and Kohli, 1988). Homology-dependent

recombination between plasmid vectors and chromosomes occurs with a 30 - 40X higher frequency than homology independent recombination.

In *S. cerevisiae*, there are essentially two mechanisms for maintenance of plasmid sequence. The first involves integration into a chromosomal sequence; the second involves autonomous replication via an autonomous replicating sequence (ARS) (Wright et al., 1986). Homologous integration of plasmids of the type seen in *S. cerevisiae* also occurs in *S. pombe* (Beach et al., 1982). However, the nature of non-ARS plasmid transformation in *S. pombe* differs from *S. cerevisiae* in two respects. Both homologous and non-homologous integration appear to be relatively frequent events and integrated plasmids are mitotically unstable. Homology-dependent events (gene conversion, integration) are more often seen when *S. pombe* is transformed with non-ARS plasmids. In addition, low frequency integration by illegitimate recombination has been observed in *S. pombe* (Grimm and Kohli, 1988; Wright et al., 1986). Homology-independent integration of the type seen in *S. pombe* is also seen in mammalian transformation systems and presumably results from a recombination system which is not present in *S. cerevisiae* (Wright et al., 1986).

It is widely assumed that eukaryotic DNA synthesis is initiated at specific sites in the chromosome called origins of replication. The best candidates for such a function in yeast are the chromosomally derived, ARS elements. In *S. pombe*, ARS elements are defined as sequences which promote high frequency transformation and yield mitotically stable transformants containing non-rearranged plasmid (Maundrell et al., 1985). ARS elements exist as specialized sequences in *S. pombe* chromosomal

DNA, occurring on average every 20 kbp in the genome (Wright et al., 1986). Autonomously replicating vectors in yeast depend on putative origins of replication and have been described in detail for *S. pombe* (Beach and Nurse, 1981; Losson and Lacroute, 1983; Maundrell et al., 1985; Heyer et al., 1986; Wright et al., 1986). The prototype *S. pombe* ARS is called ARS1 and according to Heyer (1986), plasmids containing this element behave much like 2μ *S. cerevisiae* ARS plasmids in *S. pombe* except that they tend to have a higher copy number (30 vs 2-10). It appears that ARS function is only partially conserved between *S. pombe* and *S. cerevisiae*. The region of the *S. cerevisiae* 2μ plasmid that functions as an ARS in *S. pombe* does not include the complete replication origin which is required in *S. cerevisiae* (Gaillardin et al., 1983). In fact, many elements from *S. pombe* identified on the basis of ARS activity in *S. pombe* have not exhibited the same function in *S. cerevisiae* (Maundrell et al., 1985).

It is possible to achieve high frequency transformation in *S. pombe* with non-ARS plasmids if they are co-transformed with ARS containing plasmids. Sakai et al., (1984) found a 280X increase in transformation frequency due to the presence of these sequences. Analysis of plasmid structure by Southern hybridization indicated that the establishment of cotransformants occurred by copolymerization of the ARS and non-ARS plasmids, presumably by homologous recombination. This property has been exploited to transform *S. pombe* at high efficiency with gene banks constructed in non-ARS plasmids as demonstrated by Lee and Nurse, (1987) in the cloning of a human *cdc2* from a human cDNA library.

(b) Genetic markers for use in *S. pombe*

Two selection systems have been extensively used in *S. pombe* transformations. The *S. cerevisiae* LEU2 gene coding for β -isopropylmalate dehydrogenase complements the *S. pombe* *leu1-32* mutation. The *leu1-32* mutation is stable in *S. pombe*, having a reversion frequency of less than 1 in 10^7 cells (Beach and Nurse, 1981). The second selection system utilizes the *S. cerevisiae* URA3 gene which codes for orotidine-5'-phosphate decarboxylase, is responsible for the 5-fluoro-orotic acid resistance phenotype and complements the *S. pombe* *ura4-294* mutation (Boeke et al., 1984). *Leu1-32* strains containing only a single integrated copy of LEU2 have reduced growth rates in selective media (Russell and Nurse, 1987) and the URA3 gene complements only when cloned on multi-copy plasmids indicating poor expression of both LEU2 and URA3 in *S. pombe* (Russell, 1989). In a comparative study of transcription initiation in *S. cerevisiae* and *S. pombe* by Russell (1983), it was determined that genes from one yeast are transcribed inefficiently and differently when introduced into the other and this difference appears to lie in the mechanism that determines the transcription initiation point. In the case of highly expressed genes which have strong transcriptional promoters, inefficient transcription initiation can effect expression (Russell, 1989).

Other selection systems that have been utilized in *S. pombe* are described in Table 1. These include complementation of *S. pombe* auxotrophs, resistance to antibiotics and expression of marker or reporter gene functions .

c) Heterologous expression in *S. pombe*.

The use of *S. pombe* as a host for heterologous gene expression has lagged behind *S. cerevisiae*. Inducible promoters equivalent to the *S. cerevisiae* *GAL1-10* or *PHO* promoters have not been obtained as yet for *S. pombe*. Counterparts of the *GAL* promoter are unlikely to be found in this yeast since it does not assimilate galactose. The only reported examples of the use of inducible promoters in *S. pombe* are (Russell, 1984; Russell and Nurse, 1986; and McLeod et al., 1987) based on the alcohol dehydrogenase (*adh*) gene promoter for the expression of the human alpha-1 antitrypsin gene (Russell, 1989), the *cdc2*⁺ encoded protein kinase (Carr, 1987) and the *mei3*⁺ gene (McLeod et al., 1987). Kudla et al., (1988) described the expression of the *E. coli* β -galactosidase gene and the Tn5 *ble* gene which confers resistance of *S. pombe* to bleomycin and phleomycin through the use of the 54/1 promoter. Data also presented by these authors indicated that the 54/1 promoter in the plasmid pBK02 functions constitutively in *S. pombe*. Attempts to match the 54/1 sequence to existing sequences in the EMBL and Genbank databases were unsuccessful. Translational fusion of the *Pseudomonas putida* *XyleE* gene with the 54/1 promoter was also shown

Table 1: Selective markers used in *Schizosaccharomyces pombe* transformation.

Source	Gene	<i>S. pombe</i> Gene Complemented or Gene Added	<i>E. coli</i> Gene Complemented or Gene Added	References
<i>S. pombe</i>	ade5 ade6 sup3-5 aro3 leu1 ura1 ura4	ade5 ade6 sup3-5 aro3 leu1 ura1 ura4	- - - aroD, E leuB pyrB -	Niwa et al., 1988 Niwa et al., 1988 Carr, 1987 Nakanishi and Yamamoto, 1984 Kikuchi et al., 1988 Sakaguchi and Yamamoto, 1982 Bach, 1987
<i>P. putida</i>	xylE	catechol 2,3-dioxygenase	catechol 2,3-dioxygenase	Kudla et al., 1988
<i>E. coli</i>	ble	bleomycin' pleomycin'	-	Kudla et al., 1988
Tn903	kan'	G418'	kan'	Sakai et al 1984

to allow expression of catechol 2,3-dioxygenase in *S. pombe* (Kudla et al., 1988). The pBK02 plasmid described by Kudla et al., (1988) requires the use of a helper plasmid as it contains no selectable markers for stable maintenance in *S. pombe*. No information was provided on the stability of this plasmid or any of its related cotransformants. However a fusion between 54/1 and the *lac Z* gene was found to direct up to 5% of the total *S. pombe* protein as β -galactosidase. In comparison, the *adh* promoter is expressed constitutively in glucose and glycerol/ethanol grown cells, producing between 0.5 to 2% of the total soluble protein as alcohol dehydrogenase (Russell, 1983; Russell and Hall, 1983).

Of the vectors described to date for gene expression in *S. pombe*, none address the area of protein export. The vector PEX1, described in this thesis, was constructed for this purpose.

d) Selection of reporter gene

A number of genes have been utilized for their reporter function for studies of heterologous gene expression. The best characterized and most widely used has been the full length or truncated version of the *E. coli lacZ* gene coding for β -galactosidase (Casadaban and Cohen, 1980; Casadaban et al., 1980; Gentz et al., 1981; Kroos and Kaiser, 1984; Stachel et al., 1985; Kudla et al., 1988). Genes encoding resistance to various antibiotics such as tetracycline (West et al., 1979; An and Friesen, 1979), ampicillin (An and Friesen, 1979; Palva et al., 1982; Katonaga et al., 1984; Smith

et al., 1987; Wang et al., 1987), kanamycin (An and Friesen, 1979; Reiss et al., 1984) chloramphenicol (An and Friesen, 1979; Goldfarb et al., 1981; Monkolsuk et al., 1985) have also been used rather extensively. Other reporter genes include those coding for bacterial luciferase (Legocki et al., 1986), firefly luciferase (Ow et al., 1987), tryptophanase and naphthalene dioxygenase (Ensley et al., 1983), catechol 2,3-dioxygenase (Zukowski et al., 1983), alkaline phosphatase (Hoffman and Wright, 1985; Manoil and Beckwith, 1985) and β -glucuronidase (Jefferson et al., 1986). However, these reporter genes have been used almost exclusively in bacteria and with the exception of β -galactosidase, ampicillin^R and luciferase, there is no evidence of their being functional in yeast.

The choice of a reporter gene to study protein export is important; the product should be active only when expressed correctly as well as being easily detectable and quantifiable (Wang et al., 1987). In this thesis a *Bacillus* endoglucanase was used as a reporter. Members of the Bacillaceae are Gram-positive, endospore forming bacteria which are known to secrete a wide variety of enzymes including glucanases such as amylase, cellulase, and xylanase and various proteases (Priest, 1977). The cellulolytic enzymes of Bacilli are entirely extracellular (Beguín, 1990; Fukamori et al., 1989; Robson and Chambliss, 1989). A number of publications have reported the identification of *B. subtilis* strains producing cellulolytic endoglucanases (MacKay et al., 1986; Sashihara et al., 1984; Koide et al., 1986; Robson and Chambliss, 1986). The extracellular endo- β -1,4-glucanase gene from *B. subtilis* has been cloned in *E. coli* and expressed in both *E. coli* and the yeasts *S. cerevisiae* and *Schwanniomyces*

alluvius (Seligy et al., 1987). The gene encodes a protein of 499 amino acids in length, which includes a 29 amino acid N-terminal signal peptide required for export and activation. The protein product is known to be processed in *E. coli* in a fashion similar to that in *Bacillus*, based on amino-terminal analysis and Western blot analysis of gene products, (MacKay et al., 1986; Lo et al., 1988). This gene was selected for its "reporter" function because it has been recently well characterized in both *E. coli* and in two yeasts which do not normally carry such a gene. The active product can also be assayed for easily in both organisms. In addition, the gene has been sequenced and some information on its cellular location and function is known (Seligy et al., 1987; Lo et al., 1988; Baird et al., 1990; Hefford et al., 1991).

