Chapter 3

RESULTS:

Characterisation of xprF1

A revertant strain (MEK-R4) carrying one of the *xprE1* suppressors had already been outcrossed by M.E. Katz and it was thought that one of the segregants, R4-11, expressed high milk clearing activities without carrying the *xprE1* mutation. In comparison with the wild-type strain, R4-11 colonies produced noticeably larger clear zones (halos) on plates where milk was used as the nitrogen source (fig 3.1).

The new phenotype raised many questions: How many affected genes were involved (more than one mutated gene is hard to study)? What was the nature of the mutation in the gene? Was it a gain-of-function or a loss-of-function mutation? What was the nature of the gene? Had the mutation altered a secretory gene? Was the affected gene a structural gene for one of the proteases or did it have a regulatory function? If the altered gene had a regulatory role, what type of function/nature did it have: *cis*-acting or *trans*-acting, pathway-specific or wide-domain acting, negatively acting or positively-acting? Furthermore, what was the interaction between the new mutation and the other regulatory genes already reported to affect the production of extracellular proteases? Where was the affected gene located?

A series of experiments were carried out in an attempt to answer these questions.

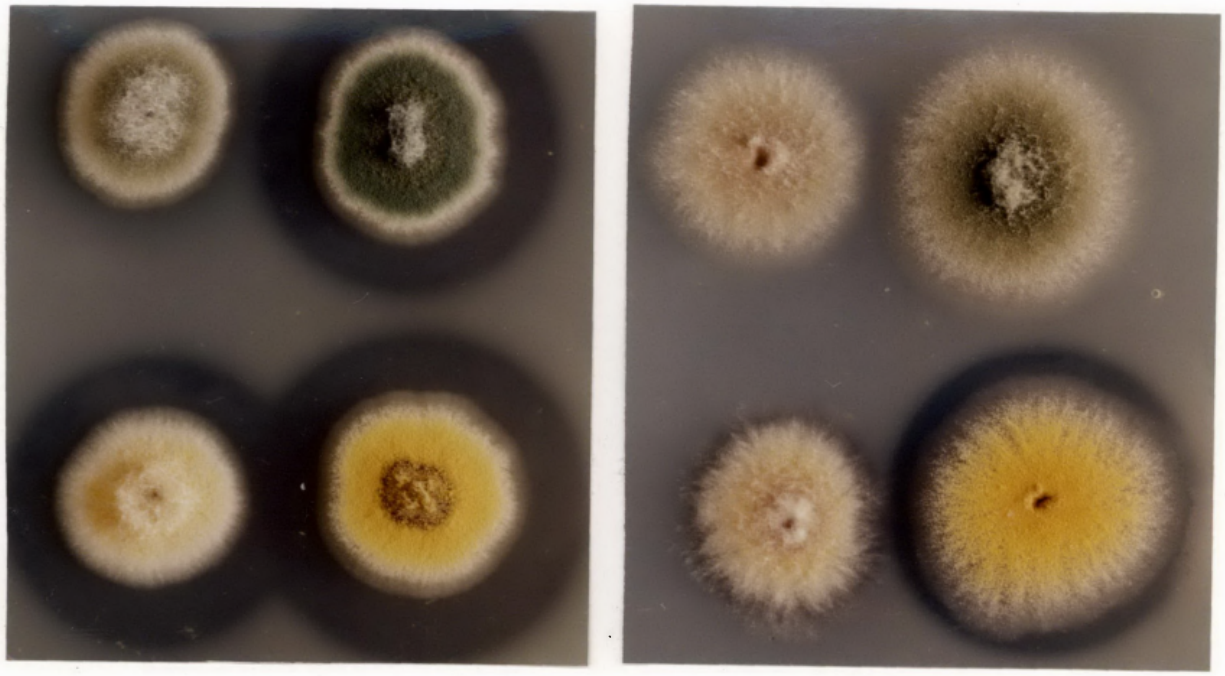

Figure 3.1. *xprE1* and its suppressor on -N (left) and -CN (right) milk plates. The strains in each photograph were: *xprE1* (top left), wild type (top right), *xprE1 xprF1* (bottom left), *xprF1* (bottom right). Photographs were taken after 3 days.

3.1. Segregation

To start the study, it was necessary to see how this new phenotype was segregated. Therefore, R4-11 was crossed to a wild-type strain (*y paba mas*) with the wild-type level of milk clearing on milk plates.

To study the effect of *xprF1* on milk clearing under different conditions, 39 segregants were tested on different milk plates where milk acted as carbon and/or nitrogen source. Two types of colonies were observed:

Table 3.1. Results from the cross R4-11 × *y paba mas*

group	milk clearing on milk plates*				remarks	number	frequency
	a	b	c	d			
1	+++	+	+++	+++	high level	17	44%
2	++	+	-	++	wild-type	22	56%

* Scores are for the size of halo relative to that of wild-type control.
a. 1% skim milk serving as the sole nitrogen source
b. 1% dialysed skim milk serving as the sole nitrogen source
c. 1% skim milk serving as the sole carbon source
d. 1% skim milk serving as both nitrogen and carbon source

There was a difference between the two phenotypic groups with respect to the level of extracellular protease activity. The milk clearing on plates where milk was used as the sole carbon source, in particular, was interesting since wild-type parent had no halo whereas R4-11 demonstrated a high level of protease activity. This different behaviour was used later in many cases to detect *xprF1* individuals.

The number of phenotypic groups was used to estimate the number of genes segregating. For any given locus, a haploid individual carries only one allele, and any pair of alleles segregating at a single locus during a cross are expected to give rise to two phenotypic groups at the ratio of 1:1. In contrast, the presence of more than two phenotypic groups implies involvement of more than one locus.

The result of this cross, however, showed that there were only two phenotypic groups. Assuming only one affected gene was involved, half of the segregants were expected to be wild type whereas the rest were expected to express the mutated phenotype. The value of chi-squared ($\chi^2 = 0.64$, calculated according to the method in Devore and Peck, 1986) was not significant at 0.05 level for the difference between expected and observed numbers ($P>0.05$). This indicated that either one mutated gene was involved in the new phenotype or if there was more than one mutated gene, they were tightly linked, and hence segregated together. Considering the effects caused by the *xprG1* mutation, it was unlikely that two (or more) linked genes were involved here (discussed in Chapter 5).

3.2. Pleiotropy

The suppressor mutation affected levels of milk clearing. It was of interest to see whether *xprF1* had any other effects. That is, was *xprF1* a pleiotropic mutation with wide-domain acting nature?

3.2.1. Effect on the Utilisation of Nitrogen Sources

The effect of *xprF1* on utilisation of different nitrogen sources was examined. In order to investigate the association between the presence of *xprF1* and any other possible effects, 43 segregants of the cross "R4-11 × *y paba mas*" were tested on uric acid, hypoxanthine, acetamide (as nitrogen source), nitrate and proline (as nitrogen source). The milk plate containing milk as the carbon source (-C milk) was used for detection of *xprF1* segregants. Four phenotypic groups were identified (table 3.2).

A fairly similar effect was observed on all nitrogen plates. Only results from only uric acid, hypoxanthine and acetamide are shown here. Colonies with abnormal morphology had a fuzzy appearance with weak conidiation whilst retaining normal growth rate. All segregants carrying *xprF1* (big halo on the milk plate) had weaker growth rate associated with a dark brown background colour deep in the agar, irrespective to whether the morphology was normal or abnormal.

Presence of four phenotypic groups suggested that two genes were involved. For this assumption the difference between expected and observed numbers was not significant ($\chi^2 = 2.49 \Rightarrow P>0.05$). Two factors were considered: weaker growth rate and, abnormal morphology. R4-11 had abnormal morphology and suffered from extremely weak growth (similar to the fourth group). In contrast, the wild-type parent expressed both normal morphology and wild-type growth rate (first group). Results in table 3.2 suggested that a second mutation was responsible for the abnormal morphology while *xprF1* was responsible for the weak growth. Presumably, R4-11 carried both mutations. Random segregation of chromosomes or chromosomal cross-over (if two unlinked genes were located on the same chromosome) between xprF1 and the second mutated gene had resulted in segregants categorised in second and third groups.

