Chapter 6.

Isolation and characterisation of mutations which affect the regulation of the extracellular proteases of *A. nidulans*.

6.1. Background.

The precise mechanism by which the extracellular protease genes are regulated is currently unknown. Gene products which mediate wide-domain regulatory responses including carbon catabolite repression, nitrogen metabolite repression, and response to environmental pH have been identified in *A. nidulans* (see section 1.2.). Less is known about sulphur metabolite repression in *A. nidulans*. Four genes, *sconA*, *sconB*, *sconC* and *sconD*, which affect sulphur metabolite repression have been identified (Natorff *et al.* 1993). Though mutations in these genes result in derepression of enzymes in the sulphur assimilation pathway, they did not result in derepression of the extracellular proteases (Katz *et al.* 1996). The response of the extracellular proteases to sulphur limitation is not mediated by any known gene.

6.2. Rationale and aims.

With its well characterised genetics and amenability to molecular analysis, the extracellular proteases of *A. nidulans* present an ideal model system in which to study gene regulation and protein secretion in a eukaryotic organism, using a classical genetic approach. In contrast to the approach described in section 5.2., where the structural genes of a system are used as a means of gaining information regarding regulatory genes, an alternative approach is to identify regulatory genes directly. One way of achieving this end, is the use of mutagenesis to identify genes which effect the expression of structural genes in the system of interest. Therefore identification of putative regulatory genes by mutagenesis was also pursued in this project as an alternative approach to the study of the regulation of the extracellular proteases of *A. nidulans*.

6.3. Results.

6.3.1. The effects of the *sB1* mutation on the production of extracellular proteases.

In *A. nidulans* there are two forms of sulphate transporter encoded by different genes. Strains carrying the *sB1* mutation grow poorly on minimal medium. With the addition of 1% thiosulphate *sB1* strains are phenotypically normal with regards to growth. Strains carrying the *sB1* mutation are deficient in sulphate permease, resulting in impaired uptake of sulphate which is the sulphur source present in minimal medium. The second transporter, capable of suphate uptake, is able to, at least partially, compensate for the *sB1* mutation when high levels of exogenous sulphate are present. When grown on medium where milk is the sole sulphur source, strains carrying the *sB1* mutation are unable to take up the sulphate present in the medium, and therefore produce extracellular proteases in response to sulphur metabolite repression. When both milk and thiosulphate are the sulphur source, the alternative transporter is able to partially compensate for the *sB1* mutation, this appears to result in partial sulphur derepression, as some extracellular protease is produced, resulting in the "fuzzy halo". This observation has allowed us to screen for mutants that effect the response to sulphur metabolite derepression.

6.3.2. The mutant screen.

6.3.2.1. Selection strategy.

As *sB1* mutants consistently produce a halo on media where milk is the sole sulphur source, it was decided to exploit this observation in the hope of isolating mutants whose response to sulphur limitation was affected. The ability to observe a partially sulphur derepressed phenotype allowed a screen to be designed in which mutations,

which resulted in either loss of the "fuzzy" halo or gain of a clear halo could be identified. To reduce the probability of isolating mutants in the already well characterised regulatory *areA* gene, it was decided to use a strain carrying the null allele *areA 19* as well as the *sB 1* allele as the parental strain in this mutagenesis screen. In order to obtain such a strain, strains MH205 (which carries the *areA 19* mutation) and MSF (which carries the *sB 1* mutation) were crossed. The phenotypes of the segregants from this cross are listed (see Table 6.1) (For full genotypes of strains see Table 2.2). It can be seen from the results (Table 6.1), that *areA 19* segregants don't grow very well on media where milk is the sole nitrogen source. The *areA 19* mutation results in a constitutively repressed phenotype with regards to nitrogen metabolite repression, consequently these strains are unable to utilise milk or alanine as a nitrogen source. The segregants carrying both the *areA 19* and *sB 1* mutations don't grow very well but produce a faint halo on media where milk is the sole nitrogen source, when thiosulphate was omitted. Strains of this phenotype are constitutively repressed with

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Table. 6.1. Growth and extracellular protease production of segregants from MH205 x MSF. ANM = *A. nidulans* minimal media (contains 1% glucose as a carbon source, salts and trace elements). Media which contained milk also contained sodium deoxycholate to induce compact colony morphology and increase the visibility of halos surrounding the colonies which is due to the production of extracellular proteases. Full genotypes of strains are given in Table 2.2. +++ = strong growth, ± = poor growth

	PHENOTYPE ON DIFFERENT MEDIA				
Relevant genotype of segregants.	ANM + alanine + vitamins + thiosulphate	ANM + NH_4 + vitamins	ANM + milk + NH_4 + vitamins + thiosulphate	ANM + milk + NH_4 + vitamins	ANM + milk + vitamins
wildtype	+++	+++	+++ no halo	+++ no halo	+++ clear halo
sB 1	+++	±	+++ fuzzy halo	+++ clear halo	++ small halo
areA 19	±	+++	+++ no halo	+++ no halo	± no halo
areA 19 ;sB 1	±	±	+++ fuzzy halo	+++ clear halo	± very small halo

regards to nitrogen metabolite repression, but the *sB1* mutation results in derepression of extracellular proteases due to sulphur metabolite derepression. Presumably these strains are unable to grow as well as wildtype strains on medium containing milk as a nitrogen source because they are unable to use amino acids as a nitrogen source due to the *areA19* mutation. The *areA19 sB1* segregant which was used as the parental strain in the mutagenesis screen was designated strain MK130.

6.3.2.2. Mutagenesis and selection.

More than 20,000 colonies of strain MK130, which had been mutagenised by exposure to ultra-violet light (10% survival), were screened for the production of halos on solid media where milk was the sulphur source. On this medium the "fuzzy halo" phenotype of *sB1* mutants can be observed. As it was difficult to score for loss of halo on the initial spread plates, approximately 200 of these colonies were rescreened using the plate test. Any colony which appeared to produce less protease on the initial plate was rescreened. Eight of the 200 colonies rescreened using the milk plate assay showed reduced protease production, and were assayed for protease production in liquid culture under sulphur-limiting conditions. Two of the 8 strains produced negligible levels of protease in the liquid culture assay (fig 6.1.). Further characterisation was carried out on these two mutants, which have been designated PVK1 and PVK2. The mutations carried by strains PVK1 and PVK2 were designated *xprI1* and *xprJ1* respectively. No colonies that produced increased levels of protease activity were identified.

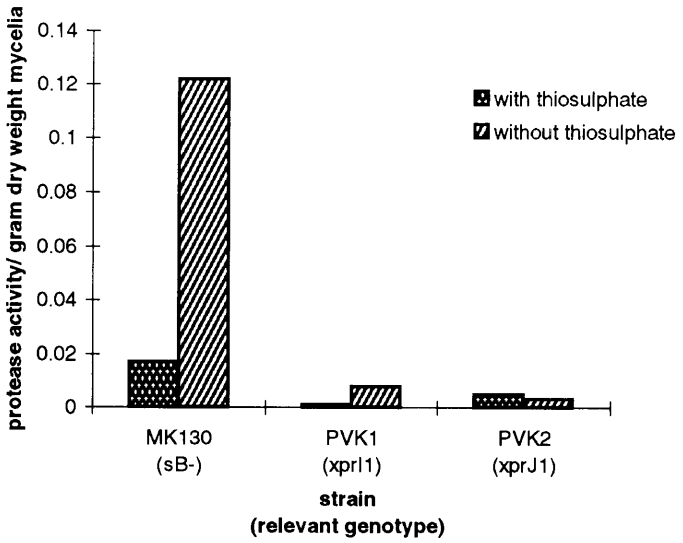

Figure 6.1. Results from the initial assay examining the effects of the mutations on the production of extracellular protease. These results show that strains carrying either the *xprI*$_1$ (PVK1) or *xprJ*$_1$ (PVK2) mutation produce negligible amounts of protease activity under sulphur derepressing conditions, and that both mutations suppress the *sB*$_1$ mutation with regards to protease production. Conidia were inoculated directly into sulphur repressing (1% glucose, 10 mM ammonium tartrate, 0.1% sodium thiosulphate) or sulphur derepressing (1% glucose, 10 mM ammonium tartrate) medium for 20 hours. All media used in this experiment was made from low-sulphur salt solution. The protease activity present in the culture filtrate was determined by assaying the degradation of casein at pH 7.2. The results of a single assay are shown. Full genotypes of the strains are given in Table 2.2.

6.3.3. Analysis of the *xprI1* mutation.

6.3.3.1. Haploidisation analysis.

A diploid strain was constructed from the original strain carrying the *xprI1* mutation and MH764 which carried complementary markers. Haploidisation analysis showed that the *xprI1* mutation was linked to both the chromosome II marker (*wA*$^+$) and the chromosome VII marker (*nicB8*) carried by the *xprI1* parental strain. 163 haploids were analysed and the parental combinations of the *wA*, *nicB* and *xprI* alleles were found in all of them, suggesting that the *xprI1* mutation was the result of a reciprocal translocation event between chromosomes II and VII (Table 6.2.).