(e) Selection of homologous protein export signal.

Nucleotide information coding for the acid phosphatase protein secretion signal was chosen for inclusion in this vector because the fission yeast produces few secreted extracellular proteins, and acid phosphatase is the best characterized of these extracellular proteins (Maundrell et al., 1985). The gene coding for acid phosphatase has been cloned and sequenced and has been shown to code for a protein 453 amino acids in length, including an 18-amino acid N-terminal secretion signal which is removed by cleavage after Ala₁₈ during secretion (Elliot et al., 1986). The report by Elliot was the first description of a gene coding for an exported protein in *S. pombe*, and according to Russell (1989), the *S. pombe* acid phosphatase signal sequence is still

one of the only native *S. pombe* controlling elements which has been well characterized. The gene is negatively regulated by intracellular phosphate levels. As a result, the promoter for this gene would not be suitable for use in an expression vector due to the requirement for low phosphate levels in the growth medium. Approximately 90% of the acid phosphatase activity is secreted, with 66% of this secreted activity being associated with the cell wall. For these reasons, the acid phosphatase signal sequence was seen as a realistic candidate sequence to direct the export of the *Bacillus* endoglucanase protein. A summary of the plasmid constructs employed or created during the course of this work is provided in Table 2.

Table 2: Plasmids used in this study.

Plasmid	Source	Reference/Description
pIRT2	P. Sunnerhagen	Russell, P., 1989
pBK02	H. Heslot	Kudla et al., 1988
pBT11.6	V. Seligy	Seligy et al., 1987
pMut31.1A	M. Hefford	Hefford, M. and V. Seligy, 1991
pIRT2Δ	This study	pIRT2 with modified multiple cloning site
pUCDI	This study	pUC118 + assembled Duplex I
pAP1	This study	pUCDI + assembled Duplex II
pEX1	This study	Constructed secretion vector
pEX21E	This study	pEX1 + <i>B. subtilis</i> endoglucanase structural gene

II. Materials and Methods

A) Growth of Bacteria and Yeasts

a) Bacteria (*Escherichia coli* K12)

Strain TG1 ($\Delta lac-pro$), *supE*, *thi*, *hsdD5* / F', *traD36*, *proA*⁺*B*⁺, *lacI*^a, *lacZ*, $\Delta M15$) was maintained on M9 medium (per litre: 6g Na₂HPO₄, 3g KH₂PO₄, 0.5g NaCl, 1g NH₄Cl, 2 ml 1 M MgSO₄, 10 ml 20% glucose, 0.1 ml 1 M CaCl₂, 15g agar) or on LB (per litre: 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar, pH 7.5). Antibiotic (ampicillin) when required, was added at a final concentration of 100 µg/ml. Strains were stored as stab cultures or streaked on agar plates and stored at 4°C and were routinely cultured at 37°C.

b) Yeast (*Schizosaccharomyces pombe*)

S. pombe strain SP1 (*rad10-198*, *ade6-250*, *leu1-32*, *ura4-294*, h⁻) was maintained on MIN (per litre: 6.7g yeast nitrogen base without amino acids, 20g glucose, 20g agar) or YEA (per litre: 5g yeast extract, 30g glucose, 20g agar) at 4°C. Liquid cultures were grown in PM [per litre: 3g phthalic acid, 1.8g Na₂HPO₄, 5g NH₄Cl, 20g glucose, 1 ml 1000 X vitamin stock (per litre: 1g sodium pantothenate, 10g nicotinic acid, 10g inositol, 10mg biotin), 1 ml 1000 X mineral stock (per litre: 5g H₃BO₃, 4g MnSO₄, 4g ZnSO₄, 2g FeCl₂·6H₂O, 1.6g H₂MoO₄·H₂O, 1g KI, 0.4g CuSO₄·5H₂O, 10g citric acid), 20 ml 50 X salts stock (per litre: 53.5g MgCl₂·6H₂O,

0.75g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50g KCl, 2g Na_2SO_4). Amino acids were added to a final concentration of 75 mg/ml when required. Strains were routinely cultured at 30°C.

c) Culture conditions for protein purification.

Parental and transformed strains of *S. pombe* strain SP1 were grown in PM liquid medium supplemented with appropriate amino acids from single colony isolates to a cell density of 5×10^7 cells/ml at 30°C in a New Brunswick gyratory shaker at 200 rpm. Culture volumes were increased in 10-fold steps from 10 ml to either 250 ml (small scale) or 2 litres (large scale) using 1/10th the total volume as an inoculum for the next step. The 2 litre culture was used to inoculate a 28 litre Microferm fermenter (New Brunswick Scientific) and the cells were grown at 200 rpm and 30°C with 12 litres of air/minute sparging, to a cell density of 5×10^7 cells/ml. Cells were harvested using a Millipore Pelicon cassette tangential flow system (Millipore Corp., 0.2µm) and were then recovered by centrifugation (5000 rpm for 30 minutes at 4°C). The cell pellet was washed twice in a volume of extraction buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF) numerically equal to its weight in grams.

d) Plasmid stability study in *Schizosaccharomyces pombe*.

Single colony isolates of *S. pombe* transformants were grown overnight in PM liquid medium supplemented with adenine and uracil to a cell density of 5×10^7 cells/ml

(200 rpm at 4°C). Baffled 500 ml Erlenmeyer flasks containing 90 ml of either PM supplemented with adenine, uracil and leucine (PM+AUL) or PM supplemented with adenine and uracil (PM+AU) were inoculated with 10 ml of the overnight culture to an initial cell density of 5×10^6 cells/ml and were grown for 3 generations under selective (PM+AU) or non-selective (PM+AUL) conditions. This was repeated 3 times (9 generations). Fifty ml of the 9th generation culture was used to inoculate 450 ml of either PM+AU or PM+AUL in 2 litre baffled flasks. The cultures were permitted to undergo one additional doubling and were then divided into two 200 ml aliquots. Cells were recovered by centrifugation (5000 rpm., 4°C) and were immediately frozen and stored at -80°C for later extraction of protein and total cellular DNA. In addition, cell samples were diluted and used to inoculate selective and non-selective PM solid media at an initial density of 100 cells/plate. The plates were examined after 5 days for viable colonies. This entire procedure was repeated resulting in samples being obtained at 10, 20, 30, 40 and 50 generations.

B) Isolation and characterization of DNA

(a) Isolation of *Escherichia coli* plasmid DNA

(i) Large scale procedure

Cells from 500 ml of *E. coli* cultures grown in LB containing 100 µg/ml ampicillin were grown overnight at 37°C. The bacterial pellet was recovered by centrifugation at 6,000 rpm for 10 minutes in a Sorvall SS-36 rotor and plasmid DNA was isolated

and purified from CsCl-ethidium bromide density gradients according to the alkali lysis procedure of Birnboim and Doly, (1979).

(ii) Mini-prep procedure

Mini-preparations of plasmid DNA were isolated by a modification of the procedure of Birnboim and Doly (1979). Overnight 5 ml cultures from a single colony were grown in LB containing 100 $\mu\text{g/ml}$ ampicillin and were incubated at 37°C and 200 rpm. Cells from 3 ml of culture were recovered by centrifugation in an Eppendorff micro-centrifuge and the cell pellet was resuspended in 100 μl of 25 mM Tris-HCl pH 8.0, 10 mM EDTA and stored on ice for 3 minutes. Two hundred μl of 0.2 N NaOH, 1% SDS were added and the tube inverted gently 10 times then placed on ice for 5 minutes. One hundred and fifty μl of 3 M sodium acetate pH 4.8 were added and the tube was inverted 10 times and then stored on ice for a further 10 minutes. After a 10 minute centrifugation at 4°C the supernatant was transferred to a new 1.5 ml centrifuge tube and the DNA was precipitated by the addition of 1 ml of ice-cold 95% ethanol and subsequently stored on dry ice for 10 minutes. The DNA was recovered by centrifugation and was dissolved in 400 μl of 100 mM sodium acetate, 10 mM Tris-HCl pH 8.0. The DNA was again precipitated on dry ice for 10 minutes after the addition of 1 ml of ice-cold 95% ethanol. The DNA pellet was recovered by centrifugation at 4°C, was washed two times with 70% ethanol and then dried down under vacuum. The dried pellet was dissolved in 100 μl of 10 mM Tris-

HCl pH 8.0, 1 mM EDTA and 0.5 μ l of RNase (10 mg/ml) was added. After a 30 minute incubation at 37°C, 200 μ l of 2 X proteinase K buffer (20 mM Tris-HCl, 10 mM EDTA, 1% SDS pH 7.8) and 1.5 μ l of Proteinase K (20 mg/ml) were added and the tube was incubated at 50°C for 60 minutes. The sample was extracted with phenol:chloroform (1:1) and the DNA was precipitated from the aqueous phase on dry ice after the addition of 20 μ l of 3 M sodium acetate pH 8.0 and 450 μ l ice-cold 95% ethanol. The DNA pellet was washed twice with 70% ethanol, vacuum dried and resuspended in 30 μ l of sterile water. DNA prepared in this manner is suitable for both restriction enzyme digests and DNA sequencing.

(iii) Rapid screening for recombinant plasmid

Recombinant plasmids were identified using the rapid screening procedure of Sekar (1987). Putative transformant colonies were randomly selected by resuspending in 25 μ l protoplasting buffer (30 mM Tris-HCl pH 8.0, 5 mM Na₂EDTA, 50 mM NaCl, 20% sucrose, 50 μ g/ml RNase, 50 μ g/ml lysozyme). Agarose gels (0.8%) were prepared in Tris-Borate-SDS buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na₂EDTA, 0.05% SDS) and the wells were pre-loaded with 4 μ l of lysis buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na₂EDTA, 2% SDS, 5% sucrose, 0.04% bromophenol blue). Ten μ l of protoplast suspension were loaded into the sample wells under the lysis solution. Electrophoresis was carried out in Tris-Borate-SDS buffer for 15 minutes at 30V followed by 2.5 hours at 120V. After completion of the

electrophoresis, the gels were stained with ethidium bromide and photographed under UV illumination (254 nm).

b) Isolation of *Schizosaccharomyces pombe* DNA

(i) Isolation of total cellular DNA

High molecular weight DNA from *S. pombe* was prepared according to the procedure of Durkacz et al. (1985) using the protoplasting enzyme Novozyme SP234 (Stephen and Nasim, 1981). Cells from 100 ml cultures were recovered by centrifugation at 10,000 rpm for 5 minutes in a Sorvall SS-36 rotor, resuspended in 5 ml 20 mM citrate-phosphate buffer pH 5.6, 40 mM EDTA, 1.2 M sorbitol, 150 mM 2-mercaptoethanol and 5 mg/ml Novo SP234 and incubated at 30°C for 30-90 minutes. Cells were harvested at 5,000 rpm for 5 minutes in the same rotor and resuspended in 15 ml of 50 mM Tris-HCl pH 7.5, 5 mM EDTA before cell lysis by mixing with 1.5 ml of 10% SDS. Five ml of 5 M potassium acetate were added and the lysate kept on ice for 30 minutes. After a 15,000 rpm, 5 minute centrifugation the supernatant was decanted into a 50 ml centrifuge tube, 20 ml of ice cold propan-2-ol were added and then left at -20°C for 5 minutes. The precipitate was recovered by centrifugation at 10,000 rpm for 10 minutes, dried under vacuum and resuspended in 50 mM Tris-HCl pH 7.5, 5 mM EDTA. After a 2 hour incubation at 37°C with 50 µg of RNase to remove RNA, an equal volume of phenol:chloroform (1:1) was added and the DNA was precipitated from the aqueous layer with 2 volumes of 95%

ethanol. The pellet was washed twice in 85% ethanol, vacuum dried and resuspended in 500 μ l of 10 mM Tris-HCl pH 7.5, 1 mM EDTA.