Table 3.2. Results from the cross R4-11 × *y paba mas* tested on different nitrogen sources

group	growth on nitrogen plates*			milk-clearing†	morphology	number	frequency
	a	b	c				
1	+++	+++	+	-	normal	14	33%
2	+	+	±	+++	normal	7	16%
3	++	++	+	-	abnormal	10	23%
4	±±	±±	<±	+++	abnormal	12	28%

* Scores are for the amount of growth relative to that of wild-type control on corresponding plates.
† Scores are for the size of halo relative to that of R4-11 control on -C milk plate.
a. 10 mM uric acid; 1% glucose
b. 10 mM hypoxanthine; 1% glucose
c. 10 mM acetamide (nitrogen source); 1% sucrose

The χ^2 value suggested, in addition, that the two mutations were unlinked.

It was of interest to study the effects of the two above mutations separately. Therefore two segregants of the above cross were isolated and crossed to a wild-type strain. AM2 (*pabaA1 yA1 acuE; xprF1; riboB2*) was a segregant thought to carry only *xprF1* whereas AM3(*yA1 acuE; riboB2*) was thought to carry only the other mutation.

a) 47 segregants of the cross "AM2 × *niiA4*" were checked on the milk plate with milk as the sole carbon source and also on uric acid, hypoxanthine, proline and acetamide (N source) media:

Table 3.3. Results from the cross AM2 × *niiA4*

group	growth on nitrogen plates*				milk-clearing†	morphology	number	frequency
	a	b	c	d				
1	++	++	+	++	-	normal	27	57%
2	±±	±±	±	±±	+++	normal	20	43%

* Scores are for the amount of growth relative to that of wild-type control on corresponding plates.
† Scores are for the size of halo relative to that of AM2 control on -C milk plate.
a. 10 mM uric acid; 1% glucose
b. 10 mM hypoxanthine; 1% glucose
c. 10 mM acetamide (nitrogen source); 1% sucrose
d. 10 mM L-proline; 1% glucose

Two groups of segregants confirmed the involvement of only one gene. The χ^2 value (1.04) was still not significant at 0.05 level for the difference between expected and observed numbers. There was a full association between the presence of *xprF1* (higher level of milk clearing), a weaker growth rate and a brown pigmentation beneath the colony on media containing hypoxanthine, uric acid, acetamide and L-proline (as nitrogen sources). These results being consistent to those of "R4-11 × *y paba mas*", suggested that the mutation in the *xprF* locus had some negative effects on the utilisation of different nitrogen sources. Besides, segregation of these pleiotropic effects together suggested that they were due to a single mutation. The latter was consistent with results from "R4-11 × *y paba mas*".

In order to pick up an *xprF1* strain to cross to J9 (to prepare a strain needed for transformation), segregants were tested for a number of markers including *pabaA1*, *riboB2*, *niiA4* and *acuE*. AM4 (*yA1 acuE; xprF1; niiA4 riboB2*) was isolated and genetically purified.

b) AM3 presumably carried only the mutation responsible for the abnormal morphology. AM3 was crossed to *niiA4* to test this assumption.

46 segregants were tested on uric acid, hypoxanthine and proline plates as well as the milk plate with milk as the carbon source. Two phenotypic groups were identified:

Table 3.4. Results from the cross AM3 × *niiA4*

group	growth on nitrogen plates*				milk-clearing†	morphology	number	frequency
	a	b	c	d				
1	++	++	+	++	-	normal	26	57%
2	+±	+±	>±	+±	-	abnormal	20	43%

* Scores are for the amount of growth relative to that of wild-type control.
† Scores are for the size of halo relative to that of AM3 control on -C milk plate.
a. 10 mM uric acid; 1% glucose
b. 10 mM hypoxanthine; 1% glucose
c. 10 mM acetamide (nitrogen source); 1% sucrose
d. 10 mM proline; 1% glucose

Results showed that the effect was due to a single gene ($\chi^2 = 0.78 \Rightarrow$ P>0.05). Also, the absence of weaker growth with the brown background on different nitrogen sources, suggested that they were caused by *xprF1*. These data were consistent with those of "R4-11 × *y paba mas*".

To conclude, results from these three crosses suggested that *xprF1* not only elevated the rate of milk clearing (on -C milk plate), *i.e.*, extracellular protease activity on milk plates, but also adversely affected the utilisation of different nitrogen sources on minimal medium (glucose as the carbon source). Therefore, the mutation was of a wide-domain acting (pleiotropic) and not pathway-specific. This implied that *xprF1* could not be a mutation in the regulatory region upstream of the coding region of a structural gene coding for an extracellular protease.

3.2.2. Effect on the Utilisation of Carbon Sources

Segregants of the cross "AM2 × *niiA4*" were also tested on different carbon sources including sorbitol, GABA, glycerol, acetamide (C source) and L-proline (C source) where ammonium was used as the nitrogen source. Hence, the effect of *xprF1* on carbon metabolism could be examined. No notable difference was observed between *xprF1* and *xprF*$^+$ segregants in respect to the growth pattern.

3.3. Dominancy

Characterisation of the interaction between *xprF1* and its wild-type allele was of basic importance, because the primary strategy for cloning *xprF1* (discussed later in this chapter) was based on the complementation of the mutation by the wild-type copy present in genomic DNA library. This strategy required *xprF1* to be recessive to the *xprF*$^+$.

The study was carried out with a diploid strain prepared from AM1 (*yA1 pabaA1 acuE; xprF1*) and a mapping strain (J3). The mapping strain was used because the diploid could be readily used later in haploidisation analysis. AM1

was a segregant from the cross "R4-11 × *y paba mas*" which did not carry the mutation resulting in abnormal morphology. AM1, J3, AM1/J3 (diploid) and *niiA4* (wild type control) were tested on the milk medium containing milk as the carbon source as well as the minimal medium containing hypoxanthine as the nitrogen source.

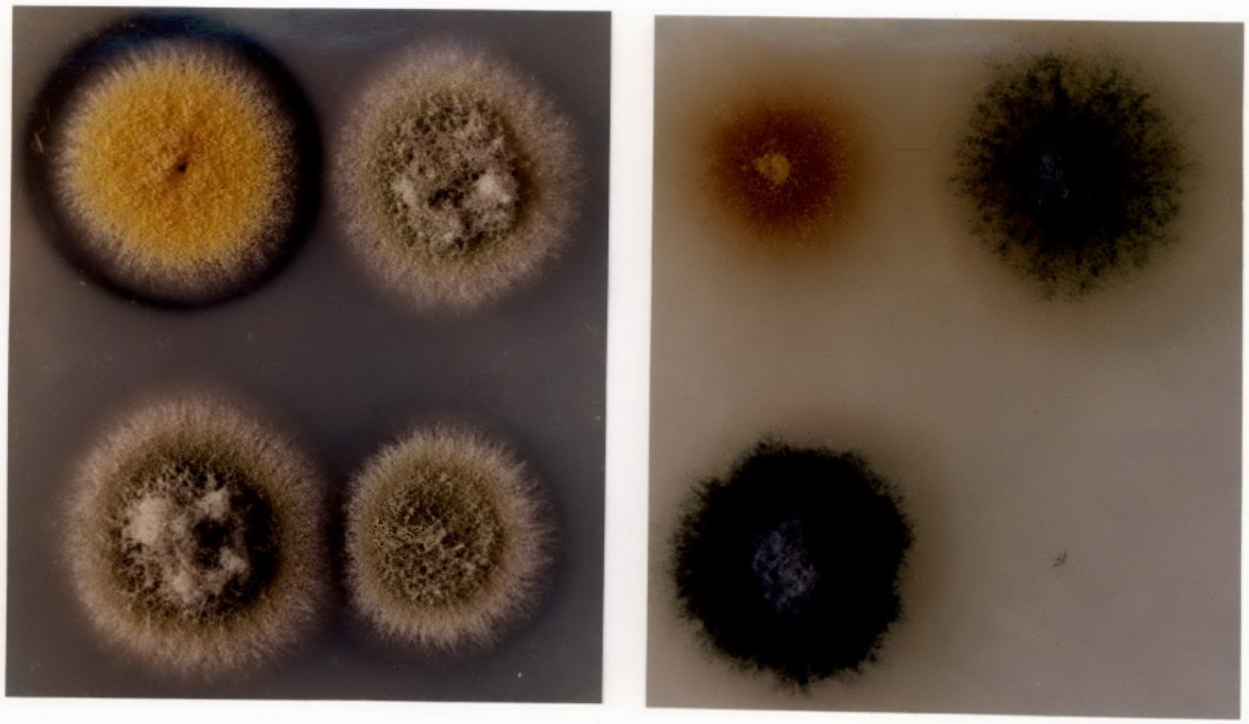

Figure 3.2. Dominancy test on -C milk (left) and hypoxanthine (right). Strains: *xprF1* (top left), *xprF1/xprF*$^+$ (top right), wild type (bottom left), mapping strain J3 (bottom right). J3 did not grow on hypoxanthine due to the *hxA* mutation.