6.3.3.2. Utilisation of alternative carbon, nitrogen, and sulphur sources by *xprI1* strains.

Strains carrying the *xprI1* mutation were tested on a variety of alternative carbon, nitrogen, and sulphur sources. Table 6.3. summarises these results. On all media tested the growth of the *xprI1* mutant was poorer than that of the wildtype controls. Though the *xprI1* colonies were smaller they were well conidiated. As the *xprI1* mutation did not affect the utilisation of any specific carbon, nitrogen, or sulphur source, it did not appear to produce pleiotropic effects with regards to the utilisation of alternative sources of carbon, nitrogen, or sulphur.

When grown on medium containing milk as the sole nitrogen source, *xprI1 areA*$^+$ strains did not produce a milk-clearing halo. Therefore, in addition to suppressing the response to sulphur metabolite derepression, it appears that the *xprI1* mutation also suppresses the response to nitrogen metabolite derepression. The effect of the *xprI1* mutation on carbon catabolite derepression was examined using protease enzyme assays (see section 6.3.3.3).

Table 6.2. Haploidisation analysis to determine the chromosomal location of the *xprI* gene. One hundred and sixty-three haploids derived from a diploid, which was constructed from strains PVK1 and MH764, were analysed to determine the linkage group to which the *xprI* gene belonged. The chromosome marker scored for chromosome I was yA_1. Due to epistatic effects, the genotype at this locus could not be scored in the wA_1 background. As all *xprI*+ segregants were wA_1, they could not be scored for the chromosome I marker (designated n/s = not scorable, on the Table).

Chromosome number	parental strain which carried the marker(s)	marker gene(s)	Number of *xprI*+ haploids	Number of $xprI_1$ haploids
I	PVK1 markers	yA_1 $su\text{-}adE_{20}$ adE_{20}	n/s	38
	MH764 markers	*yA*+ *su-adE*+ *adE*+	n/s	25
II	**PVK1 marker**	***wA*+**	**0**	**63**
	MH764 marker	**wA_1**	**100**	**0**
III	PVK1 marker	$areA_{19}$	39	38
	MH764 marker	*areA*+	61	25
IV	PVK1 marker	$pyroA_4$	33	31
	MH764 marker	*pyro*+	67	32
VI	PVK1 marker	sB_1	31	28
	MH764 marker	*sB*+	69	35
VII	**PVK1 marker**	**$nicB_8$**	**0**	**63**
	MH764 marker	***nicB*+**	**100**	**0**
VIII	PVK1 markers	$niiA_4$ *facB*+ *riboB*+	34	18
	MH764 markers	*niiA*+ $facB_{101}$ $riboB_2$	66	45
total number of haploids			100	63

Table 6.3. Growth of wildtype, *xprI*$_1$ and heterozygous diploid strains on a variety of carbon, nitrogen, and sulphur sources. The effect of the *xprI*$_1$ mutation was examined in haploids with a *sB+* genotype, the diploid used was heterozygous at the *sB* locus.

Media.			Relevant Genotype.[1]		
Carbon source	**Nitrogen source**	**Sulphur source**	***xprI*$^+$**	***xprI*$_1$**	***xprI*$^+$/*xprI*$_1$**
1% glucose	10 mM ammonium tartrate	0.1% thiosulphate	++++++	+++	+++++
0.5% glycerol	10 mM ammonium tartrate	0.1% thiosulphate	++++	++	++++
1% ethanol	10 mM ammonium tartrate	0.1% thiosulphate	++++ [2]	++	+++
50 mM GABA	10 mM ammonium tartrate	0.1% thiosulphate	++++++	++	+++++
50 mM acetate	10 mM ammonium tartrate	0.1% thiosulphate	+++++ [2]	+++	+++++
50 mM proline	10 mM ammonium tartrate	0.1% thiosulphate	+++++	++	++++
50 mM acetamide	10 mM ammonium tartrate	0.1% thiosulphate	++++ [2]	+	++
1% skim milk	10 mM ammonium tartrate	0.1% thiosulphate	++++++ no halo	+++ no halo	++++++ no halo
1% glucose	10 mM sodium nitrate	0.1% thiosulphate	++++++ [3]	++++	++++++
1% glucose	10 mM alanine	0.1% thiosulphate	+++++	++++	+++++
1% glucose	10 mM uric acid	0.1% thiosulphate	++++++	++++	++++++
1% glucose	10 mM hypoxanthine	0.1% thiosulphate	++++++	+++++	++++++
1% glucose	10 mM GABA	0.1% thiosulphate	++++++	++++	++++++
1% glucose	10 mM proline	0.1% thiosulphate	++++++	++++	++++++
1% glucose	10 mM acetamide	0.1% thiosulphate	++++++	+++	++
1% glucose	1% skim milk	0.1% thiosulphate	++++++ halo	++++ no halo	++++++ halo
50 mM acetamide		0.1% thiosulphate	++++	+++	+++
50 mM proline		0.1% thiosulphate	++++++	++++	++++++
50 mM GABA		0.1% thiosulphate	++++++	++++	++++++
1% skim milk		0.1% thiosulphate	++++++ no halo	++ no halo	++++ no halo
1% glucose	10 mM ammonium tartrate	none	++++++	++++	++++++
1% glucose	10 mM ammonium tartrate	3 mM cysteine	++++++	++++	++++++
1% glucose	10 mM ammonium tartrate	3 mM methionine	++++++	+++	+++++

[1] Fungal strains used in this experiment were the *xprI*$^+$ strains, MH2 and MH97, and the *xprI*$_1$ strains, MK169 and MK170, and MK242 as the *xprI*$_1$ / *xprI*$^+$ diploid strain.

[2] MH97 grows poorly on media containing acetamide, acetate, or ethanol as carbon sources, due to the *acuE*$_{215}$ mutation it carries, therefore the MH2 phenotype was taken as the wildtype (*xpr*$^+$) phenotype on these media.

[3] MH2 is unable to grow on nitrate as the sole nitrogen source, therefore MH97 phenotype was taken as the wildtype (*xpr*$^+$) phenotype on this media.

6.3.3.3. Biochemical analysis of *xprI1* mutants.

In protease assays, strains carrying the *sB1* mutation, such as MK130, show high levels of protease activity in the filtrate obtained from cultures grown with or without 0.1% thiosulphate as a sulphur source (fig. 6.2.). When grown in sulphur-limiting conditions strains carrying the *xprI1* mutation produced negligible levels of extracellular protease, as measured by the degradation of azocasein (fig. 6.2.). The *xprI1* mutation suppressed the *sB1* protease phenotype in both sulphur-repressing and derepressing conditions (fig. 6.2.).

Strains carrying the *xprI1* mutation and no other marker affecting protease production were also examined for the effect of the *xprI1* mutation alone. It was found that the *xprI1* strains did not produce significant levels of extracellular protease in response to carbon, nitrogen, or sulphur limitation (figs. 6.2. to 6.4. and Appendices 8-10). It was also observed that on solid media containing milk as the sole nitrogen source, strains carrying the *xprI1* mutation did not produce a halo whereas wildtype control strains did (fig. 6.3.). Therefore, the *xprI1* mutation results in a constitutively repressed phenotype with regards to extracellular protease production in response to carbon-, nitrogen-, and sulphur-nutrient-limiting conditions.

The acid and alkaline phosphatases are secreted enzymes which are regulated in response to environmental pH. Therefore, the production of these enzymes by *xprI1* strains was studied as a means of determining if this mutation affected secretion or pH regulation. As the levels of both acid and alkaline phosphatase produced by *xprI1* strains was shown to be equal to, or greater than, that produced by the wildtype strain, the *xprI1* mutation does not appear to effect secretion. The level of both alkaline and acid phosphatase secreted by *xprI1* strains was compared to that of