(ii) Rapid recovery of yeast plasmid.

Autonomously replicating, extrachromosomal plasmids were recovered from extracts of *S. pombe* according to the procedure of Hoffman and Winston (1987). Plasmid DNA was obtained from 1.4 ml of an overnight yeast cell culture recovered in a 1.5 ml centrifuge tube. Cell pellets were resuspended in 200 μ l of 2% Triton X-100, 1% SDS, 10 mM NaCl, 10 mM tris pH 8.0, 1 mM EDTA, by vortexing for 1 minute at full speed. After the addition of 2 μ l phenol-chloroform-isoamyl alcohol (25:24:1) and 0.3 g acid washed glass beads, the tube was vortexed for 2 minutes. The aqueous phase was recovered after centrifugation and a 1-5 μ l aliquot used to transform *E. coli* strain TG1 to ampicillin resistance.

(c) Characterization of DNA by restriction enzyme analysis

Total cellular or plasmid DNA was digested to completion using a variety of restriction endonucleases obtained from Bethesda Research Laboratories (BRL). Samples were typically digested for 2 hours at 37°C at an enzyme concentration of 2 units per μ g of DNA using buffers supplied by the manufacturer. Reactions were terminated by the addition of 1/10th volume of 10X stop buffer (25% glycerol, 100

mM EDTA, 0.5% SDS, 0.1% bromophenol blue) and then heat inactivated at 65°C for 5 minutes.

DNA was fractionated by horizontal gel electrophoresis using agarose (Seakem) as the supporting matrix at concentrations of 0.8-1.5% (w/v) in TAE buffer. Electrophoresis was typically carried out at either 80 V for 4 hours or 20 V for 18 hours with ethidium bromide added only to the electrode buffer at a concentration of 0.5 µg/ml. DNA fragments in the gels were visualized by fluorescence of the ethidium bromide induced by UV illumination (254nm), and recorded by photography using Polaroid type 52 or 55 film with a red filter. Polaroid negatives were developed in a solution of 18% Na₂SO₃ for 3-5 minutes and washed in running tap water for 30 minutes. Typical DNA markers included lambda DNA digested with HindIII and φX174 DNA digested with HaeIII.

(d) Purification of DNA from agarose gels

Three methods were used to purify DNA from bands visualized under long wave UV light. The first method involved electroelution of the DNA into dialysis bags as described by Maniatis et al. (1982). The second method involved direct electroelution of the DNA fragment onto NA-45 DEAE paper (Schleicher & Schuell) (Dretzen et al., 1981; Lizardi et al., 1984) placed at right angles to the direction of electrophoresis. DNA was eluted from the paper at 65°C for 1 hour in 20 mM Tris-HCl pH 7.5, 1 mM EDTA and was ethanol precipitated from 0.3M sodium acetate,

recovered by centrifugation, dried under vacuum, and resuspended in sterile water. The third method made use of a commercial kit called GENECLAN (Bio 101), which uses a silica matrix to selectively bind single or double stranded DNA.

C) DNA mediated transformations

(a) *Escherichia coli* transformation

Bacterial cells were made competent by adding 2.5 ml of an overnight LB broth culture to 100 ml fresh LB medium and incubated with good aeration at 37°C (200 rpm) until an absorbance at 550 nm of 0.4 was obtained. Cells were immediately chilled on ice and then recovered by centrifugation at 4°C at 5000 rpm for 10 minutes in a Sorvall SS-36 rotor. The cell pellet was resuspended in 50 ml ice-cold 100 mM CaCl₂ and incubated on ice for 30 minutes. Cells were again recovered by centrifugation at 4°C and resuspended in 4 ml ice-cold 100 mM CaCl₂, 15% (v/v) glycerol. Cells were distributed in 2 ml aliquots and maintained at 4°C overnight. Competent cells were subsequently frozen in a dry ice-ethanol bath and stored at -80°C. For transformations, an aliquot of frozen competent cells was thawed on ice. One microgram of plasmid DNA was mixed with 100 µl of competent cells and maintained on ice for 30 minutes. Cells were then heat shocked for 2 minutes at 42°C. One ml of LB broth was added and the cells incubated at 37°C for 1 hour. Cells were subsequently spread on LB agar plates containing appropriate antibiotics and incubated overnight at 37°C.

(b) Schizosaccharomyces pombe transformation

S. pombe transformations were performed according to Beach and Nurse, (1981) as modified by Beach et al., (1982) as follows: cells were grown at 25°C to 1×10^7 cells/ml in 200 ml PM medium supplemented with amino acids at 75 mg/l as required. The medium contained only 0.5% rather than the normal 2.0% glucose as this greatly enhanced the ease of formation of spherical protoplasts. The cells were harvested by centrifugation at 3600 rpm for 5 minutes, washed in 1.2 M sorbitol, 20 mM citrate-phosphate pH 5.6, 40 mM EDTA, 150 mM 2-mercaptoethanol and after centrifugation, were resuspended at 3×10^8 cells/ml in 1.2 M sorbitol, 50 mM citrate-phosphate pH 5.6, 30 mM 2-mercaptoethanol, 5 mg/ml Novo SP234. The cells were incubated at 30°C for 30-60 minutes until at least 50% of the cells had become spherical as determined by light microscopy. They were washed 3 times in 35 ml 1.2 M sorbitol, 10 mM Tris-HCl pH 7.6, taking great care to gently resuspend the pelleted protoplasts between each wash. The protoplasts were finally resuspended at 5×10^8 cells/ml in 1.2 M sorbitol, 10 mM Tris-HCl pH 7.6, 10 mM CaCl_2 . The cells to be transformed were dispensed in 100 μl aliquots to which plasmid DNA was added in a volume less than 10 μl . After incubation for 15 minutes at 22°C, 1 ml of 10 mM Tris-HCl pH 7.6, 10 mM CaCl_2 , 20%(w/v) PEG 4,000 (BDH) was added to each tube and left for 15 minutes at room temperature. The cells were finally pelleted and resuspended in 200 μl 1.2 M sorbitol, 10 mM Tris-HCl pH 7.6, 10 mM CaCl_2 , 0.5 mg/ml yeast extract, 5 μg /ml leucine for 60 minutes before plating directly on

minimal selective plates containing 1.2 M sorbitol and the appropriate amino acids without leucine. The appearance of putative transformant colonies were noted and scored after 3-5 days incubation at 30°C.

D) Immobilization of DNA and hybridization procedures

(a) Immobilization of DNA from agarose gels

DNA fractionated on agarose gels was transferred to nylon membranes (Biodyne A, Pall Corp.) according to the procedure of Maniatis et al., (1982). The DNA was denatured in 0.5 N NaOH, 1.5 M NaCl for 30 minutes and then neutralized in 3 M sodium acetate for 30 minutes. The DNA was transferred directly to nylon membrane by capillary action in 20 X SSC overnight. The membranes were air dried for 30 minutes, baked at 80°C for 1 hour and stored under vacuum until used.

(b) Immobilization of DNA from *E. coli* recombinants

DNA from recombinant *E. coli* colonies was transferred to nylon filters for identification by hybridization. Colonies were replica transferred, using velvet transfer pads, onto nitrocellulose filters on LB plates containing 100 µg/ml ampicillin and were incubated for 4 hours at 37°C. DNA was released by placing the nitrocellulose filters onto 3MM paper saturated with 0.5 N NaOH, 0.5 M NaCl for 5 minutes at room temperature. The filters were then transferred sequentially to 3MM

paper saturated with 1 M Tris-HCl pH 7.5, and 3MM paper saturated with 0.5 M Tris-HCl, 1.5 M NaCl pH 7.5 for 1 minute and 5 minutes respectively. The filters were dried under vacuum at 80°C for 2 hours and stored dry until used.

(c) Immobilization of DNA from *S. pombe* recombinants

DNA from recombinant *S. pombe* colonies was transferred to nylon filters for identification by hybridization. The procedure followed was the same as for *E. coli* recombinants (see section b), except that transferred colonies were grown overnight on selective medium and were then pre-incubated on 3MM paper saturated with protoplasting buffer (50 mM citrate-phosphate pH 5.6, 40 mM EDTA, 50 mM 2-mercaptoethanol, 1.2 M sorbitol, 5 mg/ml Novozyme SP234) before transfer to the NaOH/NaCl lysis solution. After baking under vacuum for 2 hours at 80°C the filters were washed in 5 X SSC, 0.1% SDS for 2 hours to allow the excess cellular debris to be wiped from the filter to reduce hybridization background.

(d) DNA hybridizations

Filters were prehybridized and hybridized in heat sealed plastic bags (Seal-a-Meal). Filters were prehybridized for a minimum of 2 hours at 55°C in 15 ml of 4 X SSC containing 5 X Denhardt's solution (per 500 ml; 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g Fraction V of bovine serum albumin), 0.1% SDS and 100

$\mu\text{g/ml}$ denatured sonicated salmon sperm DNA. The prehybridization solution was removed and the probe ($0.5 - 1.0 \times 10^6$ cpm/ml) was added and the incubation continued for 18-20 hours. Filters were washed at high stringency by first washing two times at room temperature in 4 X SSC, 0.1% SDS and then twice in the same buffer at 65°C for 15 minute. The filters were kept damp by wrapping in plastic (Saran Wrap) and were exposed to X-ray film with or without intensifying screens. If a higher stringency wash was required, the damp filter was rewashed using a combination of lower salt concentration and/or a higher temperature, followed by re-exposure of the filter.