Table 3.5. Dominancy test

strain	chromosome set	relevant - genotype	milk clearing*	growth on hypoxanthine†
AM1	n	*xprF1*	+++	+
J3	n	*xprF*$^+$	-	-
AM1/J3	2n	*xprF1/xprF*$^+$	-	+±
niiA4	n	*xprF*$^+$	-	+++

* Scores are for the size of halo relative to that of AM1 control on -C milk plate.

† Scores are for the amount of growth relative to that of wild-type control on hypoxanthine plate after 3 days.

The diploid colony expressed wild-type level of milk clearing which was clearly distinguished from the *xprF1* parent. However, on hypoxanthine plates, the diploid colony showed a morphology somewhere between those of its haploid parents. That is, although the growth was not as weak as *xprF1* parent, the deep brown background colour was visible in the diploid (fig. 3.2).

Data showed two contrasting interactions between *xprF1* and *xprF*$^+$. As far as the effect on production of extracellular proteases was involved, *xprF1* was recessive to its wild-type copy. For the effect on the utilisation of different nitrogen sources, however, *xprF1* was semi-dominant to *xprF*$^+$.

3.4. Location of *xprF1*

Localising the *xprF* locus within the *A. nidulans* genome had to be done in two steps. First, the chromosome carrying the *xprF* had to be identified. Once this was done, the locus had to be localised within the chromosome. Haploidisation studies were employed to map the *xprF* to chromosomes. Then, sexual crosses between an *xprF1* strain and a number of strains carrying different markers on the same chromosome as *xprF* had to be carried out. Given the known location of the markers used, recombination frequencies between *xprF1* and linked markers were to be analysed to localise the locus relative to them.

3.4.1. Mapping *xprF1* to Chromosomes

The location of *xprF1* was of major importance. This knowledge was particularly useful in the isolation of the wild-type gene because using chromosome-specific genomic DNA libraries, there was no need to examine clones from the entire genome. In order to address the location of the affected gene, haploidisation of a diploid strain, AM1/J3, was carried out. The markers carried by either strains are mentioned in table 3.6.

For any given chromosome, each haploid individual carried a chromosome coming from AM1 or J3. J3 carried the *xprF*+ and wild-type alleles for other AM1's markers. In contrast, AM1 carried wild-type alleles for all markers in J3. Therefore, expression of the mutant phenotype of any markers of J3 meant that the chromosome carrying that mutation had come from J3. In the same way, expression of *xprF1* meant that the corresponding chromosome was from AM1. As AM1 was wild type for all markers in J3, presence of the chromosome carrying *xprF1* was expected to be associated with the expression of the wild-type phenotype of the particular marker on the corresponding chromosome in J3. In other words, association of *xprF1* phenotype with the absence of any marker in J3, would reveal the chromosome carrying the *xprF* locus. The only exception was the case of chromosome I. J3 did not carry any marker on the latter. Instead, AM1 had *yA1*, *pabaA1* and *acuE* on chromosome I. Therefore, if *xprF1* was located on this chromosome, its presence in haploids had to be associated with the presence of markers on the chromosome I of AM1.

Table 3.6. Markers used in haploidisation studies.

chromosome	AM1*		J3	
	marker	effect†	marker	effect†
I	*yA1 pabaA1 acuE*	*yA1*: yellow conidia *pabaA1*: requires para-aminobanzoic acid *acuE*: unable to utilise acetate	-	-
II	-	-	*acrA1*	acriflavine-resistant
III	-	-	*galA1*	unable to utilise galactose
IV	-	-	*pyroA4*	requires pyridoxine
V	-	-	*hxA*	unable to utilise hypoxanthine
VI	-	-	*sB1*	unable to take up sulphate
VII	-	-	*nicB8*	requires nicotinic acid
VIII	-	-	*riboB2*	requires riboflavin

* AM1 carried *xprF1* whose location was to be determined.

† Information from Clutterbuck (1993).

64 haploids were examined on appropriate plates. Except for the 100% association between the presence of *yA1*, *pabaA1* and *acuE*, and also 100% association between the presence of *xprF1* and absence of *nicB8* (*i.e.*, wild-type growth on -nico plate), there was no correlation between presence/absence of other markers.

Since crossing-over does not occur in haploidisation, *yA1*, *pabaA1* and *acuE* were expected to be segregated together, because these three markers are located on the same chromosome. The association between the presence of *xprF1* and the absence of *nicB8* suggested that the *xprF* locus was located on the chromosome VII.

3.4.2. Location on the Chromosome VII

Results of haploidisation showed that *xprF1* was located on chromosome VII. The *xprF1* strain was crossed to a number of markers on chromosome VII including *alcAR*, *nicB*, *cnxF*, *lysD*, *gatA* and *amdA* by M.E. Katz. In addition, I tested for linkage of the *xprF* gene to *prnA* and *sconD* loci (see below). According to M.E. Katz (personal communications), linkage was observed between *xprF* and *gatA* and *amdA*. Analysis of 142 segregants by M.E. Katz showed that *xprF* is 25 map units away from the *gatA* locus.

Linkage with *prnA*

It was known that the *prnA* locus was located on chromosome VII. Therefore, as one of primary attempts, segregants of the cross "R4-11 × *y paba-mas*" were tested for the utilisation of proline as the nitrogen source. R4-11 carried *prnA457* which according to Sharma and Arst (1985, cited in Hull *et al.*, 1989) is a small deletion in the *prnA* gene. However, Hull *et al.* (1989) reported this deletion to be too small to be detected by their physical identification method and therefore, the deletion was suggested to be smaller than 0.05 kb. The *prnA* gene, according to the authors, is a positively-acting regulatory gene which mediates the proline induction. *prnA457* individuals are not able to utilise proline and do not grow on proline.

43 segregants were tested on L-proline and -C milk plates (table 3.7). R4-11 also carried the abnormal mutation (see 3.2.1). Therefore, a number of *prn*$^+$ segregants also carried this mutation. Although the latter alters the morphology of colonies, it did not prevent identification of *prn*$^+$ segregants. In addition, milk clearing activity and dark brown background on proline plates were used for discrimination of *xprF1* segregants. As it was shown before (section 3.2.1), this mutation did not appear to alter the level of milk clearing activity.

When two unlinked genes segregate, recombination results in four genotypic combinations with similar frequencies. That is, the frequency of segregants with parental phenotypes is expected to be similar to those of recombinants. In contrast, linkage between loci interferes with crossing-over and reduces the recombination frequency. The closer the two loci, the fewer recombinant individuals. It was assumed that *xprF* and *prnA* loci were not linked and it was expected to see a 1:1:1:1 ratio among segregants for each phenotypic group. Results confirmed that there is no linkage between two loci ($\chi^2 = 2.30$ for the difference between observed and expected numbers).