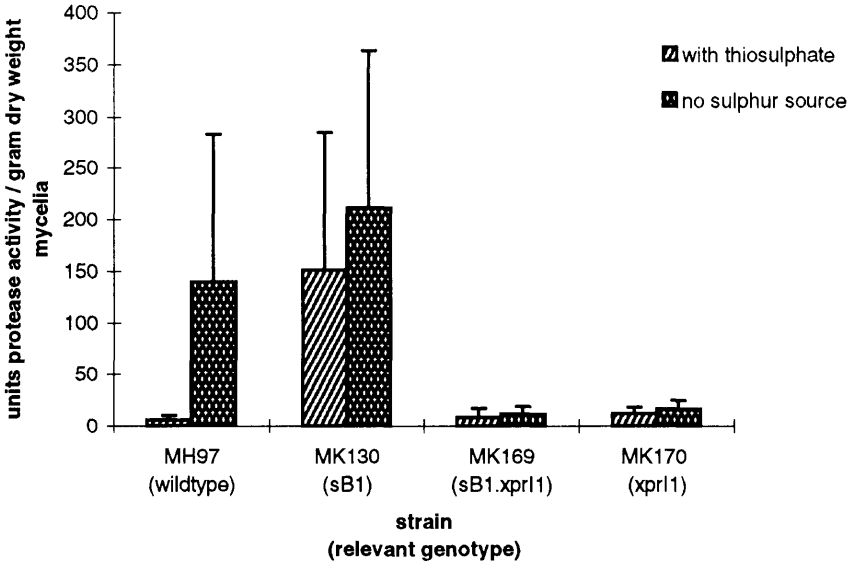

Figure 6.2. Protease assay comparing the response of the wildtype and mutant strains to sulphur-limiting conditions. These results show that strains carrying the *xprI1* mutation produce negligible amounts of protease activity under sulphur derepressing conditions, and that the *xprI1* mutation suppresses the *sB1* mutation with regards to protease production. Conidia was inoculated directly into sulphur repressing (1% glucose, 10 mM ammonium, and 0.1% thiosulphate) or sulphur derepressing (1% glucose, and 10 mM ammonium) medium for 20 hours. All media used in this experiment was made using low-sulphate containing salt solution. The protease activity present in the culture filtrate was determined by assaying the degradation of casein at pH 7.2. Protease activity was measured in arbitrary units. This assay was performed in triplicate. Raw data and analysis are contained in Appendix 9. The full genotypes of the strains are given in Table 2.2.

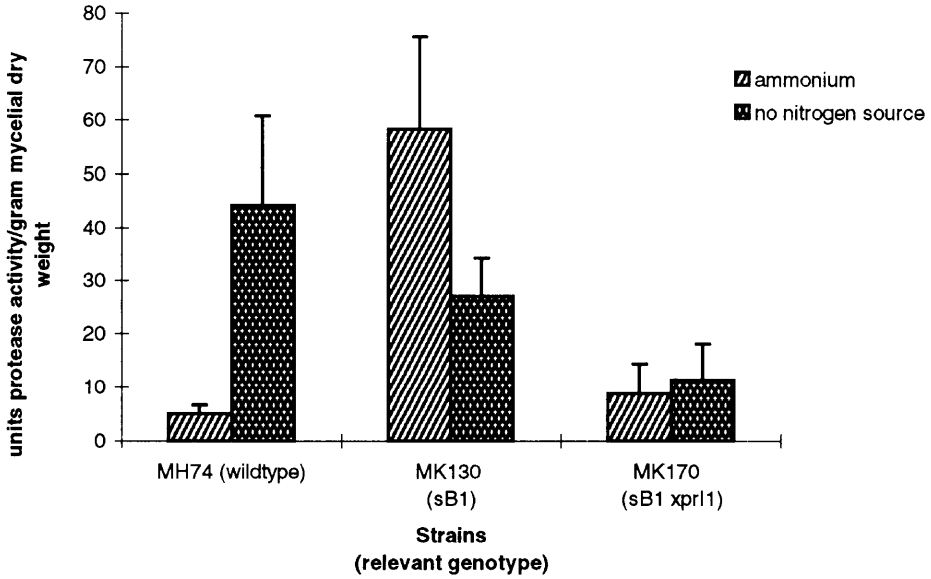

Figure 6.3. Protease assay comparing the response of the wildtype and mutant strains to nitrogen-limiting conditions. These results show that strains carrying the *xprI*$_1$ mutation produce negligible amounts of protease activity under nitrogen derepressing conditions. Conidia was grown for 16 hours in minimal medium (1% glucose, 10 mM ammonium, and 0.1% thiosulphate) prior to transfer to nitrogen repressing (1% glucose, 10 mM ammonium, and 0.1% thiosulphate) or nitrogen derepressing (1% glucose, and 0.1% thiosulphate) medium for 4 hours. All media used in this experiment was made using sulphate containing salt solution. The protease activity present in the culture filtrate was determined by assaying the degradation of casein at pH 7.2. Protease activity was measured in arbitrary units. This assay was performed in triplicate. Raw data and analysis are contained in Appendix 9. The full genotypes of the strains are given in Table 2.2.

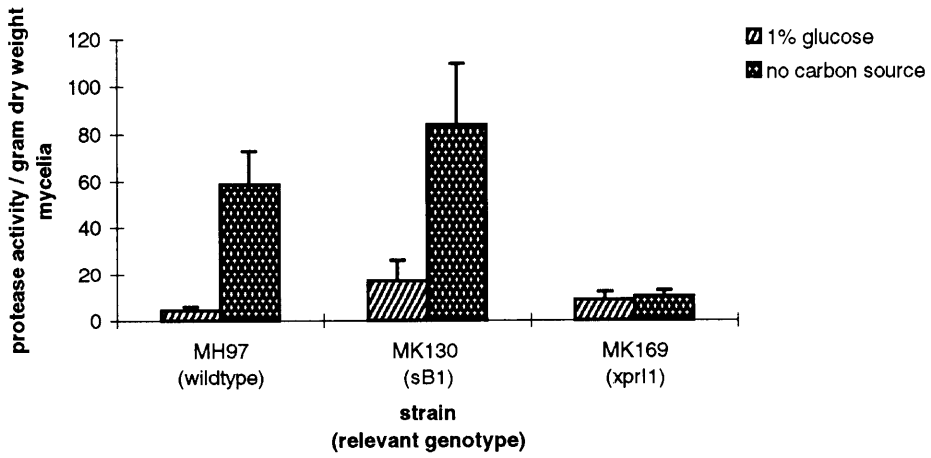

Figure 6.4. Protease assay comparing the response of the wildtype and mutant strains to carbon-limiting conditions. These results show that strains carrying the *xprI*$_1$ mutation produce negligible amounts of protease activity under carbon derepressing conditions. Conidia was grown for 16 hours in minimal medium (1% glucose, 10 mM ammonium, and 0.1% thiosulphate) prior to transfer to carbon repressing (1% glucose, 10 mM ammonium chloride, and 0.1% thiosulphate) or carbon derepressing (10 mM ammonium chloride, and 0.1% thiosulphate) medium for 16 hours. All media used in this experiment was made using sulphate containing salt solution. The protease activity present in the culture filtrate was determined by assaying the degradation of casein at pH 7.2. Protease activity was measured in arbitrary units. This assay was performed in triplicate. Raw data and analysis are contained in Appendix 10. The full genotypes of the strains are given in Table 2.2.

wildtype strains. A significant difference between *xprI*$_1$ and wildtype levels of secreted acid phosphatase, under phosphatase-repressing conditions, and alkaline phosphatase, under phosphatase-derepressing conditions, was observed (fig. 6.5. and Appendix 11). The increased levels of alkaline phosphatase produced under phosphate-derepressing conditions, and decreased levels of (predominantly alkaline) protease activity produced under protease-derepressing conditions, are not consistent with the *xprI*$_1$ mutation effecting pH regulation, but this possibility cannot be discounted.

6.3.3.4. Further genetic analysis of *xprI*$_1$.

6.3.3.4.1. Determination of the dominance relationship.

Diploid strains of the genotype *xprI*$_1$ /*xprI*$^+$ produce extracellular proteases when grown on plates where milk is the sole nitrogen source, indicating that *xprI*$_1$ is at least partially recessive with regards to its ability to suppress the extracellular proteases response to nitrogen derepression. The halo produced by the *xprI*$_1$ /*xprI*$^+$ strain is smaller than that produced by the *xpr*$^+$ /*xpr*$^+$ strain (fig. 6.6.). The difference in halo size may be due to incomplete dominance of the *xprI*$^+$ halo, or it could be due to the smaller size of the *xprI*$_1$ /*xprI*$^+$ colony. It has been observed that the most frequent class of mutations result in loss-of-function. If *xprI*$_1$ is a loss-of-function mutation in a regulatory protein, its recessive nature suggests that the *xprI* product functions in a positive manner.