Hybridization with radiolabelled oligonucleotides was carried out in 5 X SSC, 5 X Denhardt's, 0.1% Triton X-100, 100 $\mu\text{g/ml}$ denatured salmon sperm DNA at a temperature 5°C less than the melting point of the oligomer as determined by the formula $t_m = [(\text{the number of G and C bases}) \times 4^\circ\text{C} + (\text{the number of A and T bases}) \times 2^\circ\text{C}]$ (Suggs et al., 1981). Filters were washed at the calculated hybridization temperature in 5 X SSC, 0.05% Triton X-100 and autoradiographed. Wash temperatures were increased by 2.5°C increments, if higher stringency conditions were indicated.

E) Protein purification and analysis**(a) Extraction of proteins**

S. pombe cells were harvested by centrifugation, washed once in extraction buffer (10 mM Tris-HCl pH 6.5, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and stored frozen at -80°C until required. A volume of extraction buffer and a weight of acid-washed, sterile glass beads equal to the weight of cells to be extracted, were added to the cells, and the mixture sonicated (Sonic Dismembrator Model 300, Fisher Scientific) on ice for 5 minutes. The supernatant was recovered after centrifugation at 12,000 rpm for 10 minutes in a Sorvall SS-36 rotor and was stored frozen at -80°C until required.

(b) Polyacrylamide gel electrophoresis of proteins

An aliquot of yeast cell extract was mixed (1:4) with gel loading buffer (625 mM Tris-HCl pH 6.8, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 2% w/v SDS, 0.02% bromophenol blue), boiled for 5 minutes and loaded onto a 5% stacking-15% separating polyacrylamide gel and electrophoresed at 150 V in electrophoresis buffer (25 mM Tris, 200 mM glycine, 0.5% SDS pH 8.0) for 45-60 minutes employing a Mini-Protean II (BioRad) PAGE apparatus. The separating and stacking gels were prepared from an acrylamide stock of 29.2% w/v acrylamide / 0.8% w/v N,N'-methylenebisacrylamide (BRL). Separating gels contained 375 mM Tris-HCl pH 8.3,

0.1% SDS and 0.04% w/v ammonium persulfate, while stacking gels contained 125 mM Tris-HCl pH 6.8, 0.1% SDS and 0.06% ammonium persulfate. Gel polymerization was initiated by the addition of TEMED to a final concentration of 0.15% v/v.

After electrophoresis, proteins in duplicate polyacrylamide gels, were detected by staining one gel with 0.2% Coomassie Blue R-250 (Sigma) in 40% methanol/10% acetic acid for 20 minutes at 55°C. Gels were destained (10% v/v methanol, 10% v/v acetic acid) at room temperature. For western blotting, proteins from the duplicate gel were electrophoretically transferred (Towbin et al., 1979) onto BioTrace NT membranes (Gelman) at 4°C at 250 mA for 2 hours using 25 mM Tris base, 192 mM glycine, 4% v/v methanol, pH 8.3. Filters were stored in fresh buffer until immunodetection was undertaken, typically the same day.

(c) Isoelectric focusing

Proteins were separated on PhastSystem™ (Pharmacia) PhastGel IEF media (pH 5 - 8) according to the manufacturers instructions. The gels were prefocused to permit formation of a pH gradient. Samples (0.1 µg/µl) were applied and the gel developed. Protein bands were visualized by staining with Coomassie Brilliant Blue. Zymograms were generated according to the procedure of MacKenzie and Williams (1984). Typically, 0.1% carboxymethyl cellulose substrate in 25 mM potassium phosphate pH 6.0, 2% agar, prepared on GelBond (FMC) plastic film, was placed in

contact with one of a duplicate pair of IEF. CMC hydrolysis zones were visualized by staining with Congo Red and destaining with 1M NaCl.

(d) Immunodetection of endoglucanase

Proteins electrophoretically transferred to membranes were probed with rabbit anti-endoglucanase antiserum (Lo et al., 1988) utilizing an alkaline phosphatase immunodetection assay as detailed by the manufacturer (Protoplot Western Blot AP System, Promega Biotech). All reactions were performed at room temperature. Filters were quenched in TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% FCS (Gibco) for 30 minutes. The quenching solution was replaced with anti-endoglucanase antiserum (primary) diluted 1:100 in TBST and the incubation continued for 30 minutes. The filter was washed 3 times in TBST for 10 minutes each to remove unbound antibody. The filter was then incubated for 30 minutes in TBST containing a 1:7500 dilution of anti-rabbit IgG alkaline phosphatase conjugate (secondary). The filter was washed 3 times for 10 minutes each to remove unbound secondary antibody and then transferred to 10 ml AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 66 μ l NBT substrate and 33 μ l BCIP and was incubated in the dark until banding was visible. The reaction was stopped by rinsing in distilled water. The nitrocellulose filters were air dried on 3MM paper (Whatman).

(e) Detection of endoglucanase activity

Endoglucanase activity was assayed by one of three methods: (i) zymogram, for enzyme separated by PAGE (ii) agarose-CMC plate assay for enzyme associated with bacterial or yeast cells or (iii) liquid assay for enzyme in solution.

Zymograms were generated according to the procedure of MacKenzie and Williams (1984). Typically, 0.1% carboxymethyl cellulose substrate in 25 mM potassium phosphate pH 6.0, 2% agar, prepared on GelBond (FMC) plastic film, was placed in contact with native polyacrylamide or electrofocused polyacrylamide gels. Hydrolysis zones in the substrate gels were visualized by staining with Congo Red (1 mg/ml in water) and destaining with 1M NaCl.

For CMC plate assays, *E. coli* or *S. pombe* cells were transferred to appropriate solid growth media containing 0.1% CMC using sterile tooth picks and were incubated at appropriate temperatures, 48 hours at 30°C for yeast and 18 hours at 37°C for bacteria. Cellulosic digestion was visualized by staining the undigested CMC with Congo Red and destaining with 1M NaCl after removal of colonies by washing with water.

The reducing sugar, liquid assay was performed according to Somogi (1952) using glucose as a standard.

(f) Concentration of proteins

Protein in small volumes of culture supernatant or broken cells (up to 15 ml) were concentrated using Centriprep-10 (Amicon) disposable ultrafiltration concentrators according to the manufacturers instructions. Typically, protein in 15 ml of sample was concentrated 15 - 30 X using this method. According to the manufacturer, the anisotropic, hydrophilic YTM membranes employed in the Centriprep concentrators are low adsorption membranes which yield >90% solute recovery with no apparent effect on enzyme activity.

(g) Fractionation of cultures for endoglucanase activity

Both cells and culture supernatants were assayed for endoglucanase activity. The procedure of Rouenhorst et al., (1988) was used for the fractionation of cultures into supernatant enzyme, cell wall enzyme and cell-bound enzyme. Cultures (100 ml) were harvested by centrifugation at 4000 rpm and 4°C for 10 minutes in a GSA rotor. The supernatant fraction served as the source of extracellular enzyme. The liberation of cell wall associated enzyme was induced by suspension of the cells in 10 ml of enzyme release buffer (50 mM potassium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM dithiothreitol, 2 mM MgSO₄) after incubation for 1 hour at 30°C. The suspension was then centrifuged at 5000 rpm and 4°C for 10 minutes and washed twice, first with 5 ml of enzyme release buffer and subsequently with 5 ml of

sonication buffer (50 mM potassium phosphate, pH 7.0, 10 mM MgSO_4). Enzyme activities present in the supernatant after incubation of the cells in enzyme release buffer and in the supernatants after washing were designated as extracellular enzyme trapped by the cell wall. After release of the cell wall enzyme and washing, cells were suspended in 5 ml sonication buffer and sonicated at 4°C for 5 minutes on ice. Cell debris was removed by centrifugation at 15,000 rpm for 15 minutes at 4°C in a Sorvall SS-34 rotor and suspended in 5 ml sonication buffer. Enzyme activities present in cell-free extract and resuspended debris were taken as cell-bound enzyme.

F) Preparation of [^{32}P] radiolabelled DNA probes

(a) Nick translation procedure

Double stranded DNA was radiolabelled with [α - ^{32}P] dATP by nick translation using the method of Rigby et al., 1977 as described by Maniatis et al., 1982. DNA (0.5-1.0 μg) was combined with 60 μM each of dCTP, dGTP and dTTP and 50-100 μCi [α - ^{32}P] dATP (Amersham, 800 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{l}$) in 50 mM Tris-HCl, 10 mM MgSO_4 , 100 μM DTT and 50 $\mu\text{g}/\text{ml}$ BSA (Sigma). One μl of a 10^4 dilution of a 2 mg/ml DNase I stock and 10 units of *E. coli* DNA polymerase I Klenow fragment (Pharmacia) were added and the reaction incubated at 15°C for 1.5 hours. The reaction was terminated by heating at 65°C for 10 minutes. Nick translated DNA was separated from the unincorporated dNTP'S utilizing an Elutip-d column (Schleicher

& Schuell) according to the manufacturers instructions. To calculate the percent incorporation of label into DNA, 1 μ l of pre- and post-Elutip-d treated solutions were added to a scintillation vial with 100 μ l water and 5 ml Aquasol and total cpm were calculated by scintillation counting using a Beckman LS 7000 liquid scintillation counter (Berger, 1984). Typically, probes were obtained with specific activities of 1.5×10^7 dpm/ μ g with 25-30% of the label being incorporated.

(b) 5'-end labelling procedure

Deoxyoligonucleotides were 5'-end labelled according to the procedure of Maniatis et al., (1982). Typically, 10 pmole of deoxyoligonucleotide, 20 to 65 bases in size, were labelled with 100 μ Ci [γ - 32 P] ATP (Amersham, 3000 Ci/mmol, 10 μ Ci/ μ l) in 10 μ l of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 5 mM DTT containing 10 units T4 DNA polynucleotide kinase (Pharmacia). After incubation at 37°C for 30-60 minutes the reaction was terminated by heating at 90°C for 5 minutes. Unincorporated nucleotides were removed using Elutip-d columns (Schleicher & Schuell) according to the manufacturers instructions.

G) Preparation and separation of *Schizosaccharomyces pombe* chromosomes

Intact DNA of *S. pombe* chromosomes were separated according to the method of Avdalovic and Furst, (1988). All glassware and buffers were heat sterilized and all utensils were rinsed in 95% ethanol just prior to use. Cells were cultured in PM medium (0.05% glucose) supplemented with appropriate amino acids, recovered by centrifugation and washed twice in ET buffer (1 mM Tris-HCl pH 8.0, 50 mM EDTA). Cells (4×10^8) were resuspended in 5.6 ml ET buffer to which 5.6 ml of 1% LMP agarose (FMC Corporation) mixture (1% agarose, 0.2 ml lyticase) in ET buffer was added. The cell suspension was pipetted into refrigerated plug molds. The plugs were expelled into 20 ml 10 X ET buffer containing 0.15 ml lyticase and were incubated at 37°C for 48 hours with one change in buffer. The plugs were transferred to 20 ml of 10 X ET buffer containing 1% sodium lauryl sarcosinate 1 mg/ml Proteinase K (Sigma) and incubated at 50°C for 48 hours, with one change of buffer and enzymes during the incubation period. Plugs were stored in ET buffer until use, typically less than 1 week.