Table 3.7. Results from the cross R4-11 × *y paba mas* tested on L-proline

group	presumed genotype	milk clearing*	growth on proline†	number	frequency
a^i	*xprF*$^+$ *prnA*$^+$	-	++	10	23%
b	*xprF*$^+$ *prnA457*	-	-	15	35%
c	*xprF1 prnA*$^+$	+++	+	9	21%
d^{ii}	*xprF1 prnA457*	+++	-	9	21%

* Scores are for the size of halo relative to that of R4-11 control on -C milk plate.
† Scores are for the amount of growth relative to that of wild-type control.
i The phenotype similar to that of *y paba mas*.
ii The phenotype similar to that of R4-11.

Linkage with *amdA*

The *xprF1* locus was also mapped relative to the *amdA* gene. Linkage between these two loci was identified by M.E. Katz. In order to obtain more

precise results more segregants of the cross between R4J3 (carrying *amdA7*) and AM1 (carrying *xprF1*) were tested. *amdA7* caused elevated acetamidase activity and vigorous growth on acetamide. *amdA* is one of several regulatory genes involved in the regulation of *amdS*, the structural gene for acetamidase. Arst and Cove (1973, cited in Hynes, 1978) showed that mutations in *amdA* resulted in strong growth on acetamide. Hynes (1978) reported that *amdA7* resulted in high acetamidase activity and strong growth on acetamide both as carbon or nitrogen source. Accordingly, *amdA7* also allowed the individual to utilise non-inducing acrylamide as the sole nitrogen source.

144 segregants were tested on -C milk medium and acetamide (N source). Four phenotypic groups were obtained.

Table 3.8. Results from the cross AM1 × R4J3

group	presumed genotype	milk clearing*	growth on acetamide†	number	frequency
a	*xprF*$^+$ *amdA*$^+$	-	+ (wild type)	26	18%
b^i	*xprF*$^+$ *amdA7*	-	+++	41	28%
c^{ii}	*xprF1 amdA*$^+$	+++	±	59	40%
d	*xprF1 amdA7*	+++	++±	18	12%

* Scores are for the size of halo relative to that of AM1 on -C milk plate.
† Scores are for the amount of growth relative to that of AM1.
i This phenotype was similar to that of R4J3. Among this group, 26 were green and 15 yellow. $\chi 2 = 2.95$, not significant at 0.05 level, assuming there is no contamination with parental conidia.
ii This phenotype was similar to that of AM1. Among this group, 31 were green and 28 yellow ($\chi 2 = 0.08$, not significant at 0.05 level, similar comments as that of '*i*').

The presence of *xprF1* caused weaker growth on acetamide plate. If it is assumed that *amdA* and *xprF* loci are not linked, a frequency of 25% is expected for each phenotypic group. Frequencies of four phenotypic groups in table 3.5 were significantly different from 1:1:1:1 ($\chi^2 = 27.17$, significant at 0.001 level). This suggested that *xprF1* and *amdA7* were close enough to reduce the recombination frequency. The genetic distance between two loci was calculated:

total number of segregants:	144
segregants with parental phenotypes:	100
recombinants:	44

$$44 \div 144 = 0.301$$
$$= 30.1\%$$

xprF1 is, according to these results, located 30.1 map units away from *amdA7* on chromosome VII. According to Clutterbuck (1993), *gatA* and *amdA* loci are about 5 map units apart. It had already been shown by M.E. Katz that the *xprF* was 25 units away from the *gatA* locus. Since the sum of *xprF-gatA* and *gatA-amdA* distances is consistent with the distance between *xprF* and *amdA* loci, it can be concluded that the *xprF* lies in the same direction as *gatA*, relative to the *amdA* locus.

Linkage with *sconD*

It was suspected that *xprF* and *sconD* loci might be linked. *xprF1* was crossed to *sconD6* which was 21 map units left of *amdA7* (Natorff *et al.*, 1993 and Clutterbuck, 1993). According to Natorff *et al.* (1993), *sconD* is one of four regulatory genes mediating sulphur metabolite repression (SMR). A mutation in this gene, *sconD6*, leads to insensitivity to SMR and accumulation of sulphur-containing compounds synthesised from sulphate, even in the presence of methionine (repressive sulphur source). 144 segregants were tested for *xprF1* and *sconD6* effects. As the first approach for detection of *sconD6* individuals, 24-hour colonies grown on complete plates were flooded with 30 mM indoxyl sulphate in 0.5 M Tris-HCl; pH8.0. *sconD6* colonies were expected to turn blue (Natorff *et al.*, 1993). Results were not as expected since no colonies turned blue. As the second approach for identification of *sconD6* colonies, segregants were tested on sulphur-free -N minimal medium containing 1 mM sodium selenate and 5 mM methionine (A. Paszewski, personal communication to M.E. Katz). Sodium selenate is a toxic substance whose uptake is repressed in *sconD*$^+$ individuals when methionine is present as a repressive sulphur source. Due to sulphur derepression in *scon* mutants, sodium selenate is taken up resulting in growth inhibition. The second approach was also unsuccessful for identification of *sconD6* individuals. The

second approach worked well for *sconA* mutants (M.E. Katz, personal communication). It could be suggested that *sconD6* does not have derepressing effects as extreme as those of *sconA* mutations.

3.5. Genetic Interactions Between *xprF1* and Some Other Regulatory Mutated Genes

areA102

The *areA* gene mediates the nitrogen metabolite repression in *A. nidulans* (Arst and Cove, 1973). The product of this gene, according to Davis and Hynes (1991), is a positively-acting protein and is required for the function of many enzymes and permeases involved in the metabolism of different nitrogen sources. A number of mutations in the *areA* locus had already been studied. To investigate whether there was any possible interaction between *xprF* and *areA* gene, an *xprF1* strain was crossed to ac102. The latter carried the *areA102* mutation which is due to a conservative single amino acid substitution in the zinc finger region of the *areA* protein and results in elevated level of acetamidase activity (Hynes, 1973, cited in Kudla *et al.*, 1990). *areA102* individuals were identified by their vigorous growth on acetamide (as N source) plate.

46 segregants were tested on -C milk plate as well as on acetamide (N source). Four groups were observed (Table 3.9).

The presence of four groups, particularly wild-type segregants, eliminated any possibility of the two mutated genes being alleles. This was consistent with the result of mapping as *xprF* was located on chromosome VII whereas *areA* was located on chromosome III (Clutterbuck, 1993).

Interaction between loci was studied in *areA102 xprF1* double-mutants (group d). The growth on acetamide (*areA102* phenotype) was not significantly affected by the presence of *xprF1* and the level of milk clearing on -C milk plate (*xprF1* phenotype) was not affected by *areA102* at all (fig. 3.3). This

observation suggested that there was no noticeable genetic interaction between these two mutations.

Table 3.9. Results from the cross AM1 × ac102

group	presumed genotype	milk clearing*	growth on acetamide†	number	frequency
a	*areA*$^+$ *xprF*$^+$	-	+	14	30%
b	*areA102 xprF*$^+$	-	+++	12	26%
c	*areA*$^+$ *xprF1*	+++	±	5	11%
d	*areA102 xprF1*	+++	++±	15	33%

* Scores are for the size of halo relative to that of AM1 control on -C milk plate.
† Scores are for the amount of growth relative to that of AM1 control.
N.B. $\chi^2 = 5.30$ for the difference between expected and observed numbers for each group assuming no linkage or contamination with parental conidia/mycelia is present.

areA217

H17A12 carried *areA217,* also known as *areA*$^-$, which resulted in repression of all activities under *areA* control including production of extracellular proteases. *areA217* is a loss-of-function mutation and is due to a single amino acid substitution adjacent to the finger (Kudla *et al.*, 1990). It was desirable to study the interaction between these mutations in double-mutants.

43 segregants of a cross between an *xprF1* strain and H17A12 were tested on -N and -CN milk plates and also on the minimal medium containing alanine as the sole nitrogen source. *areA217* individuals are not able to grow when alanine is the only nitrogen source in the medium. Four groups were identified (Table 3.10).

Segregants in group '4' carried both *xprF1* and *areA217* mutations. The *areA217 xprF*+ segregants showed no milk clearing on -N milk plate after 48 hours, whereas in *areA*$^+$ *xprF1* individuals, the *xprF1* mutation caused elevated levels of milk clearing. In other words, *xprF1* did not suppress the *areA*$^-$ mutation under nitrogen limiting conditions.