6.3.3.4.2. Crosses to other mutations effecting extracellular protease production.

The *xprI*$_1$ mutation was shown to be linked to chromosome VII as are the *xprG* and *xprF* genes. To determine whether *xprI*$_1$ was an allele of *xprG*, an *xprI*$_1$ strain was

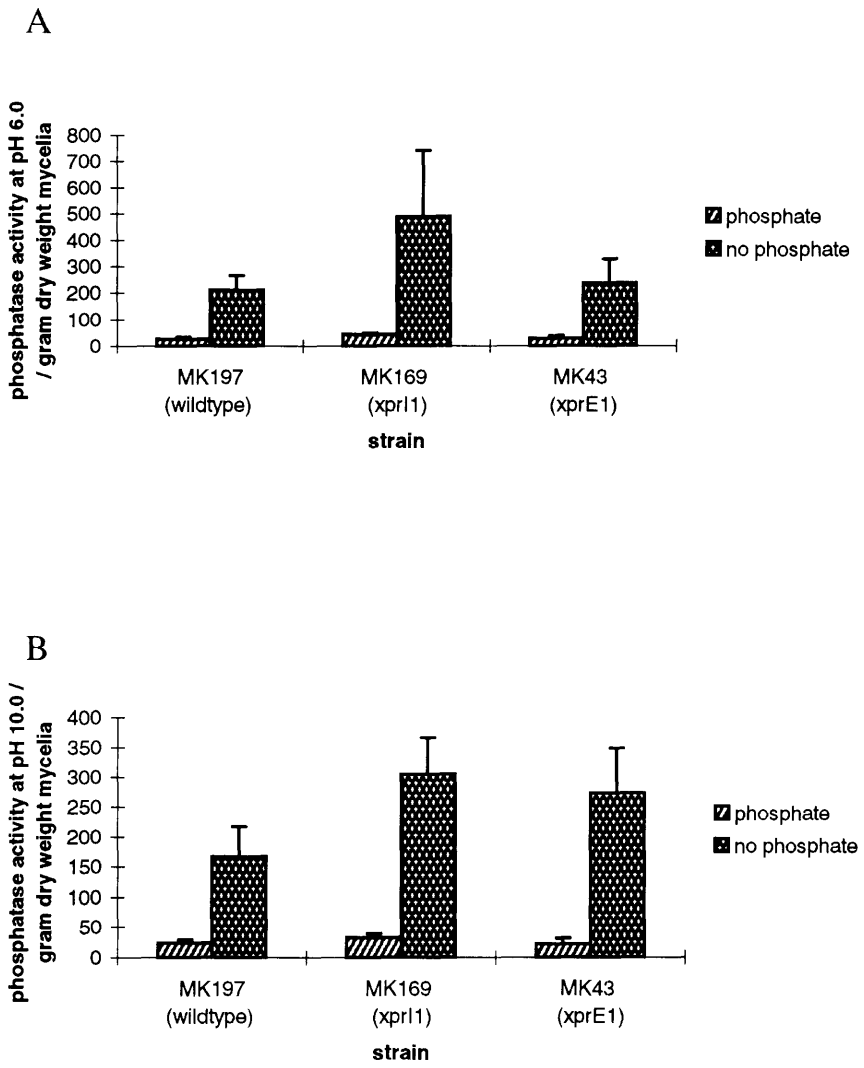

Figure 6.5. Phosphatase activity of mutant and wildtype strains at pH 6.0 (A) and pH 10.0 (B). These results show that the strains carrying the *xprl*$_1$ (MK169) or *xprE*$_1$ (MK43) mutations produce acid and alkaline phosphatase, indicating that these mutations do not effect secretion. Phosphatase activity was measured in arbitrary units. See Appendix 11 for raw data and statistical analysis. See Table 2.2. for full strain genotypes.

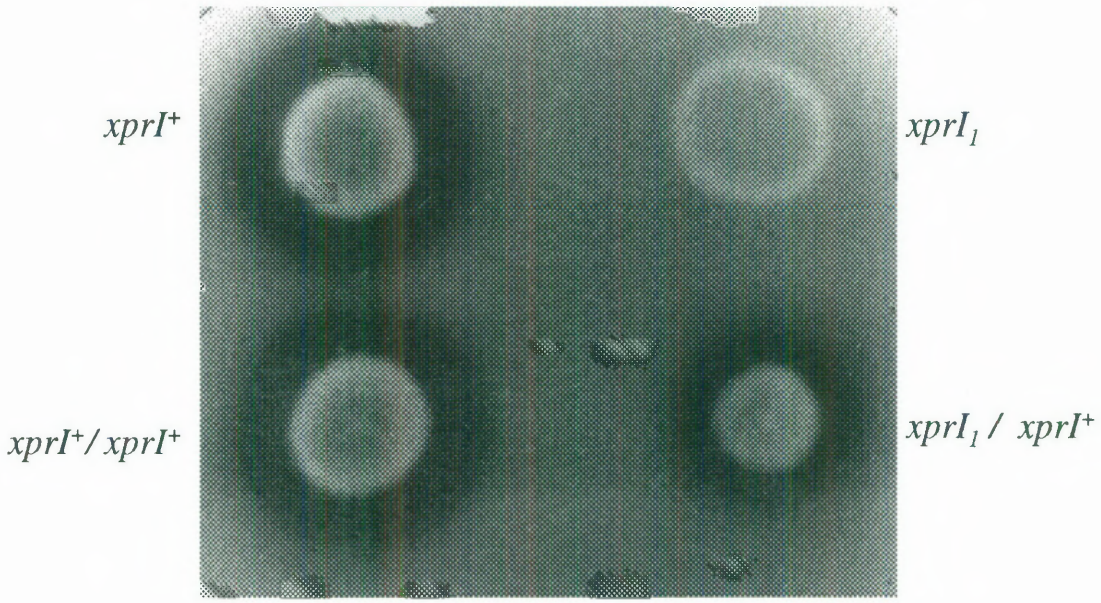

Figure 6.6. Growth on solid media containing milk as the sole nitrogen source. It can be seen that on media where milk is the sole nitrogen source that the wildtype haploid strain produces protease, while the *xprI*$_1$ strain does not. The wildtype diploid strain produces protease, as does the heterozygous diploid, showing that *xprI*$_1$ is recessive. The relevant genotypes are shown on the figure. Strains used were, left to right, top row *xprI*+ (MH2), *xprI*$_1$ (MK169), bottom row *xprI*+/*xprI*+ (MK241), *xprI*$_1$ /*xprI*+ (PVK14).

crossed to a strain carrying an allele of *xprG* (*xprG*$_2$) which results in a protease-deficient phenotype. If the two mutations were allelic all progeny would lack halos when grown on plates where milk was the sole nitrogen source. If the two mutations were in different genes, wildtype progeny, which would produce a halo when grown on plates where milk was the sole nitrogen source, would be expected. The proportion of wildtype progeny observed would depend on the linkage relationship between the two genes. The cross between a strain carrying *xprI*$_1$ and a strain carrying *xprG*$_2$ produced a high proportion (at least 1/4) of colonies which produced aerial hyphae and were colourless, though conidia could be seen when these colonies were viewed under a dissecting microscope. These colonies shall be referred to as "fluffy". Of 103 segregants analysed (64 normal and 39 "fluffy"), 29 (28.2%) produced halos when grown on milk as the sole nitrogen source. Statistical analysis indicated that these results did not differ significantly from what would be expected if *xprI*$_1$ and *xprG*$_2$ were unlinked (Appendix 12.). Therefore, these two mutations are in two unlinked genes. Supplementation of the media with adenine restored the ability to conidiate normally to only a small number of the "fluffy" segregants, and these segregants were excluded from the analysis. Of 32 "fluffy" segregants analysed, 16 produced halos when grown on plates where milk was the sole nitrogen source. This class of segregants is believed to constitute at least one class of partial aneuploid which are believed to have arisen due to the translocation mutation which is linked to, and likely to be the cause of the *xprI*$_1$ mutation (fig. 6.7.). "Fluffy" haploids were not recovered from the haploidisation experiment, probably because fluffy sectors in haploidisation analysis usually correspond to sectors which have not yet achieved a haploid state, and were therefore avoided. *xprI*$_1$ and *xprG*$_2$ were found to be unlinked. As more than 1/4 of the "fluffy" segregants produce extracellular proteases, when grown on medium containing milk as the sole nitrogen source, it seems likely that the "fluffy" segregants correspond to one of the classes of segregants with unbalanced chromosomes (see fig. 6.7.). The "fluffy" segregants which do produce protease must be *xprG*$^+$, carry a translocation chromosome, and carry a normal chromosome which carries the *xprI*$^+$