For chromosome separation 0.6% or 0.8% LMP agarose gels (50 ml) in 10 mM Tris-HCl pH 8.0, 20 mM NaCl and 100 mM EDTA, pre-equilibrated to 50°C, were cast in the manufacturer-supplied mould. Agarose plugs containing lysed cells were loaded into the pre-cast wells and sealed with agarose. The gel was transferred to the transverse alternating field electrophoresis (TAFE) apparatus (Beckman) and electrophoresed for either 144.5 hours (0.8% agarose gels; Stage 1: 30 minutes

duration, 4-second pulses, 50 mA constant current, Stage 2: 72 hours duration, 90-minute pulses, 35 mA constant current, Stage 3: 72 hours duration, 90-minute pulses, 35 mA constant current) or 48 hours (0.6% agarose gels; Stage 1: 3 hours duration, 45 minute pulses, 40 V constant voltage, Stage 2: 21 hours duration, 20 minute pulses, 60 V constant voltage, Stage 3: 24 hours duration, 20 minute pulses, 60 V constant voltage) at 10°C.

Gels were stained in ET buffer containing 0.5 mg/ml ethidium bromide for 30-60 minutes and the chromosomes visualized by photography under short wave (254 nm) UV illumination.

The gel was treated with 200 ml 0.2 N HCl (5 minutes), 0.4 N NaOH (60 minutes) and then neutralized in 200 ml 1 M Tris-HCl pH 8.0, 1.5 M NaCl for 30 minutes. After a short wash in 20 X SSC, DNA in the chromosomes was transferred to Biotrans A membrane (Pall Corp.) by capillary transfer with 20 X SSC for 24 hours. Membranes were probed with specific radiolabeled DNA.

(H) Deoxyoligonucleotide synthesis and purification.

Deoxyoligonucleotide fragments were synthesized utilizing an Applied Biosystems 380A DNA synthesizer. The α -protected deoxyoligonucleotides were deblocked in NH_4OH at 55°C for 24 hours, dried under vacuum and resuspended in distilled water. Individual oligomers were isolated and purified by electrophoresis through a 16% polyacrylamide/8M urea gel. The purified deoxyoligonucleotides were desalted on

C₁₈-Sep Pak columns (Waters). The columns were washed sequentially with 5 ml HPLC grade methanol (Caledon, 0.023% H₂O) and 10 ml deionized water. The deoxyoligonucleotide was loaded onto the column, washed with 10 ml deionized water, recovered in 3 ml 0.1M TEAB pH 7.3 : methanol (1:1), dried and resuspended in 100 μ l deionized water. Concentration (μ g/ml) was determined using the formula $A_{260} \times \text{dilution} \times 20$ as recommended by Maniatis et al., (1986). Deoxyoligonucleotides were stored at -20°C until required.

III. Results

A) Construction of the *S. pombe* expression/secretion vector pEX21E

The expression vector was assembled from material constructed in four separate steps: Step 1 involved the deletion of non-essential restriction enzyme sites from the plasmid vector pIRT2 into which the final expression cassette was to be inserted; Step 2 involved the isolation and modification of the 54/1 promoter originating from plasmid pBK02; Step 3 involved the synthesis and assembly of oligonucleotides encoding the acid phosphatase secretion signal; Step 4 involved the ligation of all components to generate plasmid pEX1.

(a) Modification of plasmid pIRT2

Plasmid pIRT2, (Hindley, 1987) is a derivative of pUC118 which contains a 1.2 kbp fragment with ARS1 function derived from one of the three *S. pombe* chromosomes (source unknown), inserted into the *Eco*RI site, and a 2.2 kbp DNA fragment containing the *S. cerevisiae* LEU2 gene (Beggs, 1978) from *S. cerevisiae* chromosome III (Mortimer and Hawthorne, 1966) inserted into the *Hind*III site (Figure 1A). For this plasmid construct, only the *Sph*I site was required. To remove the non-essential cloning sites while at the same time retaining the *Sph*I site, pIRT2 was digested with *Sac*I and the DNA ends containing the *Sac*I site were made blunt by treatment with T₄ DNA polymerase. *Sph*I linkers (5'-GGCATGCC) (Gibco BRL)

Figure 1: Construction of plasmid pIRT Δ .

(A) Restriction map of plasmid pIRT2 (Hindley, 1987). pIRT2 was digested to completion with the restriction endonuclease *SacI*. The DNA ends defining the *SacI* site were made blunt by treatment with T₄ DNA polymerase and attached to double stranded, *SphI* linkers (5'-GGCATGCC-3') by ligation. Subsequent digestion with *SphI* followed by self ligation in the presence of T₄ DNA ligase produced plasmid pIRT2 Δ .

(B) Restriction map of plasmid pIRT2 Δ . (C) Agarose (1%) gel electrophoresis of restriction endonuclease digested pIRT2 (see lanes a, c, e, g) and pIRT2 Δ (see lanes b, d, f, h). Digestions with *EcoRI* (a,b), *SacI* (c,d), *SphI* (e,f) and *HindIII* (g,h) were carried out to confirm the deletion of the 0.06 kbp *SacI*-*SphI* fragment in the multiple cloning site of pIRT2.

were kinased as described in methods and ligated to the blunt ended *SacI* sites of pIRT2. Following phenol extraction and ethanol precipitation, the ligated plasmid was re-suspended in *SphI* buffer and digested with *SphI*. After a second round of phenol extraction and ethanol precipitation, the plasmid was resuspended in ligation buffer containing T₄ DNA ligase and was allowed to circularize and self-ligate overnight at 12°C. The newly constructed plasmid, designated pIRT2Δ, was recovered by transformation of *E. coli* strain TG1. Of the ten transformants picked at random and examined by restriction enzyme digestion with *EcoRI*, *SamHI*, *SphI* and *HindIII*, 7 isolates had the 0.06 kbp multiple cloning site deleted, with the *EcoRI*, *SphI* and *HindIII* sites intact. Analysis of restriction fragments of one plasmid and the parental plasmid pIRT2 is shown in Figure 1C. pIRT2Δ (Figure 1B) contains selectable markers for both yeast and *E. coli* transformation to leucine prototrophy and ampicillin resistance, respectively and contains a unique *SphI* site into which the assembled expression cassette will be subsequently inserted.

(b) Isolation and modification of the 54/1 promoter.

Engineering of the *S.pombe* 54/1 promoter is shown in Figure 2A to C. Plasmid pBK02 (Kudla et al., 1988), was digested with *XmaI* and *XhoI* to release a 1.2 kbp fragment containing the sequence of the 54/1 promoter. After electrophoresis through a 0.8% agarose gel the 1.2 kbp *XmaI-XhoI* fragment was recovered using a

Figure 2: Isolation of the *S. pombe* 54/1 promoter.

Plasmid pBK02 (Kudla et al., 1988) was digested with *Xho*I and *Xma*I to release a 1.2 kbp *Xma*I-*Xho*I fragment containing the 54/1 promoter as illustrated schematically in (A). The schematic shows the promoter-polylinker region of pBK02 which contains the promoter (nucleotide position 180 to 1290) and the 59 bp, multiple cloning site (MCS, nucleotide position 1230 to 1289). The T at position 1250 was altered by site-directed mutagenesis to G (ATG -> AGG) by Kudla et al., (1988) to replace the ATG start codon with a unique *Stu*I site to permit subcloning of the promoter. The fragment was recovered after agarose gel electrophoresis, and the *Xho*I and *Xma*I sites were filled in using *E. coli* polymerase I Klenow fragment. Single stranded *Sph*I linkers (5'-GGCATGCC-3') were ligated onto the blunt ends. The *Sph*I linkered fragment was digested with *Bam*HI and the site was filled-in using Klenow enzyme. Digestion with *Sph*I produced a fragment with a 5'-*Sph*I site and a 3'-blunt-end that is in-frame with the 54/1 promoters' modified ATG (AGG) start codon at nucleotide position 1239-1241, as illustrated in (C).

A.

B.

C.

commercial reagent (Geneclean™, Bio 101). This fragment contains all the upstream regulatory sequences as well as the transcribed, non-translated sequences located upstream or 5' from the ATG codon, and the polylinker region from plasmid pFR10 (Shapira et al., 1983). Details of this construction, shown in Figure 2A are also described in Kudla et al., (1988). The *XhoI* and *XmaI* sites were subsequently filled in using Klenow fragment. The synthetic *SphI* linker was attached after phosphorylation of the ends by annealing the ends and ligating to the filled-in *XmaI*-*XhoI* ends. The *SphI* linkered fragment was subsequently digested with *SphI* and *BamHI* and the 1.2 kbp fragment containing the 54/1 promoter was ligated to pUC118 which had been digested with the same restriction enzymes. Recombinant plasmids (designated pUC54/1) were identified using the rapid screening method of Sekar, (1987) and DNA was isolated by use of a small scale, mini prep method. Plasmid pUC54/1 was digested with *BamHI*, and the *BamHI* site filled-in with Klenow fragment. After phenol extraction and ethanol precipitation, the resuspended plasmid was digested with *SphI* and the modified 1.2 kbp fragment containing the 54/1 promoter was released and recovered after electrophoresis through 0.8% agarose using the Geneclean™ method. As shown in Figure 2C, this generated a fragment with a 5'-*SphI* site and a 3'-blunt end containing the 54/1 promoter.

(c) Construction and isolation of the acid phosphatase secretion cassette components.

The process leading to construction of the secretion cassette is summarized in Figure 3. Twelve synthetic deoxyoligonucleotide fragments corresponding to a portion of the *S. pombe* acid phosphatase sequence published by Elliot et al., (1986), were synthesized, purified and phosphorylated as described in methods. Duplex-I was formed by annealing 10 pmole of each kinased oligonucleotide PA1-6 and PB1-6 whose sequences are shown in Figure 3A. The duplex was added to *SphI* linearized pUC118 which had been dephosphorylated with calf intestinal alkaline phosphatase and then purified by phenol extraction and ethanol precipitation. Then to the annealed mixture was added 2 units T4 DNA ligase together with ligase buffer and ATP (to 10 mM). After an 18 hour, 15°C incubation, recombinant plasmids, designated pUCDI, were recovered by transformation of *E. coli* TG1, and identified by colony hybridization using 5'-labelled oligonucleotide PA4 as a probe. Duplex-II coding for a multiple cloning site with unique sites for *Bam*HI, *Pst*I, *Pvu*II, *Sac*I and *Xho*I restriction enzymes was formed by annealing 10 pmole of the two oligomers 5'-GGGAGCTCGAGGATCCAGCTGCAGTCGAC and 5'-GTCGACTGCAGCTGGATCCTCGAGCTCCC) after phosphorylation using T4 polynucleotide kinase. The annealed duplex was then ligated into the unique *Sma*I site of the pUCDI construct and recombinants were recovered by transforming *E. coli* strain TG1. Putative recombinants containing duplex-II were identified by colony

Figure 3: Synthesis and assembly of the *S. pombe* secretion cassette components.