Table 3.10. Results from the cross AM6 × H17A12

group	presumed genotype	milk clearing* a	milk clearing* b	growth on L-alanine†	number	frequency
1	*areA*$^+$ *xprF*$^+$	++	+	++	15	35%
2	*areA217 xprF*$^+$	-	-	-	8	19%
3	*areA*$^+$ *xprF1*	+++	+++	+±	10	23%
4	*areA217 xprF1*	-	green: +++ yellow: +	-	10	23%

* Scores are for the size of halo relative to that of AM6 control. The milk clearing activity on -CN plate in green double-mutants differed from that of yellow ones.

† Scores are for the amount of growth relative to that of AM6 control.

a. 1% glucose; 1% skim milk (nitrogen source)

b. 1% skim milk (carbon and nitrogen source)

N.B. $\chi^2 = 2.49$ for the difference between expected and observed numbers for each group assuming no linkage or contamination with parental conidia/mycelia is not present is not significant at 0.05 level.

areA217 xprF$^+$ segregants expressed no milk clearing activity on -CN milk plates. *areA217 xprF1* segregants, however, showed extracellular protease activity. The size of halo on the -CN plate varied between green and yellow segregants which could be due to the different amount of growth of these segregants on the plate. The phenomenon can be explained by the presence of *adE20 suAadE20* mutations in the yellow *areA217* parent. The *adE20* mutation, isolated by MacDonald and Pontecorvo (1953) (in *adE* locus tightly linked to the *yA* locus on chromosome I), results in an adenine requiring phenotype. According to Pritchard (1955), *adE20* individuals have weak growth with aconidiated morphology. The *suAadE20* mutation (Pritchard, 1955) has been mapped to the same chromosome but unlinked to *adE*. It suppresses the effect caused by the *adE20* mutation. Arst and Cove (1973) showed that in individuals carrying *adE20, suAadE20* and *areA*$^-$ mutations, the suppressor effect of *suAadE20* is interferred with by the *areA*$^-$ mutation. This results in weak growth of these individuals on complete medium. In this cross, due to extremely poor growth of *adE20 suA*$^+$ individuals, it is likely that all yellow colonies picked have only *adE suAadE20* genotype. This explains why the yellow double-mutant in figure 3.4 is weaker than the green double-mutant.

Two double-mutants (one green and one yellow) were later tested on -C milk (for milk clearing activity). Similar to -CN milk plate, the green double-mutant expressed milk clearing activities at the same level of the *xprF1* parent

whereas the yellow one expressed weaker growth accompanied by smaller halo (figure 3.4). The effect of *adE20* mutation is visible in the yellow double-mutant in this figure.

It seemed that *xprF1* could suppress the *areA*$^-$ mutation when no glucose was present. This observation showed that extracellular proteases can be produced without the activator function of the *areA* gene product. So this implies that the production of these enzymes could be under the control of more than one regulatory circuit in response to different nutritional contexts. The other implication could be that the *xprF1* affects the regulatory system involved in carbon regulation of protease production.

creA204

The creA gene is the major gene known to be involved in regulation of carbon metabolism. The *creA204* mutation, characterised by Hynes and Kelly (1977), results in high sensitivity to allyl alcohol in the presence of sucrose which is due to derepressed metabolism of carbon sources. According to authors, this mutation affects all activities under *creA* control. *creA204* has no effect on the production of extracellular proteases. Allyl alcohol was used to identify *creA204* mutants. Catabolism of the latter releases a toxic substance which inhibits the growth.

Segregants of a cross between an *xprF1* (AM5) strain and a *creA204* (SA4B17) strain were replicated on a medium containing sucrose as the repressive carbon source plus allyl alcohol. Wild-type individuals were not affected by allyl alcohol (due to carbon repression caused by sucrose) and grew normally. In contrast, *creA204* individuals did not grow as, due to derepression, they utilised allyl alcohol (table 3.11). SA4B17 was the strain carrying *creA204* and crossed to AM5.

The study on double-mutants (fig. 3.5) showed no notable genetic interactions. *xprF1* had no effect on the shape of colony on different media whereas *creA204* caused a compact appearance. *creA204 xprF1* double mutant colonies were fairly similar to the *creA204* strain. That is for morphology, *xprF1* did not affect *creA204*. For the milk clearing, however, *creA204 xprF1* individuals behaved somewhat like the *xprF1* strain. The only exception was

on the milk plate with milk serving as the nitrogen source where double-mutants expressed the milk clearing activities similar to that of *creA204* parent (where the difference between *xprF1* and *creA204* was not considerable).

Table 3.11. Results from the cross AM5 × SA4B17

group	presumed genotype	milk clearing*			growth on allyl alcohol†	number	frequency
		a	b	c			
1	*creA*$^+$ *xprF*$^+$	++	++	-	++	7	16%
2	*creA204 xprF*$^+$	++	+++	-	-	12	27%
3	*creA*$^+$*xprF1*	+++	+++	+++	++	15	34%
4	*creA204 xprF1*	++	+++	+++	-	9	21%

* Scores are for the size of halo relative to that of AM5 control.
† Scores are for the amount of growth relative to that of AM5 control.
a. 1% glucose; 1% skim milk (nitrogen source)
b. 1% skim milk (nitrogen and carbon source)
c. 10 mM ammonium; 1% skim milk (carbon source)
N.B. $\chi^2 = 3.40 \Rightarrow P>0.05$ for assuming there was no contamination with parental conidia/mycelia.

creB15

The *creB* gene was reported to be involved in the regulation of carbon metabolism (Hynes and Kelly, 1977). A mutation in this locus (*creB15*) was described to elevate the level of milk clearing. *creB15* individuals were reported to be sensitive to allyl alcohol in the presence of repressive carbon sources such as sucrose. The *creB15* mutated gene expresses a variety of effects on activities related to carbon catabolism. It also has a considerable effect on milk clearing, particularly when milk serves as the sole carbon source. This cross was carried out to study any possible interaction between *creB15* and *xprF1*. *creB15* individuals were identified by their failure to grow on medium containing sucrose plus allyl alcohol.

Table 3.12. Results from the cross AM5 × SA15B12

group	presumed genotype	milk clearing* a	b	c	growth on allyl alcohol†	number	frequency
1	*creB*$^+$ *xprF*$^+$	++	++	-	++	13	32%
2	*creB15 xprF*$^+$	+++	++	++	-	6	12%
3	*creB*$^+$ *xprF1*	++	++	++‡	++	11	27%
4	*creB15 xprF1*	++++	+++	++++	-	11	29%

* Scores are for the size of halo relative to that of AM5 control.
† Scores are for the amount of growth relative to that of AM5 control.
‡ Faint halo
a. 1% glucose; 1% skim milk (nitrogen source)
b. 1% skim milk (nitrogen and carbon source)
c. 10 mM ammonium; 1% skim milk (carbon source)
N.B. $\chi^2 = 2.61 \Rightarrow P>0.05$ for assuming there was no contamination with parental conidia/mycelia.

During the study of double-mutants, it was realised that the *creB15* strain (SA15B12) had a negative effect on the utilisation of hypoxanthine as a nitrogen source. Hynes and Kelly reported that *creB15* has no effect on the growth on hypoxanthine which is different here. The morphology of the *creB15* strain on hypoxanthine was similar to that of AM5. As shown in table 3.12, *creB15* has a significant effect on the level of milk clearing when compared to wild-type (fig. 3.5). Interestingly, *creB15 xprF1* double-mutants expressed amplified effects on both milk clearing and morphology on hypoxanthine. Double-mutants were weaker than both parents on hypoxanthine and have much higher milk clearing activities on milk plates.

Two *creB15 xprF1* double mutants were tested on "-N milk + ammonium" plate to examine whether these two mutations together derepress the milk clearing activity. Results showed that in fact, they did so. Double-mutants produced halos on the milk plate while none of the parents showed a similar effect. Under these conditions, where every nutrient is provided, the production of extracellular proteases is expected to be repressed, which is the case for *xprF1* and *creB15* parents. Derepressed levels of milk clearing is another aspect of the genetic interaction between these two mutations.

creC27

In addition to *creB*, *creC* was reported to be involved in the carbon metabolism (Hynes and Kelly, 1977). *creC27* is a mutation in the latter gene characteristics of which, particularly effects on milk clearing and sensitivity to allyl alcohol, were similar to those of *creB15*. Results of testing 42 segregants from this cross are presented in table 3.13.