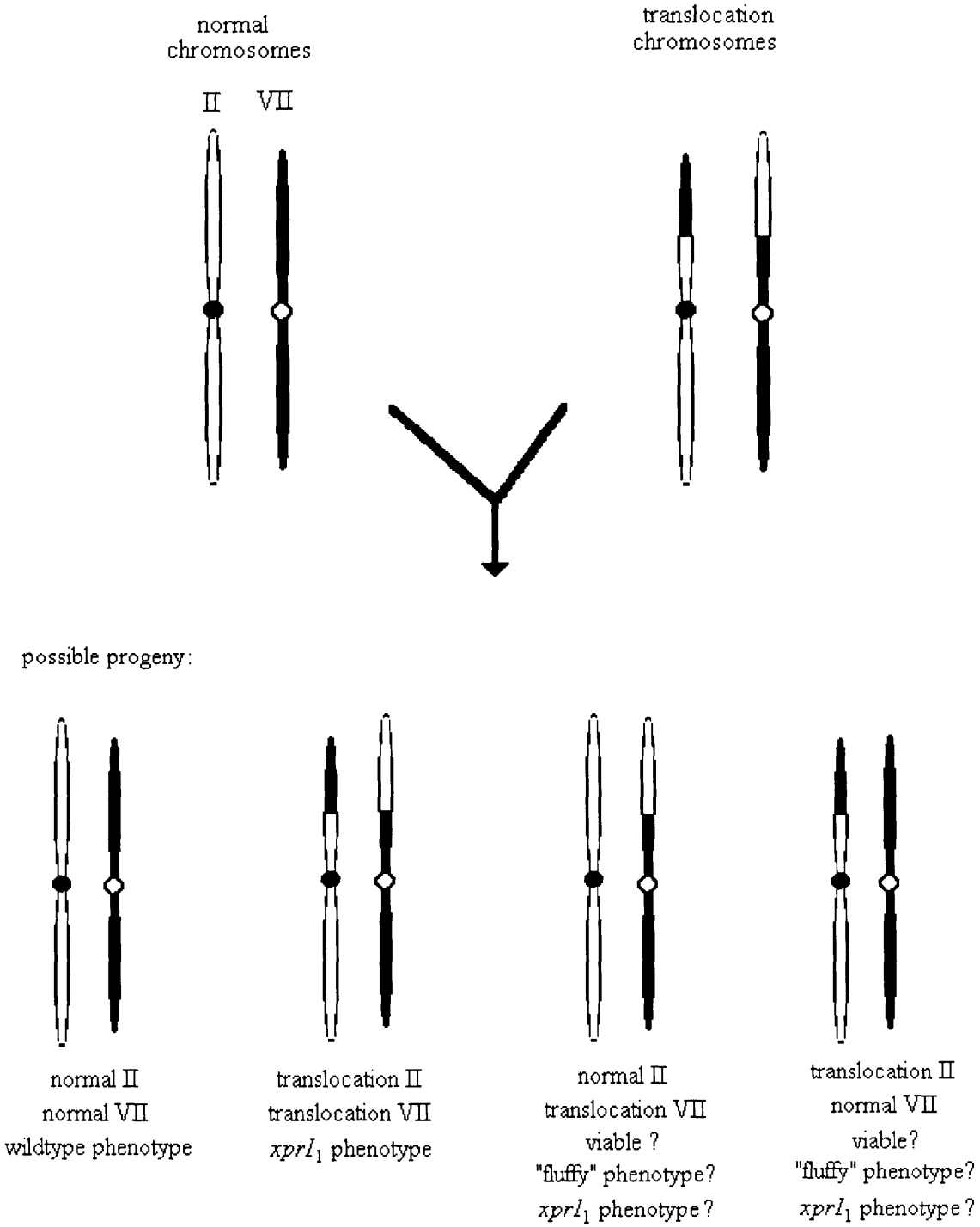

Figure 6.7. Schematic diagram showing how segregants with a "fluffy" phenotype may have arisen from a cross where one strain is carrying a balanced translocation. "fluffy" segregants were observed in a cross between MK170 and MK199. Neither haploid strain shows the "fluffy phenotype". Haploidisation analysis indicated that the *xprI1* mutation is linked to a translocation between chromosomes II and VII. It is thought most likely that one class of segregants carrying unbalanced chromosomes results in the "fluffy" phenotype.

allele. Assuming the *xprI*$_1$ allele is the result of the translocation event, and not just linked to it, "fluffy" segregants which produce protease could be used in haploidisation analysis, or pulse-field gel electrophoresis experiments, to determine whether chromosome II or VII carries the *xprI* gene. An *xprI*$_1$ strain could then be crossed to a suitable mapping strain to further localise the gene.

6.3.4. Analysis of *xprJ*$_1$.

6.3.4.1. Genetic analysis of *xprJ*$_1$.

Strains PVK2 and MH764 were used to construct a diploid. Very few *xprJ*$_1$ haploids were obtained from this diploid. This was believed to be due to the very weak growth displayed by the *xprJ*$_1$ mutant. The *xprJ*$_1$ mutation did not appear to be linked to any of the markers in the diploid, indicating that it is probably linked to the only chromosome for which the diploid did not carry a marker, chromosome V (Table 6.4). As no *areA*$^+$ *xprJ*$_1$ haploids were isolated, the effect of the *xprJ*$_1$ mutation on protease production under carbon-, and nitrogen-limiting conditions was unknown.

To determine whether the *xprJ*$_1$ mutation was able to suppress mutations which resulted in the production of extracellular proteases in levels higher than those produced by wildtype strains, a strain carrying the *xprJ*$_1$ mutation was crossed to strains carrying *xprF*$_1$, and *xprG*$_1$. In all three crosses, the *xprJ*$_1$ mutation did not segregate in a manner consistent with Mendelian genetics. To score the *xprJ*$_1$ mutations effect on protease production, segregants must also have the *sB*$_1$ mutation. Therefore in crosses heterozygous for the two genes one would expect 1/2 of the *sB*$_1$ segregants to also have the *xprJ*$_1$ mutation. In the cross to a *xprF*$_1$ strain 5 of 40 segregants had the *xprJ*$_1$ phenotype, and in the cross to a *xprG*$_1$ strain 4 of 33

Table 6.4. Haploidisation analysis to determine the chromosomal location of the *xprJ* gene. One hundred and seven haploids derived from a diploid, which was constructed from strains PVK2 and MH764, were analysed to determine the linkage group to which the *xprJ* gene belonged. The yA_1 phenotype can only be scored in a wA$^+$ background.

Chromosome number	parental strain which carried the marker(s)	marker gene(s)	Number of *xprJ*+ haploids	Number of $xprJ_1$ haploids
I	PVK2 marker	yA_1 *su-adE*$_{20}$ *adE*$_{20}$	35	6
	MH764 marker	*yA*+ *su-adE*+ *adE*+	29	2
II	PVK2 marker	*wA*+	41	8
	MH764 marker	wA_1	56	2
III	PVK2 marker	*areA*$_{19}$	13	6
	MH764 marker	*areA*+	84	4
IV	PVK2 marker	*pyroA*$_4$	3	4
	MH764 marker	*pyroA*+	94	6
VI	PVK2 marker	sB_1	27	10
	MH764 marker	*sB*+	70	n/s
VII	PVK2 marker	*nicB*$_8$	38	6
	MH764 marker	*nicB*+	59	4
VIII	PVK2 marker	*niiA*$_4$ *facB*+ *riboB*+	67	4
	MH764 marker	*niiA*+ *facB*$_{101}$ *riboB*$_2$	30	6
total number of haploids			97	10

segregants had the *xprJ1* phenotype (Appendix 13). Though few double mutants were obtained from these crosses, the *xprJ1* mutation appeared to suppress the *xprG1* mutation and not affect or be affected by the *xprF1* mutation. The aberrant results observed in the two crosses could be explained by a significant reduction in the viability of the *xprJ1* mutant compared to *xprJ*$^{+}$ strains, which correlates with the difficulty in recovering *xprJ1* mutants from the haploidisation. Non-Mendelian segregation of the *xprJ1* allele could also be due to the fact that the phenotype attributed to this mutation, is the result of more than one mutation, *i.e.* it is possible that the *xprJ1* phenotype is not a single gene effect.

6.3.5. Do any of the mutations which result in a reduction in protease production effect secretion?

An additional experiment was used to determine whether any of the protease deficient mutants isolated in our laboratory effected secretion. The level of protease activity present in mycelia and culture filtrate was compared under both repressing and derepressing conditions. If any of the mutations blocked secretion of extracellular proteases, I expected to see high levels of extracellular proteases present in extracts obtained from mycelia grown under derepressing conditions.

Four mutations (*xprE1*, *xprG2*, *xprG3*, *xprI1*) isolated in this laboratory render *A. nidulans* protease deficient. Mutations in one of the genes (*xprG*), can also result in an increased production of extracellular protease under derepressing conditions. The proteases present in the mycelia and culture filtrate of the four mutant strains, and one wildtype strain, were examined after growth under carbon-limiting and non-limiting conditions (fig. 6.8.). No proteolytic activity was observed in the culture filtrate of any strain grown on 1% glucose. Comparatively low levels of intracellular protease were detected in the mycelia of all strains. After 16 hours incubation in carbon-limiting growth conditions all strains showed an increase in the number of