(A) Sequence of oligomers which assemble to form duplex-I. (B) Sequence of the final secretion cassette showing junction of the re-engineered 54/1 promoter and the synthetic oligomers. Indicated are the postulated mRNA start and stop sequences (S1, S2, S3), the codons for the secretion signal amino acids (three letter codes) and the multiple cloning site (duplex-II) sequences, (C) dot blot detection of *E. coli* colonies containing recombinant plasmid with assembled secretion cassette (arrow indicates clone chosen for subsequent work).

hybridization using 5'-labelled MCS1 as a probe (Figure 3C). *SmaI* digestion of putative recombinants was used to identify the correct duplex-II construct, since the *SmaI* site in the plasmid pUCD1 is reconstituted only if duplex-II has been inserted in the correct orientation. Subsequent restriction enzyme digestion with those enzymes having sites unique to the duplex-II sequence, confirmed sequence fidelity. The plasmid was designated pAP1. Plasmid pAP1 was digested with *NlaIII* and the protruding 3'-overhang was made blunt ended using T₄ DNA polymerase. Further digestion with *SphI* released the assembled 257 bp fragment containing the *S. pombe* acid phosphatase secretion signal, multiple cloning site and transcriptional stop codons (see Figure 3B).

The 1.2 kbp 54/1 promoter (*SphI*--> blunt end) fragment and the 257 bp synthetic secretion signal fragment (blunt end--> *SphI*) were blunt-end ligated using T₄ DNA ligase to produce a 1.46 kbp fragment flanked by *SphI* restriction sites. The structure of the resulting fragment, from position 1230 of the original 54/1 promoter, is illustrated in Figure 3B. This fragment was recovered by ligation with plasmid pIRT2Δ which had been previously digested with *SphI* and treated with CIAP. Recombinant *E. coli* transformants containing the expression cassette were identified by the rapid screening procedure of Sekar, (1987). Mini prep DNA was prepared from putative recombinants for DNA sequencing. Dideoxy sequencing of putative Duplex I transformants confirmed the sequence fidelity of the assembled Duplex I oligomers (Figure 4). The orientation of the expression cassette was determined by restriction endonuclease double digests using the enzymes *EcoRI* and *BamHI*. The

Figure 4: Dideoxy-sequence data from a segment of the synthetic signal sequence in plasmid pUCDI.

Autoradiogram shows nucleotide sequence ladder that spans the *Sma*I and *Sph*I sites.

Smal
CCCGGCTGAGCGTTGGCACAACGACGGAAAAAGCCAAGGAATAAATTTGCAAGAACAT
TGAACGCTACCTTAAAAAGTTGTAGGAATCACTAAAAGTTTGGTTGTAACCAAAGAAGCAG
AGTCCGAACATGAGACCTTAAATACCACGGTAGACCATCAATTTCTCATCAAAAACCATGG

Spb1

presence of a 1.4 kbp *EcoRI-BamHI* fragment after agarose (1 %) gel electrophoresis of the digest confirmed the orientation of the cassette. The organization of the resulting expression plasmid, designated pEX1, is given in Figure 5.

(d) Isolation and cloning of the *B. subtilis* endoglucanase structural gene in plasmid pEX1.

Plasmid pMUT31.1a provided by Hefford and Seligy (1991) was digested to completion with *PstI* and the 1600 bp fragment containing the endoglucanase structural gene was recovered after electrophoresis through 1 % agarose and purified using GeneClean™. Pertinent structural features of the original construct are given in Figure 6. The gene was cloned into plasmid pEX1 by ligation into the unique *PstI* site located in the synthetic multiple cloning site using T₄ DNA ligase. Putative *E. coli* recombinants were identified by assaying for the endoglucanase phenotype i.e. halos around colonies on CMC containing plates (data not shown). Presence of a 1600 bp fragment corresponding to the cloned endoglucanase fragment was confirmed by *PstI* digestion of plasmid DNA isolated from putative recombinants using the rapid plasmid isolation procedure of Sekar (1987). This plasmid was designated pEX21E.

Figure 5: Organization of expression plasmid pEX1.

The plasmid is capable of replication in *E. coli* (ORI) and *S. pombe* (ARS1) with selectable markers for ampicillin resistance (Ap) and leucine prototrophy (LEU 2), respectively. The f1 origin of replication (f1-ORI) permits recovery of single-stranded DNA on co-infection with a helper phage in *E. coli*. The multiple cloning site (MCS) contains unique restriction endonuclease sites for *Bam*HI, *Pst*I, *Pvu*II, *Sal*I, *Sma*I and *Sst*I. 54/1 and PHO indicate *S. pombe* specific promoter and acid phosphatase secretion signal.

Figure 6: Structural features of the *Bacillus subtilis* endoglucanase gene.

(A) Restriction map of the 3.1 kbp *Pst*I fragment containing the endoglucanase structural gene. Site-directed mutagenesis (A -> T) at position 817 (M.A. Hefford and V.L. Seligy, unpublished data, 1991) generates a *Pst*I site at the C-terminus of the secretion signal and the N-terminus of the endoglucanase structural gene at position A₃₉ (Ala). The large unfilled arrow pointing to the right indicates the starting position and polarity of the mRNA transcript. The hatched segment represents the 29-amino acid secretion signal which ends at Ala₂₉. The location of the 500 bp *Acc*I/*Mlu*I portion of the structural gene used as a gene probe in this study is also indicated. Abbreviated codes used to identify codons for specific restriction site positions are (P) *Pst*I, (H) *Hind*III, (Av) *Ava*II, (Ac) *Acc*I, (E) *Eco*RI and (Mu) *Mlu*I. Abbreviated codes used to identify amino acid positions are M (methionine), A (alanine) and N (asparagine). Small black arrows indicate position and orientation of two flanking open reading frames. The ⊥ and ♀ symbols indicate positions of rho⁻ transcription terminator and stop codons, respectively. (B) Restriction map of the 13.6 kbp plasmid pBTI1.6 containing the *Bacillus subtilis* endoglucanase gene (EG). A 2.8 kbp *Hind*III fragment containing the EG gene was obtained from a pUC8 plasmid and ligated with *Hind*III (partial digest) cut pBTI-1 cosmid vector (11.1 kbp) as described by Seligy et al., 1987. This plasmid contains the LEU2 gene for selection in yeast, the β-lactamase (A') and tetracycline resistance genes (T') for selection in *E. coli* and 2μ yeast plasmid sequences for high copy replication in *S. cerevisiae*. The 200 bp λ cos sequence can be used to direct assembly of cosmid constructs (> 36 kbp) into bacteriophage particles.

B) Endoglucanase gene detection and stability.

S. pombe was transformed with plasmids pBT11.6 (Seligy et al., 1987) and pEX21E according to the procedure of Beach et al., (1982). Both plasmids were capable of transforming a *leu*⁻ host (SP1) to leucine prototrophy. Calculated transformation frequencies for pBT11.6 and pEX21E were 3×10^3 and 4.5×10^5 transformants/ μ g of DNA, respectively.

Colony hybridization analysis, using the 500 bp *AccI*-*MluI* fragment of the endoglucanase gene as a probe (see Figure 6), revealed the presence of the endoglucanase gene in 45 out of 48 (94%) and 47 out of 48 (98%) of individual colonies examined from the pBT11.6 and pEX21E transformations, respectively. Attempts to rescue plasmid DNA from the pBT11.6 isolates were unsuccessful. Plasmid DNA was rescued from the pEX21E transformants, however, and *S. pombe* cells transformed with recovered plasmid were shown to express the endoglucanase phenotype.

Total DNA was prepared from SP1 and isolates SP1-E30, E42, E48 and E50, four putative pBT11.6 transformants which exhibited the endoglucanase phenotype. The DNA was digested to completion with *HindIII* and examined by Southern hybridization employing the 500 bp *MluI*-*AccI* fragment, from the coding region of the endoglucanase gene, as a probe. This probe hybridizes to the structural endoglucanase gene exclusively (see Figure 6). As is evident in Figure 7, all transformants examined exhibited a 2.8 kbp fragment, which hybridized to the probe.

Figure 7: Southern hybridization analysis of pBT11.6 *S. pombe* transformants.

Total DNA (10 μ g) was digested to completion with HindIII, electrophoresed through 1 % agarose, transferred to nitrocellulose and probed with the [32 P]-labelled 500 bp *MluI*-*AccI* fragment of the endoglucanase structural gene. Isolates are as follows: SP1 untransformed control (a) and pBT11.6 transformants, isolate number E30 (b), E42 (c), E48 (d) and E50 (e). The 2.8 kbp fragment indicated in the margin corresponds to the size expected for the fragment containing the endoglucanase gene.

Additional hybridization bands were also present possibly due to incomplete digestion products, concatenation or integration of the plasmid into *S. pombe* chromosomal DNA. No hybridization signal was observed in the SP1, non-transformed control, (see lane a) indicating that the hybridization signal was due to the presence of the endoglucanase gene. Southern analysis (Figure 8) of *Pst*I digested plasmid DNA rescued from pEX21E transformants revealed a 1.6 kbp fragment which hybridized to the probe. This fragment corresponds to the size expected for the endoglucanase structural gene.

Data on plasmid stability (Figure 9) indicated a rapid loss of plasmid under non-selective conditions (presence of leucine in the medium) in the case of pBT11.6 transformants which was not observed with cells transformed with plasmid pEX21E.

C) Endoglucanase expression studies in *S. pombe*

The transformants were examined for the production of extracellular endoglucanase by employing the Congo Red plate assay. As shown in Figure 10, all pEX21E transformants tested produced zones of clearing on Congo Red assay plates. pBT11.6 transformants produced little if any clearing.

Cells from 100 ml cultures of *S. pombe* transformants were examined according to the procedure of Rouwenhorst et al. (1988), as described in methods, to locate enzyme activity. The assay results (Figure 11) indicate that the endoglucanase activity was almost exclusively cell wall associated in the case of pEX21E

Figure 8: Southern hybridization analysis of pEX21E transformed *S. pombe*.

Plasmid DNA (1 μ g) isolated according to the procedure of Hoffman and Winston (1987) was digested to completion with *Pst*I, electrophoresed through 1% agarose and stained with ethidium bromide. (a) pEX1 and (b) pEX21E. The DNA was transferred to nitrocellulose and probed with the [32 -P]-labelled 500 bp *Mlu*I/AccI endoglucanase probe. Autoradiography demonstrated that pEX21E (d) had a 1.6 kbp band hybridizing to the probe whereas pEX1 (c) did not.