An additive effect between *xprF1* and *creC27* was observed particularly on the utilisation of hypoxanthine. *creC27 xprF1* double-mutants were as weak as *xprF1 creB15* individuals on hypoxanthine (figure 3.6, shows only *xprF1 creC27* double-mutants). On milk plates, in contrast, the effect was not as strong as that of *xprF1 creB15* but still the dditive effect was considerable specially on the -N milk plate. Hynes and Kelly (1977) characterised the *creC27* mutation and explained that its effects are similar, though not as extreme, to those of *creB15*. Results from testing *xprF1 creC27* double-mutants when compared with those of *xprF1 creB15* were consistent with this comment.

Table 3.13. Results from the cross AM5 × SA27D8

group	presumed genotype	milk clearing*			growth on allyl alcohol†	number	frequency
		a	b	c			
1	*creC*$^+$ *xprF*$^+$	++	+	-	++	12	29%
2	*creC27 xprF*$^+$	++	++	-	±	8	19%
3	*creC*$^+$ *xprF1*	+++	++	++‡	++	15	35%
4	*creC27 xprF1*	++++	+++	+++	±	7	17%

* Scores are for the size of halo relative to that of AM5 control.
† Scores are for the amount of growth relative to that of AM5 control.
‡ Faint halo
a. 1% glucose; 1% skim milk (nitrogen source)
b. 1% skim milk (nitrogen and carbon source)
c. 10 mM ammonium; 1% skim milk (carbon source)
N.B. $\chi^2 = 3.91 \Rightarrow P>0.05$ for assuming there was no contamination with parental conidia/mycelia.

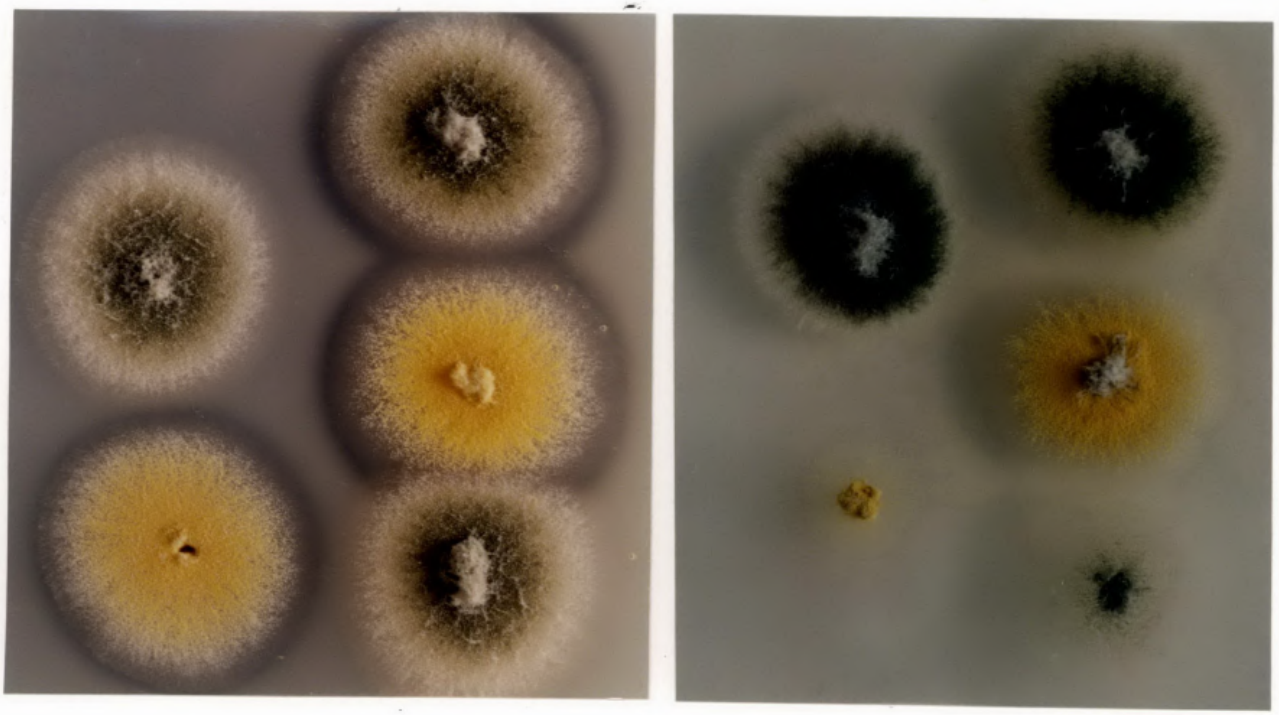

Figure 3.3. *xprF1 areA102* double-mutants on -C milk (left) and acetamide (right). Strains: *areA102* (top left), *xprF1* (bottom left), *xprF1 areA102* (top and middle right), wild type (bottom right).

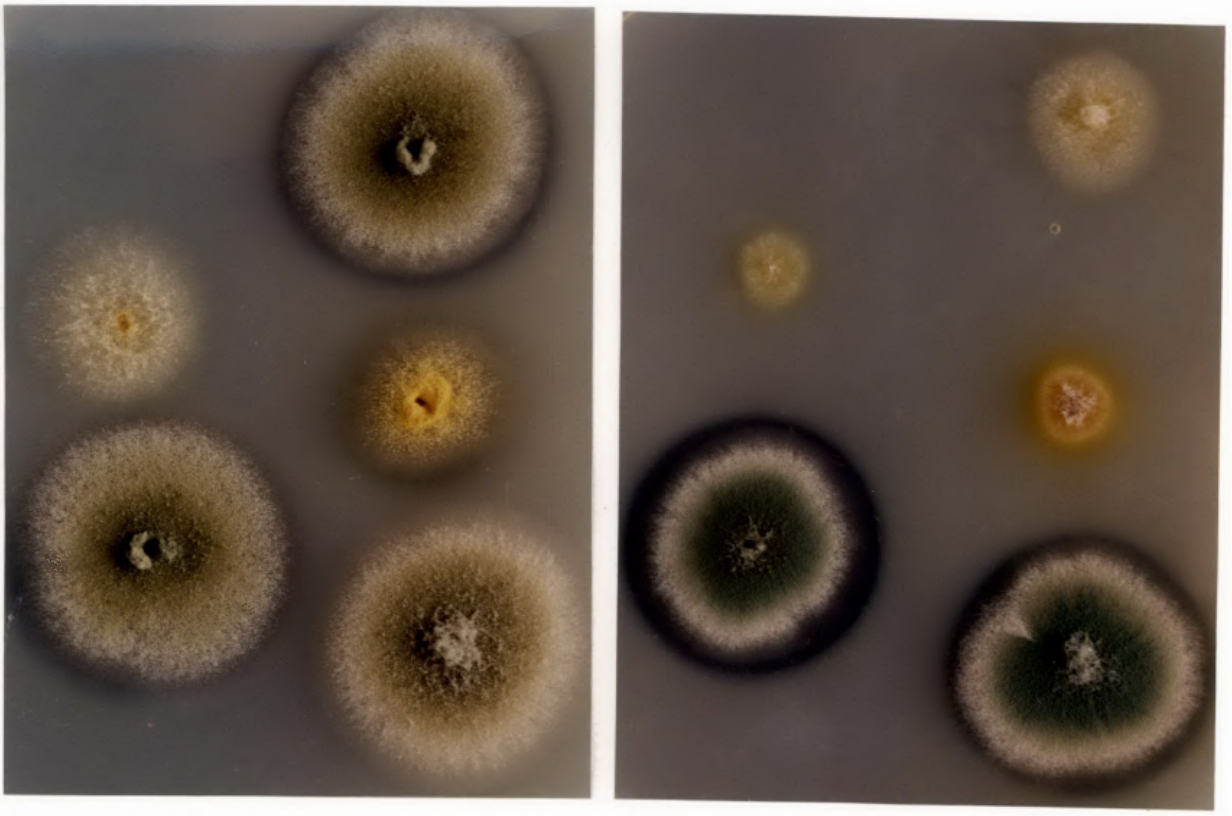

Figure 3.4. *xprF1 areA217* double-mutants on -C milk (left) and -N milk (right). Strains: *areA217* (top left), *xprF1* (bottom left), *xprF1 areA217* (top and middle right), wild type (bottom right).