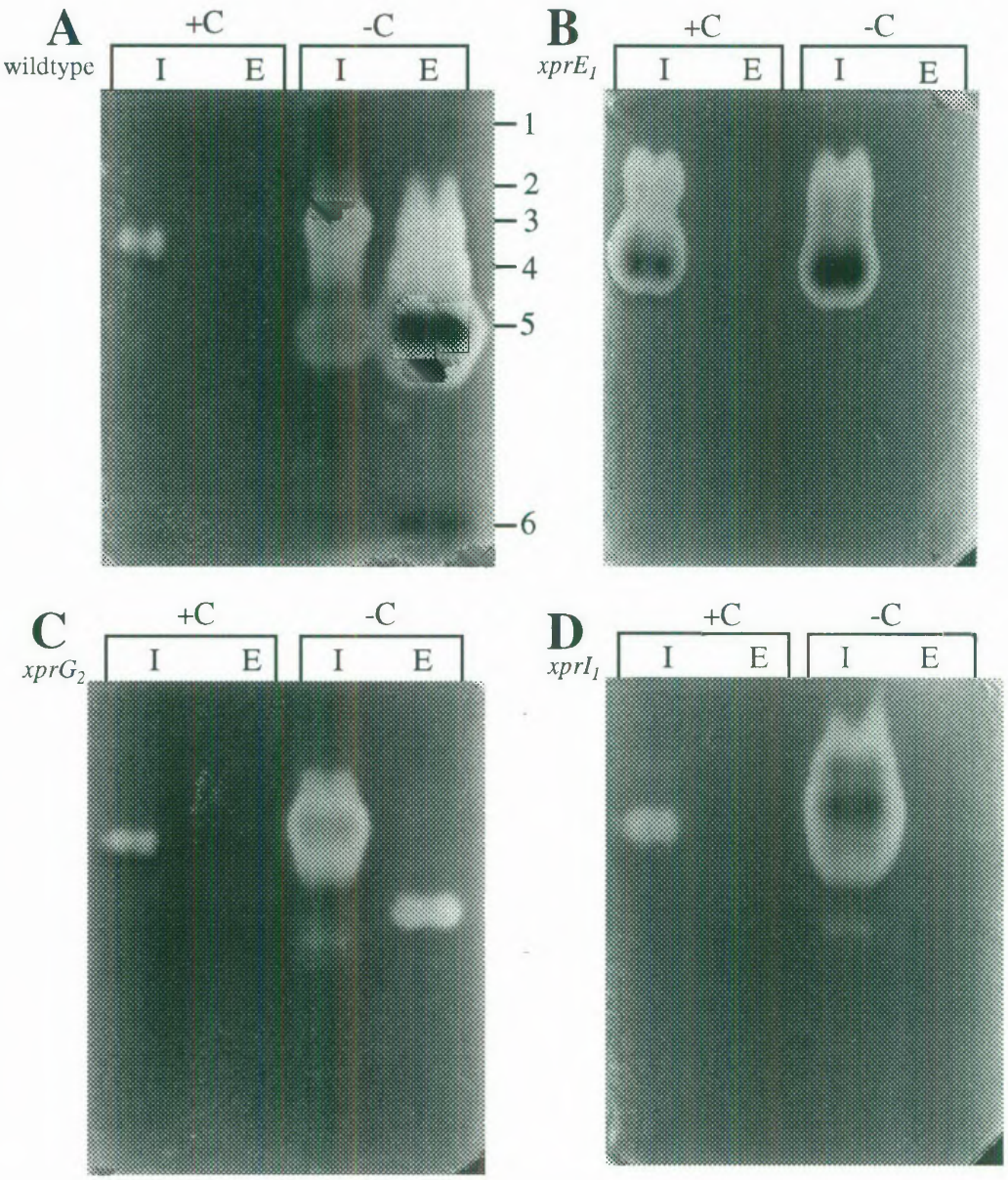

Figure 6.8. Comparison of proteases present in intracellular and extracellular samples obtained from strains grown under carbon repressed and carbon derepressed conditions. Strains shown are A. MH2 (wildtype), B. MK43 ($xprE_1$), C. MK199 ($xprG_2$) and D. MK169 ($xprI_1$). All samples contained 4% of the total volume of filtrate (intracellular or extracellular) obtained from each strain. The result for MK200 ($xprG_3$) was almost identical to MK199 ($xprG_2$) and is not shown. I = intracellular, E = extracellular, +C = carbon repressed, -C = carbon derepressed. The proteases found in the culture filtrate from wildtype grown under carbon derepressed conditions are labelled as in Chapter 3. The mycelium dry weights for each culture are as follows: MH2 +C = 795 mg, -C = 315 mg; MK43 +C = 456 mg, -C = 187 mg; MK199 +C = 390 mg, -C = 361 mg; and MK169 +C = 390 mg, -C = 153 mg.

bands of proteolytic activity observed in mycelia. In the mutants the bands were predominantly allozymes of the intracellular serine protease, though bands known to correspond to the serine extracellular proteases were faintly visible in some mutants and clearly visible in the wildtype/control mycelial samples. All four *A. nidulans* proteases were clearly visible in the culture filtrate sample of the wildtype strain. The *prtA* gene product was the predominant band observed in the culture filtrate of strains carrying the $xprG_2$ and $xprG_3$ mutations, after growth in carbon-limiting conditions. In the wildtype strain (fig. 6.8.A.), the intracellular sample contains PrtA predominantly in the form with slower electrophoretic mobility. It can be seen in figure 6.8.C. that the PrtA allozyme present in mycelial extract from the $xprG_2$ strain, MK199, has a greater electrophoretic mobility than the PrtA allozyme observed in the extracellular sample from this strain. It is unknown why the PrtA allozyme of faster mobility is not observed in the intracellular sample obtained from the wildtype strain after incubation in carbon-derepressing conditions. Cohen noted that enzyme γ (PrtA), occurs as an active precursor, designated δ, which showed greater electrophoretic mobility. It is probable that the PrtA allozyme which is less electrophoretically mobile is a glycosylated form of PrtA. No regions of proteolytic activity were observed in the culture filtrate samples obtained from the strains carrying $xprI_1$ or $xprE_1$ mutations grown in carbon-limiting conditions.

6.4. Discussion.

In this study I have isolated two mutations, which affect the production of extracellular protease in *A. nidulans*. The $xprI_1$ mutation is defective in its response to carbon-, nitrogen-, and sulphur-nutrient-limiting conditions, resulting in what appears to be a constitutively repressed phenotype. Experimental evidence suggests that the $xprI_1$ mutation affects the regulation of the extracellular proteases.

The limited characterisation of the mutation designated *xprJ1* tentatively positioned this gene (or genes) on chromosome V. The initial protease assay (fig. 6.1.) showed that this mutation suppressed the extracellular protease phenotype of the *sB1* mutation. Non-Mendelian segregation of the *xprJ1* mutation may be due to decreased viability of strains carrying the *xprJ1* mutation. Alternatively, the phenotype attributed to this mutation may be due to mutations in more than one gene. Before any further characterisation of this mutant is conducted, it is important to establish whether this phenotype is attributable to a single gene.

Detection of the proteolytic activity in extracts run on 1D native PAGE was used to examine the possibility that the mutants with reduced levels of protease production, due to the *xprE1*, *xprG2*, *xprG3*, or *xprI1* mutations, were defective in the secretory pathway. It was observed in the intracellular samples from wildtype strains, that both the number and intensity of the bands increased after 16 hours incubation in medium lacking a carbon source. Though a similar increase in the intensity of the intracellular protease bands was observed for all the mutants examined, bands attributable to the extracellular proteases did not appear or appeared very faintly in the intracellular extracts of these mutants. The increase in intracellular protease upon carbon derepression would seem to indicate that the signal transduction pathway involved in sensing carbon derepression is functioning in all the mutant strains, as the intracellular protease responds to the change in nutritional status. It was anticipated that a mutant with a blockage in the secretory pathway would show elevated levels of both intracellular and extracellular proteases in the intracellular sample obtained after growth in carbon-limiting conditions. No result corresponding to these expectations was obtained, indicating that it is unlikely that any of these mutations result in a blockage of the secretory pathway. The response of the intracellular protease indicates that (at least some of) the factors which mediate the response to carbon-limiting conditions differ between the extracellular and intracellular proteases. The degree of extracellular protease production in response to carbon limitation varied. The *xprE1*

mutation produced no detectable level of extracellular protease. A low level of the extracellular serine protease, PrtA, was detected in the intracellular sample of the *xprI1* mutant, and in the intracellular and extracellular samples of the *xprG* alleles tested. This suggests that the *xprG* alleles, and to a lesser extent the *xprI1* mutation, are leaky.

It has been established that the bands of extracellular protease activity observed, using PAGE and milk overlays, are the products of at least four different *A. nidulans* genes (section 3.3.4.). Protease gene-disruption mutants have been obtained for a number of extracellular protease genes in a number of different species, including the aspergillopepsin A gene of *A. niger*, and the serine protease gene of *A. fumigatus*. Such experiments resulted in a 15-20% reduction in the extracellular protease activity of the *A. niger* mutant compared to wildtype (Berka *et al.* 1990, Mattern *et al.* 1992), and an almost total loss of elastase activity in the *A. fumigatus* mutant when compared to wildtype (Tang *et al.* 1992). Disruption of the major protease gene of *A. nidulans*, *prtA*, resulted in almost total loss of extracellular protease activity, as detected by degradation of azocasein, under nitrogen- (4 hours) or sulphur- (20 hours) nutrient-limiting conditions, but 37-65% of the wildtype protease levels after 16 hours growth under carbon limiting conditions (section 5.3.3.). Therefore it is unlikely that the negligible protease production under carbon-, nitrogen-, and sulphur-limiting conditions seen in a *xprI1* mutant could result from a mutation in a structural protease gene of *A. nidulans*.