←1.6

c d

Figure 9: Determination of plasmid stability in *S. pombe* transformants.

S. pombe transformed with either pEX21E or pBT11.6, were grown in the presence and absence of leucine for 50 generations. The % LEU⁺ phenotype was determined as follows: number of viable LEU⁺ cells grown under selective conditions (i.e. in the absence of leucine) per number of viable cells grown under non-selective (i.e. in the presence of leucine) X 100%. Symbols: *S. pombe* SP1 containing plasmid pEX21E (--- ■---) or pBT11.6 (—+—).

Figure 10: Congo Red plate assay.

64

Individual *S. pombe* colonies transformed with either pEX1 (1A), pBT11.6 (2A) or pEX21E (1B to 1F, 2B to 2F) were toothpick transferred to PM agar plates containing 0.1 % CMC and the appropriate amino acids and were incubated for 48 hours at 30°C. The colonies were washed off the plates with sterile distilled water, stained with 1 mg/ml Congo Red and destained with 1 M NaCl to visualize clearing zones. Isolates containing pEX21E showed clearing zones, indicating hydrolysis of the CMC substrate. The negative pEX1 transformed control (1A), showed no clearing zone as expected. The pBT11.6 (isolate E30) containing cells (2A) produced a negligible clearing zone.

a b c d e f g

1

2

Figure 11: Cellular location of endoglucanase activity.

Aliquots (25 μ l) of the (a) culture supernatant, (b) wall bound and (c) wall associated fractions from 100 ml cultures of either pBT11.6 (Row 1) or pEX21E (Row 2) transformed *S. pombe* SP1 were spotted on GelBond films of 0.1 % CMC substrate in 25 mM potassium phosphate buffer pH 6.6 solidified with 1 % agarose and incubated for 4 hours at 37°C. The films were stained for 15 minutes at room temperature with Congo Red and destained by several washes in 1 M NaCl. pBT11.6 transformants showed zones of clearing only in the wall bound (1b) fraction whereas pEX21E transformants showed activity in both the wall bound (2b) and the wall associated (2c) fractions. There was no detectable activity associated with the supernatant fractions from either pBT11.6 or pEX21E isolates, 1(a) and 2(a), respectively.

transformants and wall bound in the case of pBT11.6 transformants. In order to determine the type of interaction which existed between the enzyme and the cell wall, 100 ml cultures of the pEX21E containing cells were prepared and broken with glass beads. Duplicates of the broken cell preparations were extracted and sonicated in 250 μ l of buffer as follows: (i) 50 mM Tris-Maleate pH 7.6, 5 mM EDTA $\pm$ 0, 0.25M, 0.5M, 1.0M NaCl and 1 mM PMSF, (ii) 50 mM Tris-Maleate pH 5.0, 6.0, 7.0, 8.0 $\pm$ 0.1% Triton X-100 (v/v), (iii) 50 mM Tris-Maleate pH 7.6, 5 mM EDTA $\pm$ 0.1% (w/v) deoxycholic acid (Sigma). Treatments varying either pH or molarity had no effect on releasing endoglucanase from the cells. In fact, enzyme activity was lost between 0.25 and 0.5 M NaCl. Activity could be restored after dialysis against extraction buffer. Treatment with deoxycholate however, was effective in releasing enzyme activity from the cell wall fraction indicating association of the endoglucanase enzyme with cell membranes.

Western analysis (Figure 12) of total protein from pEX21E transformants indicated a 35 kDa band which associated with the anti-endoglucanase antibody but which was not present in the untransformed control. An additional band with a molecular mass of approximately 32-33 kDa was also present and constituted the major protein product detectable by the anti-endoglucanase antibody. Recent work by Hefford et al., (1991) to determine the C-terminal boundary of the primary sequence of the *B. subtilis* PAP115 endo- β -1,4-glucanase required for stable catalytic activity, suggest that this degradation product should also be active. Electrofocusing (Figure 13) indicated that

Figure 12: Western analysis of total cellular protein from *S. pombe* transformants.

Aliquots (15 μ l) of total protein were electrophoresed through a 5% stacking - 15% separating polyacrylamide gel and transferred electrophoretically onto nitrocellulose membrane. The endoglucanase associated protein was detected using a rabbit anti-endoglucanase antiserum as a probe and was visualized with alkaline phosphatase-conjugated anti-rabbit IgG using NBT/BCIP as substrate. Lane (a) is a 35 kDa control purified from *E. coli* containing a 33 kDa degradation product. A 35 kDa band was detectable in both the pEX21E (c) and pBT11.6 (d) isolates that was not present in the pEX1 control (b). An apparent 33 kDa degradation product is present in both pEX21E (c) and pBT11.6 (d) extracts and judging from the relative intensities appears to be the major product formed in both yeasts.

35 kDa

a

b

c

d

Figure 13: Isoelectric point comparison of endoglucanase produced in pEX21E and pBT11.6 transformed cells.

One μ l of total cellular protein was applied to duplicate Pharmacia PhastTM IEF (pH 5-8) gels and was electrophoresed. (A) Polyacrylamide gel stained with Coomassie Brilliant Blue and (B) zymogram (incubated in contact with the IEF gel at 37°C for 4 hours) with stained with 1 mg/ml Congo Red and destained with 1 M NaCl. Samples (a) pI markers (top to bottom: 4.65, 5.1, 6.0, 6.5, 7.0, 8.2, 8.4, 8.6, 9.6) and proteins isolated from *S. pombe* transformed with (b) pBT11.6, (c) pEX1 and (d) pEX21E. A band corresponding to a pI of 6.4 is visible in lanes B(b) and B(d) corresponding to the pBT11.6 and pEX21E extracts, respectively. This band is not present in the pEX1 control lane B(c). The majority of proteins in lanes b-d of (A) are acidic in nature based on comparison with the pI markers.

A

a

b

c

d

B

b

c

d

the endoglucanase protein isolated from the pBT11.6 and pEX21E transformants have a similar pI of 6.4. The pEX21E transformants seem to be producing either more of or a more active form of endoglucanase as judged by the relative differences in the sizes of halos when equivalent amounts of total protein extracted under identical cultural conditions are examined.

Yeast containing pEX21E were able to direct the endoglucanase to the cell wall but no significant activity was detectable in the cell free medium. Results from reducing sugar assays (Somogi, 1952) indicated a 100-fold difference in activity in pEX21E containing cells when compared with cells harbouring pBT11.6 (0.1U vs 0.001U where one unit (U) = 1 μ g glucose released per minute at 42°C). Several attempts were made to obtain N-terminal sequence of the gel purified endoglucanase to determine whether the secretion signal was processed correctly. All efforts proved negative due to apparent blockage of the N-terminus. In one case, N-terminal analysis revealed a yeast ubiquitin-like sequence (Met-Glu-Ile-Phe-Val..) which could not be purified away from the endoglucanase activity.

IV. Discussion

In this thesis a plasmid vector, pEX1, containing a *S. pombe* promoter, an *S. pombe* secretion signal for the extracellular acid phosphatase protein, and a multiple cloning site (MCS) with unique cloning sites was constructed.

Plasmid pEX1, makes use of a fusion between a constitutive *S. pombe* promoter and a *S. pombe* secretion signal to direct protein expression and effect protein export. It is novel in that it is the only expression vector of this type for this yeast developed to date. pEX1 contains selectable markers for both yeast and *E. coli* and unique *Sma*I, *Xho*I, *Bam*HI, *Pvu*II, *Pst*I and *Sal*I cloning sites within the MCS region downstream of the promoter-secretion signal fusion. The presence of the f1-ORI should also permit production of single-stranded DNA on co-infection with helper phage as was demonstrated by Vernet et al, (1987) with expression vectors developed for *S. cerevisiae*. This feature however, was not confirmed. The potential utility of a synthetic *Schizosaccharomyces pombe* expression cassette was examined with respect to its ability to effect the expression and direct the export of a *Bacillus subtilis* endo- β -(1,4)-glucanase gene. When compared to the native gene and its controlling elements, differences were observed in the fate of the transforming DNA as well as in the cellular destination of the encoded protein product as determined by Western and protein activity analysis.

Construction of the vector pEX1 was undertaken in 4 stages: (i) modification of the plasmid pIRT2 to generate plasmid pIRT2 Δ , (ii) isolation and modification of the

S. pombe 54/1 promoter, (iii) synthesis and assembly of the acid phosphatase secretion signal coding sequences and (iv) assembly of the secretion cassette by ligation into plasmid pIRT2Δ to create the expression plasmid pEX1.

The *B. subtilis* endoglucanase gene used as the reporter, was isolated as a 1.6 kbp *Pst*I fragment from plasmid pMut31.1a. This fragment contains the endoglucanase structural gene lacking its native promoter and secretion signal sequence (Hefford and Seligy, 1991). Insertion of the endoglucanase gene into the unique *Pst*I site within the MCS of the expression plasmid pEX1, created plasmid pEX21E which was used to examine the utility of pEX1 as a secretion vector for use in *S. pombe*. For comparative purposes, plasmid pBT11.6 (Seligy et al., 1987) containing the native *Bacillus* promoter, secretion signal and structural gene was used. The plasmids differ from each other in several fundamental properties.

E. coli transformed with pEX21E demonstrated the endoglucanase phenotype. This would suggest that some portion of the *S. pombe* promoter and signal sequence apparently permits expression and export from *E. coli*. *E. coli* transformed with pBT11.6 had previously been shown to express the endoglucanase phenotype (Seligy et al., 1987). This observation was confirmed in the present study.

Both plasmids were shown to be capable of transforming both *E. coli* and *S. pombe* respectively to ampicillin resistance and to leucine prototrophy. However there were observed differences in yeast transformation efficiencies. For example, pEX21E was the most efficient transforming plasmid by two orders of magnitude (4.5×10^5 vs. 3.0×10^3 transformants per microgram). This increased frequency is thought to be

due in part, to the presence of an *S. pombe* ARS1 sequence in pEX21E although differences in the size of the two plasmids (pBT11.6, 8.3 kbp; pEX21E, 10.3 kbp) may also have played a role. ARS sequences have been shown to promote high transformation frequencies in *S. pombe*. In addition, *S. cerevisiae* ARS function has been shown to be only partially conserved in *S. pombe* which might also help to explain the lower pBT11.6 transformation frequencies.