Figure 3.5. *xprF1 creB15* double-mutants on -C milk. Strains: *xprF1* (top left), *creB15* (top right), wild type (bottom left), *xprF1 creB15* (bottom middle and right)

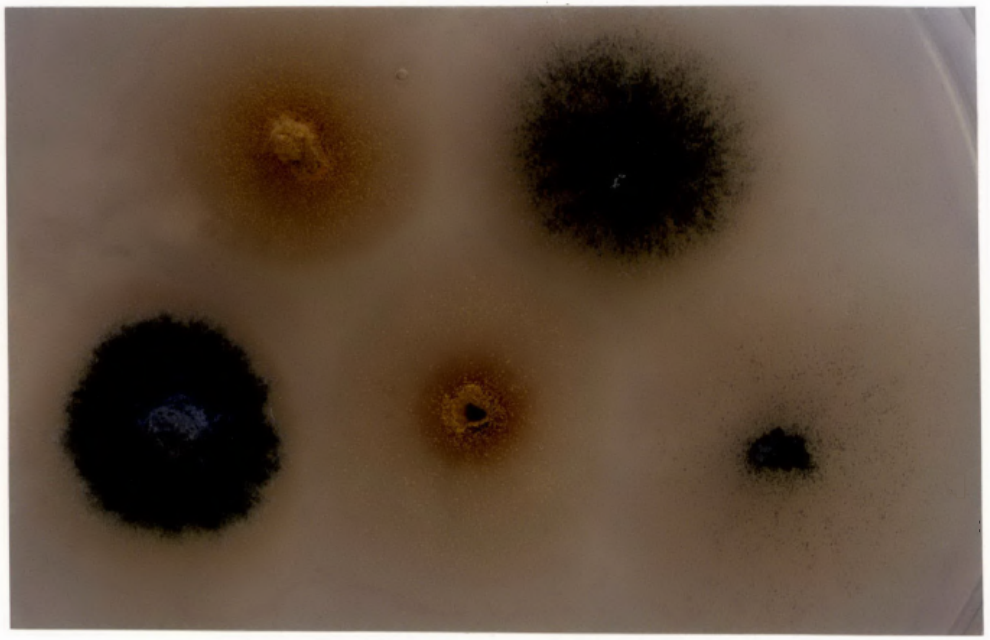

Figure 3.6. *xprF1 creC27* double-mutants hypoxanthine. Strains: *xprF1* (top left), *creC27* (top right), wild type (bottom left), *xprF1 creC27* (bottom middle and right)

Derepressed levels of milk clearing were also observed for the *xprF1 creC27* double-mutants.

3.6. Interactions between *xprF1* and Major Regulatory Circuits in *A. nidulans*

In parallel with with the study of extracellular protease activities on milk plates, extracellular protease assays were carried out to obtain a more accurate estimate of the effect of *xprF1* on the production of these enzymes under different regulatory conditions. Nitrogen metabolite repression, carbon catabolite repression and sulphur metabolite repression are the major regulatory circuits which control the metabolism of corresponding nutrients in *A. nidulans*. Since the production of extracellular proteases is controlled in response to carbon, nitrogen and/or sulphur nutrients availability (Cohen, 1973b), it was of interest to have a closer look at any influence of these regulatory systems on the production of extracellular proteases in strains with *xprF1* background.

N.B. An arbitrary unit is used for expression of extracellular protease activities in all assays. That is the activity read from the change in OD in each determination standardised to present the activity per gram of dry mycelia (section 2.5). Each assay was performed at least twice. Data presented in this section show Means with Standard Errors (SE). The number of determinations in each case (n) is presented in parantheses. SE values were calculated as follows (according to the method in Devore and Peck, 1986):

$$SE = \sigma_{(n)} / \sqrt{n}$$

where 'n' stands for the number of determinations and σ is the standard deviation.

3.6.1. Sulphur Regulation

In order to study the effect of sulphur regulation on *xprF1* strains, mycelia from an *xprF1* strain were grown under limited and unlimited sulphur source conditions and the results were compared with those of the wild-type strain (table 3.14).

Table 3.14. Extracellular protease activities under limited/non-limited sulphur supply

strain	sulphur source	activity
wild type	0.63 mM (0.01%) sodium thiosulphate	10.99±2.78 (4)
	none	226.43±17.01 (4)
xprF1	0.63 mM (0.01%) sodium thiosulphate	12.77±1.31 (4)
	none	260.01±36.34 (4)

Under non-limiting conditions, the *xprF1* strain showed low levels of enzyme activity close to those of the wild-type control. This suggests that the mutation does not result in constitutive production of the enzyme(s) since when no enzyme is required, the production is kept at the basal level. It also suggested that the *xprF1* does not result in production of an enzyme (at wild-type level) with higher enzyme activity since if it did, significantly higher levels of activities would be expected under non-limiting conditions. The latter implies that the *xprF* gene is somehow involved in the regulation of extracellular protease production.

Results presented in table showed no significant difference between levels of activity in *xprF1* and wild-type strains under limited sulphur conditions. As shown, sulphur limitation prompts a high level of protease activity in both strains which suggests that *xprF1* probably has no effect on sulphur regulation.

3.6.2. Nitrogen Regulation

In order to study the interaction between the *xprF1* mutated gene and nitrogen metabolite repression (NMR), two approaches were employed.

a) Mycelia from *xprF1* and wild-type strains were subjected to 4-hour and 16-hour starvations for nitrogen nutrients and results were compared with those of non-limiting conditions. These assays were carried out to study the effect of nitrogen regulation of extracellular protease production in *xprF1* background, and vice versa. Ammonium was used as a strong repressive nitrogen source to stimulate nitrogen metabolite repression (NMR) (table 3.15).

b) A second approach examined the effect of the *xprF1* mutation on protease production when NMR was not operating. L-alanine was used as a non-repressive nitrogen source. Mycelia from *xprF1* and wild-type strains were grown using either ammonium or L-alanine to study the response of *xprF1* and *xprF*$^+$ to the nitrogen metabolite repression without nitrogen starvation (table 3.16).

Table 3.15. Extracellular protease activities under limited/non-limited nitrogen supply

strain	**nitrogen source**	**activity**	
		4 hour	16 hour
wild type	10 mM ammonium	5.76±0.61 (5)	9.58±1.64 (6)
	none	67.80±9.48 (5)	103.01±9.47 (6)
xprF1	10 mM ammonium	7.84±1.27 (5)	11.65±2.78 (6)
	none	162.50±13.58 (5)	115.45±11.24 (6)

Under repressing conditions (table 3.15), the *xprF1* strain expressed low level of activities. This was consistent with the result from 20-hour growth under limited/unlimited sulphur source and confirmed that the extracellular protease activity is repressible in *xprF1* individuals. After 16 hours under the same nutritional conditions, both strains had slightly higher protease activity.

When starved for nitrogen, after 4 hours, *xprF1* caused a higher level of protease activity than what was seen in the wild-type. After 16 hours, activities

in both strains reached a similar level. This, therefore, suggests that the *xprF1* mutation allows a rapid response to nitrogen starvation than in the wild-type strain. However, the overall level of protease activities is no higher than wild type and, given enough time the wild-type strain shows a similar level.

Table 3.16. Extracellular protease activities under the effect of different nitrogen sources.

strain	nitrogen source	absolute activity
wild type	10 mM ammonium	11.15±2.19 (6)
	10 mM L-alanine	101.67±5.75 (6)
xprF1	10 mM ammonium	11.39±2.00 (6)
	10 mM L-alanine	110.66±24.77 (6)

Data presented in table 3.16 showed that higher levels of extracellular protease activities were observed in both strains when L-alanine was used as the sole nitrogen source. These data indicated that after the 20-hour incubation period, no notable effect was caused by the *xprF1* mutation.