The *xprI1* mutation did not effect the utilisation of any other carbon, nitrogen, or sulphur source tested, nor did this mutation effect secretion. The absence of pleiotropic effects due to this mutation indicates that the *xprI1* gene product is not required for any general metabolic process.

The *sB1* mutation results in a non-functional sulphate transporter. Unless the media on which strains carrying this mutation are grown contain organic sulphur-containing

compounds, such strains exhibit high levels of extracellular protease which is attributable to sulphur derepression. In addition to suppressing the response to both carbon and nitrogen limitation, *xprI1* suppresses the *sB1* phenotype with regards to extracellular protease production, resulting in a constitutively repressed phenotype. The (at least partially) recessive nature of the *xprI1* mutation suggests that if this gene does code for a regulatory protein it is likely to be a positive-acting one and required for the expression of the extracellular protease genes under carbon-, nitrogen-, and sulphur-nutrient limiting conditions. It has been proposed that the DNA-binding protein product of the *pacC* gene may possess a role such as this in the mediation of pH regulation (Tilburn *et al.* 1995). While the *xprI1* mutation is the result of a translocation between chromosomes II and VII, the *pacC* gene is located on chromosome VI. It has been postulated that there are other genes (including *palA*, *B*, *C*, *F*, *H*, *I*) which are involved in mediating the signal produced in response to alkaline environmental pH (Tilburn *et al.* 1995). The *palF* gene is on chromosome VII (Dorn 1965). Mutations in this gene have been described as acidity mimicking, and as such would be expected to constitutively repress the expression of alkaline protease genes, such as those detected in the culture filtrate of *A. nidulans*. Unfortunately the effect of mutations in the *pal* genes on the production of extracellular proteases has not been examined. It has been shown that nitrogen starvation overrides repression by external pH, in the case of the *prtA* gene of *A. nidulans*, and the *pepA* gene of *A. niger* (section 4.3.7.2., Katz *et al.* 1996). Therefore it would seem unlikely that mutations in genes involved in pH regulation would result in the constitutive repression of the extracellular proteases genes. In addition, the *xprI1* mutation appears to have opposing effects on the production of alkaline phosphatase and alkaline proteases, which is not consistent with a role for *xprI* in pH regulation. Taking this into consideration, it seems more likely that if *xprI* encodes a regulatory protein, it is a regulatory protein specific to the extracellular protease production pathway.

Chapter 7.

General Discussion

7.1. Summary.

At least four proteases, which can be distinguished on the basis of electrophoretic mobility and susceptibility to inhibitors, were found in culture filtrate from *A. nidulans* mycelia grown under conditions of carbon-, nitrogen-, or sulphur-limitation (section 3.3.3.). Three of these proteases are serine proteases. The other is a metalloprotease. One of the serine proteases, appears to be produced in a predominantly mycelium bound manner.

All four proteases were produced under carbon-, nitrogen-, and sulphur-nutrient limiting conditions. Filtrate from cultures starved for carbon for 16 hours contained easily detectable levels of all four proteases identified, while filtrate from cultures starved for nitrogen for 4 hours, or 20 hours for sulphur, contain predominantly the serine proteases corresponding to bands 5 and 6 (section 3.3.2.). It is unknown if the differences in the ratio of the proteases produced under the different conditions is a genuine reflection of differences in regulation. If regulation under all three nutrient-limiting conditions results in an identical ratio of proteases being produced, but this ratio changes over time, then my observations could be the result of each of the three nutrient-deficient conditions being the equivalent of sampling at different time points. The production of extracellular proteases after mycelia had been subjected to 4 or 24 hours of nitrogen-limitation showed that, proteases which were less prominent at 4 hours, accounted for a greater proportion of the protease activity present in filtrate from cultures which had been subjected to 24 hours starvation (section 5.3.3.). Proteolytic enzymes produced at levels below the threshold of detection, produced under different conditions (*e.g.* pH), or with poor substrate affinity for casein, may not have been detected. The methodology utilised in this study was geared towards identification of the major enzymes responsible for the proteolytic activity observed in the methods by which this system is currently studied, and appears to have achieved this end.

One of the serine proteases is found in mycelial extracts, indicating that its predominant location is mycelium bound. Under carbon-limiting conditions, the levels of the predominantly mycelium bound protease appear to increase (6.3.5.). Cohen (1973a) noted increased levels of the more active forms of the mycelium bound protease under carbon-, nitrogen-, sulphur-, or phosphorous-limiting conditions. It has been shown in *A. niger* that the steady state transcript levels of the mycelium bound proteases, *pepC* and *pepE*, remain constant under conditions which are limiting for either carbon, or nitrogen (Jarai *et al.*, 1994c). It is possible that the *A. nidulans* mycelium bound proteases are regulated in response to nutrient-limiting conditions, and the *A. niger* mycelium bound proteases are not. Alternatively it is possible that the regulation of the mycelium bound proteases in both species occurs post-trancriptionally, either at the level of translation or at the level of activation of protease enzyme-precursors.

Two gene-replacement mutants were generated by transformation of protoplasts. Biochemical analysis of the *prtAΔ* mutants showed that this enzyme accounts for 9/10, 4/5, and 1/2 the protease activity detected in culture filtrate obtained from sulphur-, nitrogen-, and carbon-limited cultures respectively (section 5.3.3.). The predominant protease, as determined by degradation of milk protein at pH 5.3, was found to be the product of the *prtA* gene (section 5.3.3.6.) Haploidisation analysis localised the *prtA* gene to chromosome V, and analysis of meiotic recombination localised it between the *lysE* and *hxA* genes (section 5.3.4.). As *A. nidulans* produces a number of extracellular proteolytic enzymes it was expected that the absence of any one such enzyme would not affect the phenotype of colonies grown on solid media where milk was the sole nitrogen source. This was true when the media was pH 6.5, but on media at pH 4.5 wildtype strains produced a visible halo, whereas the *prtAΔ* mutants did not (section 5.3.3.1.). This result indicated that the serine protease, PrtA, is produced and is functional below pH 6, and supports the RNA expression data which has shown that this gene is expressed under nitrogen starvation conditions, regardless of the pH of the medium (Katz *et al.*, 1996). The extracellular proteases of *A. niger* have been shown

to be regulated in response to environmental pH. Northern blot analysis has shown that expression of the *pepA*, *pepB*, and *pepF* acid protease genes, of *A. niger* is easily detected in RNA from cultures grown under acidic conditions, but not under some alkaline conditions (Jarai and Buxton, 1994, van den Hombergh *et al.*, 1994). However, RT-PCR analysis of the *A. niger pepA* gene has shown that under nitrogen-limiting conditions this gene is expressed in culture medium buffered to pH 3 and pH 8 (section 4.3.6.1). Thus, my results indicate that the response to nitrogen starvation conditions overrides regulation in response to environmental pH in both *A. nidulans* and *A. niger*.

Examination of the promoter region of the *prtA* gene has revealed the existence of a region, of approximately 100 bp, that is highly conserved in the promoter regions of *Aspergillus* extracellular serine proteases (section 5.3.5.1.). Sequence specific DNA-protein interactions were observed in binding reactions containing, nuclear protein extracts, obtained from cultures subjected to 20 hours sulphur-repressing or derepressing conditions, and fragments containing this highly conserved region (HCR) (section 5.3.5.2.). This result suggests that sites for the binding of trans-acting factors involved in sulphur regulation are located in the HCR.

Though I was unable to detect the presence of an *A. nidulans* acid protease using biochemical methods (section 3.3.4.), molecular methods allowed the isolation of a gene with homology to the aspergillopepsin class of proteases (section 4.3.5.). This structural protease gene was designated *prtB*. Comparison of the putative amino acid sequence of PrtB showed that it is highly conserved with 70.7% identity to the putative *A. fumigatus* PepF aspergillopepsin. The two active site regions are conserved. Putative protein comparisons also showed that there is what appears to be an 8 amino acid deletion in the region prior to the second active site (section 4.3.6.1.). The region deleted from PrtB corresponds to part of an external β-sheet region of the 3D crystal structures of the homologous proteins from *P. janthinellum*, and *R. chenensis*. I was

not able to detect, using either Northern blot analysis or RT-PCR, the *prtB* transcript in RNA samples obtained from mycelia grown under nitrogen-limiting conditions (section 4.3.7.). The conditions used were suitable for the production of transcript from the serine protease encoding gene, *prtA*, as well as the *A. niger* aspergillopepsin, therefore the *prtB* gene may be expressed under different conditions (such as carbon-limiting conditions), or may not be expressed at all.