Colony hybridization analysis of putative pBT11.6 and pEX21E transformants using the 500 bp *MluI*-*AccI* endoglucanase probe, confirmed the presence of the gene in both sets of yeast transformants. Southern analysis of total DNA digested to completion with either *HindIII* (pBT11.6) or *PstI* (pEX21E) revealed bands hybridizing at 2.8 kbp and 1.6 kbp, respectively. The additional hybridization bands present in the pBT11.6 transformants were possibly due to integrated, concatemeric or rearranged plasmid. Preliminary data using clone E30 suggest that pBT11.6 may have integrated into chromosome III (data not shown). Sakai et al., (1984) have speculated that intensive recombination activity is intrinsic to *S. pombe* and that polymerization of plasmids occurs at fairly high frequency. This possibility can not be ruled out at this point in the study. An integration mechanism has been suggested to partly explain the replication and expression of heterologous genes lacking *S. pombe* ARS sequences in this yeast (Russell, 1989). Southern hybridization analysis of chromosomal blots seemed to indicate that there might be endoglucanase sequences associated with chromosome III, however due to the weak hybridization signal, this was not definitively established. If chromosomal integration did occur, the copy number

appears to be low. The observation that pBT11.6 plasmid could not be rescued by *E. coli* from these transformants lends some credence to the integration conjecture.

After transformation of *S. pombe* with the plasmids pBT11.6 and pEX21E, putative transformants were examined for their ability to express and secrete the endoglucanase protein. Transformants were initially screened for extracellular endoglucanase by plating isolates onto CMC-containing agar followed by staining with Congo Red. Only 4 of the 250 pBT11.6 transformants examined were observed to produce halos. These halos were not apparent after serial transfers of the four putative halo (+) producing clones. Further examination of the clones for active intracellular endoglucanase revealed that only the E30 clone was able to consistently test positive for endoglucanase activity after the cells had been broken open. The initial observations showing putative extracellular enzyme expression may have been due to unstable expression and leakiness of the cell wall due to cell lysis or death. Unstable transient expression of the *B. subtilis* endoglucanase gene has been observed in the yeasts *S. cerevisiae* and *S. alluvius* (Seligy et al., 1987). It is possible that the transformants carried a transcriptionally inactive form of the endoglucanase gene indicating possible modifications to the gene resulting from replication in the yeast.

By contrast, pEX21E transformants were consistently able to produce halos on indicator plates. The pBT11.6 data would suggest that *S. pombe* is able to recognize, to some degree, the endoglucanase promoter, but it may be unable to either correctly initiate protein synthesis or utilize the *B. subtilis* secretion signal sequence to export the protein through the cell wall. However, Rouhonen et al., (1987) were able to

demonstrate secretion of a *B. amyloliquifaciens* α -amylase using its native signal sequence in the yeast *S. cerevisiae*. The active, secreted α -amylase was glycosylated and its signal peptide was apparently processed. This indicates that in the yeast *S. cerevisiae*, a prokaryotic signal sequence has been shown to lead to genuine secretion. The normal secretory pathway from the endoplasmic reticulum to Golgi and via secretory vesicles to the cell surface was utilized. A partial signal peptide was non-functional. Similarly, two different *Trichoderma reesei* cellobiohydrolases, CBHI and CBHII, carrying their native signal sequences under the control of the *S. cerevisiae* (PGK) promoter from the phosphoglycerate kinase gene were produced in this yeast in an enzymatically active form and were secreted into the culture medium (Penttilä et al., 1988). The membrane bound endoglucanase enzyme produced in the pBT11.6 transformants could be trapped in the endoplasmic reticulum, Golgi apparatus or vesicles.

Protein localization studies suggest that the activity associated with the pBT11.6 transformants was membrane associated, that is, cytoplasmic. The activity associated with the pEX21E transformants is primarily associated with the cell wall. Little activity was detected in the cell free supernatant. This observation is somewhat surprising since *S. pombe* secretes up to 40% of its native acid phosphatase protein as a cell free form (i.e. into the growth medium). This secretion is directed by the *S. pombe* acid phosphatase secretion signal used in this study.

The observation that active endoglucanase was able to be dissociated from the cell wall only by deoxycholate and not by ionic alteration indicates a rather tight

association between the protein and the cell wall. This suggests that there may also be structural features inherent to the endoglucanase protein that are not amenable to efficient secretion in *S. pombe*. Studies with the *Bacillus subtilis* endo- β -1,4-glucanase gene by Lo et al., (1988) in *E. coli*, indicated that a stable, enzymatically active 35 kDa species of the enzyme was made and could be isolated. Results presented indicated that a large proportion (70%) of the endoglucanase activity was found extracellularly, in contrast to similar studies by Koide et al., (1986) and Robson and Chambliss (1986) which found only 25% and 1%, respectively. It was suggested by Lo et al., (1988) that there may be an unidentified property associated with the PAP115 strain of *B. subtilis* endoglucanase that gives rise to this altered excretion. The primary sequence of these two *B. subtilis* endoglucanases differ by 5 - 8% in comparison to PAP115, the enzyme used here. Seligy et al., (1987) in their studies with the *B. subtilis* endo- β -1,4-endoglucanase in *E. coli*, and the yeasts *S. cerevisiae* and *S. alluvius* suggest that the gene is processed differently in each cell type. Unfortunately the secretory pathway in *S. pombe* has not been elucidated and as a result no comment can be made on the apparent inability of *S. pombe* to utilize heterologous protein secretion sequences which have been functional in other yeasts. The analysis of the secretory pathway in *S. pombe* has just started and some important areas of study such as secretion and glycosylation mechanisms, remain virtually untouched (Yamamoto, 1989).

The protein secretory signals recognized by *S. pombe* differ from that of *B. subtilis* secretion signal used in this study. Acid phosphatase like the *Bacillus*

endoglucanase, has a signal peptide sequence that is normally removed during secretion. The cleavage site is between amino acid 18 and 19. The 18-amino acid signal peptide is structurally similar to other eukaryotic N-terminal secretion signals (Elliott et al., 1986) in that it has a 9-amino acid stretch of hydrophobic amino acids that ends 4 amino acids from the cleavage site. The sequence Ala-Asn-Ala which occurs immediately before the native acid phosphatase cleavage site is similar in structure to the Ala-X-Ala sequence found by Perlman and Halvorson, (1983) to be the most frequent sequence preceding the signal peptidase cleavage site. The 29-amino acid *B. subtilis* endoglucanase signal sequence is typical of most secretion signals; it has a 6-residue hydrophilic amino-terminus followed by an 18-residue hydrophobic core region containing several Leu and Ile residues just before the signal peptide cleavage site of Ala-Ser-Ala₂₉ (MacKay et al., 1986). The 31 amino acid signal peptide of the *Bacillus* α -amylase is cleaved after Ala₃₁ of the sequence Thr-Ser-Ala. There may be sufficient structural dissimilarity between the signal peptides of bacteria and yeast that would lead to inefficient signal processing in *S. pombe*. Since mutation or deletions of amino acids in the N-terminal portion of the endoglucanase leads to dramatic loss of activity (V. Seligy, unpublished data) detection of activity in *S. pombe* indicates some processing near or at Ala₃₀ must have taken place.

To gain more information about how the endoglucanase was being expressed in *S. pombe*, purification and analysis of the enzyme product was attempted. The endoglucanase protein was purified to homogeneity, as judged by a single band by

reducing and non-reducing PAGE and isoelectric focusing. Numerous attempts were made thereafter to obtain N-terminal amino acid sequence of the isolated protein in order to determine if the signal sequence of the endoglucanase protein was processed correctly. This lack of success may be due to a possible blockage of the N-terminus, perhaps by a yeast ubiquitin or due to the low concentration of endoglucanase produced. Amino acid sequence data corresponding to the N-terminus of a yeast ubiquitin was obtained when PAGE purified active endoglucanase protein was used as substrate for amino acid sequencing. Damaged and otherwise abnormal proteins are metabolically unstable. Recent biochemical and genetic evidence by Varshevsky et al., (1989), indicates that covalent conjugation of ubiquitin to short-lived intracellular proteins is essential for their selective degradation. The inability to detect free extracellular endoglucanase activity is a question which will require further study.

The development of expression vectors for use with *S. pombe* is only now beginning to evolve. The only reported examples to date have been based on the sequences of the *S. pombe* alcohol dehydrogenase gene promoter and the *S. pombe* 54/1 promoter. Expression of the alpha-1 antitrypsin and both the *E. coli* β -galactosidase and the *Pseudomonas putida* catechol 2,3-dioxygenase was effected in *S. pombe* using these two promoters, respectively. The pBK02 construct (Kudla et al., 1988) employing the 54/1 promoter requires co-transformation with a helper plasmid due to its lack of a selectable auxotrophic or resistance marker.

It has been shown here that the designed construct was able to effect expression of the heterologous protein as well as to partially direct the expressed protein to the

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cell's exterior. Western blots suggest that the 35 kDa endoglucanase product produced is similar to the most stable product produced in *E. coli* and *B. subtilis* as determined by Lo et al., (1988). Product sizes of 55, 43, 31 and 25 kDa were also evident. According to site-directed mutagenesis studies by Hefford et al., (1991) and Seligy (unpublished data) loss of amino acid sequence at either the N- or C-terminus (shorter than amino acid 295), would result in an inactive protein. The 25 and 31 kDa species would not be expected to be active.

No enzymatically active high molecular weight species were detected by zymogram or Western blot analysis which could have been indicative of N- or O-linked glycosylation of the protein. Interestingly, there are two potential N-linked glycosylation sites (Asn-X-Ser/Thr) within the predicted endoglucanase sequence at positions Asn₁₀₅-Pro₁₀₆-Ser₁₀₇ and Asn₂₉₂-Leu₂₉₃-Ser₂₉₄. N-linked glycosylation of bacterial proteins with potential N-glycosylation sites has been observed in *S. cerevisiae* in studies by Rouhonen et al., (1987) and Curry et al., (1988) using *B. amyloliquifaciens* α -amylase and a *Cellulomonas fimi* exoglucanase, respectively. Both glycosylated proteins retained their enzymatic activity. It is also interesting to note that the secretion of the *B. amyloliquifaciens* α -amylase was effected by a construct containing the native *Bacillus* secretion signal but not by similar constructs containing a partial or complete removal of the secretion signal. The limited information available on secreted proteins in *S. pombe* indicate that they may undergo glycosylation in a manner similar to *S. cerevisiae*. Acid phosphatase is a cell-surface glycoprotein that has been shown to be synthesized as a membrane-bound precursor,

which is processed to a membrane-bound, partially glycosylated precursor which is in turn further glycosylated to the final secreted form of the enzyme. (Maundrell et al., 1985). It would appear that the degree of glycosylation may affect the cellular fate of the protein.

V. Conclusions

(1) An expression plasmid pEX1, has been constructed containing an engineered *S. pombe* secretion cassette (promoter, synthetic *S. pombe* acid phosphatase mRNA start signal, secretion signal sequence, and transcription termination sequences).

(2) The native *Bacillus subtilis* controlling elements were utilized by *Schizosaccharomyces pombe* to direct synthesis of the endoglucanase protein.

(3) The vector pEX1 was able to effect both the expression and export of the reporter gene product.

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