While isolation and analysis of the structural genes is a useful approach in the study of a system, isolation of mutants which affect regulation of the structural genes of a system is a more direct method in gaining knowledge of the factors involved in gene regulation. Ultimately both approaches are complementary. Therefore, a mutagenesis screen was also instigated as part of this project. It was observed that strains carrying the *sB1* mutation, which affects sulphate uptake from the environment, produce detectable levels of extracellular protease when grown on solid media where milk is present as a sulphur source (section 6.3.1.). On medium such as this, there is sufficient inorganic sulphur to suppress extracellular protease production by wildtype colonies. *sB1* mutants are unable to utilise the inorganic sulphur adequately and respond to the sulphur-limitation by producing extracellular protease. This observation was exploited as a means by which mutations affecting sulphur regulation of the extracellular proteases could be isolated. This strategy resulted in the identification of two mutations which affect production of extracellular protease. The mutations affecting the response to sulphur limitation were designated *xprI1* and *xprJ1*.

Haploidisation analysis showed that the *xprI1* mutation co-segregated with the mutant parental markers linked to chromosomes II and VII, suggesting that the phenotype of interest was caused by (or at least linked to) a translocation event (section 6.3.2.1.). Biochemical analysis showed that the *xprI1* mutation results in constitutive repression of the extracellular protease structural genes, *i.e.* negligible levels of proteolytic activity are detected in the filtrate of cultures grown under carbon-, nitrogen-, or

sulphur-nutrient-limiting conditions (section 6.3.3.3.). Biochemical analysis of the mycelium bound and extracellular proteases and the acid and alkaline phosphatases indicated that this mutation does not appear to affect secretion (section 6.3.3.3. and section 6.3.5.). Though the growth of the *xprI1* strain is reduced when compared to that of wildtype colonies grown on a variety of carbon, nitrogen, and sulphur sources, the ability to utilise any specific nutrient source tested did not appear impaired. These results suggest that the effects of the *xprI1* mutation are specific to the extracellular protease system. Genetic analysis also showed that *xprI1* is not allelic to the *xprG* gene which effects extracellular protease production and is located on chromosome VII (6.3.3.4.1.).

The *xprJ1* mutation has been tentatively localised to chromosome V. Preliminary genetic analysis of this mutation resulted in the observation of non-Mendelian segregation of the phenotype attributed to this mutation (section 6.3.4.). It is unknown if this is due to reduced viability of the *xprJ1* mutants, or to the fact that this phenotype being due to mutations in more than one gene.

7.2. Future direction for the study of the extracellular proteases of *A. nidulans*.

As I have determined that the four proteolytic enzymes easily observed in the filtrate of *A. nidulans* cultures are the products of three serine protease genes and a metalloprotease gene, attempts can be made to clone these genes. Heterologous probing has proven to be a useful method to clone the structural genes from this system. For the study of this system it would be useful to isolate as many of the structural genes as possible. Preliminary experiments in our laboratory indicate that the *A. nidulans* genome contains a metalloprotease gene that cross-hybridises with the 42 kDa metalloprotease gene from *A. fumigatus*. Likely candidates for homologues of the serine proteases corresponding to band 6 and the band 2, 3, 4 cluster have been cloned from *A. niger*. It would be a fairly straight forward procedure to clone these

genes, if they are in fact homologous to the *A. niger pepC* and *pepD* genes. It is possible that *A. nidulans* produces extracellular proteases in addition to those detected in these experiments.

If the gene encoding the mycelium bound serine protease was isolated, it could be determined at what level this gene is regulated in response to carbon-limitation (6.3.5.). If, as with the *A. niger* mycelium bound proteases, the steady state transcript levels of this gene do not alter under carbon-limiting conditions, it would suggest that regulation of this gene occurs post-transcriptionally. Additional experiments could then be designed to determine if regulation occurs at the level of translation or enzyme activation. For example, protein synthesis could be blocked using inhibitors such as cyclohexamide. If the level of active mycelium bound protease still increased after carbon-limitation in the presence of inhibitors of protein synthesis, it would suggest that regulation occurs at the level of enzyme activation.

Once additional extracellular protease genes are cloned, Northern analysis and/or RT-PCR analysis could be used to examine the expression of these genes. The ability to detect the transcripts of a number of protease structural genes would also enable us to determine if the disruption of other protease structural genes results in a compensatory increase in transcript level of other protease genes, as was observed in *A. flavus* (Ramesh and Kolattukudy 1996). As gene-disruption of the serine protease structural gene, *prtA*, proved informative, other protease structural genes could be studied in this manner. It would be interesting to determine the chromosomal locations of the structural protease genes to see if they occur in clusters or are scattered throughout the genome. Mapping the ectopic copy of the marker gene used in the disruption strategy is one way of determining the disrupted gene's chromosomal location. It could also prove informative to determine the effects of disruption of a number of structural protease gene in the one strain.

Expression of the aspergillopepsin gene, *prtB*, was not detected under nitrogen-limiting conditions. Examination of the promoter suggests that it is possible that this gene may be expressed under carbon-limiting conditions, therefore experiments to examine the expression of *prtB* under conditions of carbon- or sulphur-limitation are warranted. It would also be interesting, from a biochemical perspective, to determine if the *prtB* product is a functional protease. To achieve this end (if conditions under which *prtB* is expressed are not found), a construct, in which a well characterised *A. nidulans* promoter is used to express the coding region of the *prtB* gene, could be transformed into an *A. nidulans* strain to produce the PrtB protein. Methods of detecting proteolytic activity would need to be optimised for the detection of acid protease activity. This could be achieved by optimising our methods, for the detection of *A. niger* PepA, which is a functional aspergillopepsin that is produced in high levels. Such experiments would show if the 8 amino acid deletion renders the PrtB protein non-functional. If PrtB was shown to be a functional protease, studies could be carried out to determine the conditions under which *prtB* is expressed and the regulation of this gene could be compared to that of other structural protease genes.

The preliminary analysis of the *prtA* promoter region revealed putative trans-acting factors which bound to a DNA fragment containing the HCR. Further analysis of the promoter region using gel mobility shift assays may localise binding sites for factors produced under different conditions, or other regions of the promoter involved in regulation. It would be of interest to determine if the putative AreA binding sites function as such *in vivo*. As many 5'-GATA-3' binding proteins have been shown to occur in *A. nidulans* culture filtrate (Peters and Caddick, 1994), an AreA fusion protein produced in *E. coli* would be more suitable for such experiments. As *creA* mutations have not been shown to affect extracellular protease production, gel mobility shift assays could be used to isolate proteins involved in the regulation of the extracellular protease structural genes in response to carbon catabolite repression. Such an approach would be complemented by a study examining the effects of deleting

sequences from the *prtA* promoter of a *prtA*-lacZ gene fusion. The response of the reporter gene to carbon-, nitrogen-, and sulphur-limiting conditions, and the effects on regulation in response to environmental pH could be determined. Such a study would determine regions of the promoter required for regulation under the different conditions.

DNA footprinting could be used to study the DNA-protein interactions already identified and would provide valuable information, as little is known of sulphur regulation in *A. nidulans*. Any other DNA-protein interactions identified could also be studied by DNA footprinting. Knowing the precise sites at which protein-DNA interactions occurs will enable us to determine what interactions are required for the gene to respond to different conditions. Another approach which could prove useful in isolating DNA binding proteins involved in the regulation of the extracellular proteases would be the screening of cDNA expression libraries constructed from RNA obtained from mycelia grown under the different nutrient-limiting conditions, as well as protease derepressing conditions, for factors which bind the *prtA* promoter. Such a screen could be conducted using large fragments from the promoter. Once the binding sites required for specific protein-DNA interactions are determined, the specific binding sites could be used to clone the genes coding for the relevant DNA-binding protein.

Regarding the future analysis of the two mutants isolated in this study, it is important to determine whether or not the phenotype attributed to the *xprJ1* mutation is due to a mutation in a single gene. If so, further analysis of this mutant would be valid. The results of another cross suggest that the phenotype of interest is in fact due to two mutations, as approximately 1/4 of the segregants had the *xprJ1* poor growth phenotype (Katz, pers. comm.). Given this result, the *xprJ1* mutation does not appear to warrant further study.

Further genetic analysis of the *xprI*$_1$ mutation may provide some interesting information. Of particular interest is the relationships between *xprI*$_1$ and other putative regulatory mutations which effect the extracellular proteases of *A. nidulans*. The epistatic relationships of such mutations may allow us to elucidate the hierarchy by which these genes operate to control the expression of the structural extracellular protease genes. Once it is known on which chromosome the *xprI* gene is located, it will be easier to clone this gene. As the *xprI*$_1$ mutation is linked to a translocation event, between chromosomes II and VII, the *xprI* gene could be cloned using Southern blot analysis. Cosmid clones from the chromosome specific library, corresponding to either chromosomes II or VII, could be used to probe blots containing DNA from wildtype and *xprI*$_1$ strains. If the clone used as a probe spanned, or was near to, the translocation breakpoint, then it would hybridise to fragments of different sizes in a wildtype and *xprI*$_1$ strain. This would enable clones spanning the translocation breakpoint to be identified. It could be determined whether the clones carry the *xprI* gene by seeing if they were able to complement the *xprI*$_1$ mutation. Complementation of the mutation could also be used as a strategy to pinpoint the location of the *xprI* gene within the clone.

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