To conclude, under conditions of this part of the experiment, the effect caused by *xprF1* appeared as a rapid response to nitrogen limitation. Given enough time, no significant difference from wild type was observed.

3.6.3. Carbon Regulation

A similar approach to that used in the previous section was employed. In the first approach, the effect of *xprF1* and its interaction with the carbon regulation of extracellular proteases was examined. D-glucose was used as the preferred carbon source. Experiments were carried out using both 4 hour and 16 hour starvations.

In addition, the effect of the *xprF1* mutation was tested under conditions where a derepressing carbon source was used as the sole carbon source. Mycelia were grown for 20 hours and glycerol was used to eliminate the carbon starvation whilst retaining derepressing conditions (table 3.18).

Table 3.17. Extracellular protease activities under limited/non-limited carbon supply

strain	**carbon source**	**activity**	
		4 hour	16 hour
wild type	1% D-glucose	5.76±0.61 (5)	9.58±1.64 (6)
	none	10.43±1.05 (5)	60.58±15.38 (6)
xprF1	1% D-glucose	7.84±1.27 (5)	11.65±2.78 (6)
	none	72.55±17.08 (5)	137.37±18.57 (6)

Under non-limiting conditions, low levels of extracellular protease activity were observed in both strains (similar to nitrogen starvation). When starved for carbon nutrients the wild-type strain was not responsive after 4 hours. After 16 hours activities were elevated. However, the *xprF1* strain appeared to be much more responsive to carbon limitation even after 4 hours. The rapid response in *xprF1* was similar to the one observed in response to limiting nitrogen conditions. The level of activities in the *xprF1* strain after 4 hours of carbon starvation was lower than the levels in nitrogen starvation. After 16 hours, unlike the nitrogen limiting conditions, levels of activities were still higher than those of wild type.

Glycerol is a derepressing carbon source and mycelia when grown on glycerol are expected to show derepressed levels of activities under *creA* control. Data in table 3.18 show that the level of protease production in the wild-type strain was only increased by only 2.5 folds (compared with around 7 folds in 16-hour carbon starvation). This is suggestive that protease production is not directly controlled by the *creA*-mediated carbon repression. This is in agreement with previous findings of Hynes and Kelly (1977). If so, other regulatory mechanism(s) must be involved in regulation of protease production in response to carbon nutritional conditions.

Table 3.18. Extracellular protease activities under the effect of different carbon sources.

strain	carbon source	activity
wild type	1% D-glucose	8.69±0.62 (4)
	0.5% glycerol	20.10±0.51 (4)
xprF1	1% D-glucose	15.01±3.22 (4)
	0.5% glycerol	50.43±6.40 (4)

A similar effect was observed in the *xprF1* strain (3-fold increase compared with 11-fold increase during 16-hour carbon starvation) showing that the *xprF1* mutation does not express its effect through interaction with the *creA* gene.

To conclude, results in this section showed that the pattern of elevation in extracellular protease activities in response to carbon starvation differs from that of nitrogen starvation. This implies that different mechanisms may be involved in the regulation of protease production under carbon and nitrogen limiting conditions. In addition, the *xprF1* mutation expresses a considerable effect on protease production in response to carbon starvation (and not to carbon derepressing conditions). In agreement with results from test plates on *xprF1* and *creA204*, no interacting effect on protease activities was observed here. The *xprF1* mutation causes a rapid reaction, in comparison to wild type, and high levels of activities are progressive over long term starvation (unlike in nitrogen starvation).

3.6.4. Interactive Carbon and Nitrogen Regulations

In this chapter, the effect of *xprF1* was described while either carbon catabolite repression or nitrogen metabolite repression was operating. It was, therefore, of interest to examine the behaviour of *xprF1* when both of these regulatory circuits were active.

Both strains' responses to either 4 or 16-hour starvations were fairly similar to their responses to carbon starvation rather that nitrogen starvation.

Table 3.19. Extracellular protease activities under limited/non-limited carbon and nitrogen supplies

strain	**carbon/nitrogen source**	**activity**	
		4 hour	16 hour
wild type	10 mM ammonium; 1% D-glucose	5.76±0.61 (5)	9.58±1.64 (6)
	none	15.22±4.29 (3)	59.40±15.16 (4)
xprF1	10 mM ammonium; 1% D-glucose	7.84±1.27 (5)	11.65±2.78 (6)
	none	49.70±5.15 (2)	126.90±29.30 (4)

This firstly, may support the idea of involvement of different mechanisms for elevation of extracellular protease production in response to carbon and nitrogen limitation and secondly may imply that when the organism is starved for both carbon and nitrogen, the mechanism presumably involved in the response to carbon limitation overrides the second mechanism involved in nitrogen regulation.

A brief summary of the whole section gives the following impressions about the effect of *xprF1*:

- *xprF1* has no effect when preferred carbon and nitrogen nutrients are available,

- data suggest that *xprF1* is at least indirectly involved in the regulation of extracellular proteases production,

- *xprF1* has no effect on sulphur regulation,

- *xprF1* expresses itself as a rapid response to both carbon and nitrogen limitation (4-hour starvation),

- in response to combined carbon and nitrogen limitation, the pattern of extracellular protease activities in the *xprF1* strain (like the wild-type strain) is similar to that of carbon starvation and not to that of nitrogen starvation.

Chapter 4

RESULTS:

Molecular Isolation of the xprF *Gene*

Some genetic aspects of the *xprF1* mutated gene were investigated (Results from Chapter 3). It was found that the *xprF* locus was located on the chromosome VII and the mutation was recessive (for the effect on protease production) to the wild-type allele. Cloning the gene, in long term, could help provide more detailed information about the nature of the gene and its function at molecular level in the future. In order to isolate the gene, transformation of *xprF1* strain with the wild-type copy using a chromosome-specific wild-type genomic DNA library (Brody *et al.*, 1991) formed the back-bone strategy.

Due to the unselectable nature of the effect caused by *xprF*$^+$ gene, it was not possible to select *xprF*$^+$ transformants. Therefore, a selectable marker was co-transformed with *xprF1*. pAN222 was the co-transforming DNA used in the isolation of *xprF1*. This recombinant DNA carried wild-type *prnD* and *prnB* genes (Hull *et al.*, 1989) and when introduced to the cell, was able to complement the *prn*Δ *309* deletion. J9 (*pabaA1; prn*Δ *309*) carrying *prn*Δ *309* was crossed to AM4 (*yA1 acuE; xprF1; niiA4 riboB2*) to add this deletion to the *xprF1* strain. AM5 (*yA1 acuE; prn*Δ *309; xprF1; niiA4 riboB2*) was purified and subjected to co-transformation experiments. AM6 (*pabaA1; prn* Δ *309; xprF1; niiA4*) was isolated, in addition, as a substitute.

4.1. Transformation

203 Lorist2 and 265 pWE15 cosmid clones were to be tested by transformation. Among these cosmid clones it was hoped to isolate one clone which carried the wild-type copy of *xprF*. Transformations were carried out for Lorist2 clones in two stages, as follows:

stage A. Transformations were carried out for all Lorist2 (kanamycin-resistant) clones pooled into 9 cosmid pools. Two wild type transformants were isolated and genetically purified which were designated as AM5-1 and AM5-2, respectively That is, among all *prn*$^+$ transformants which were isolated from proline plates (all pAN222+cosmid treatments) and screened on milk plates, only two colonies appeared to show wild-type levels of milk clearing. Since these transformants were obtained from the pool 73(1), the last one in Lorist2 cosmid series, transformation with the rest of pools, *i.e.*, pWE15 clones, was discontinued.

stage B. At this stage, transformation was carried out for all individual cosmid clones in the 73(1) pool. Using the same method for screening, four transformants (AM5-3, AM5-4, AM5-5 and AM5-6) were isolated from pAN222+**L32F12** treatment. This indicated that the insert in L32F12 carried the wild-type copy of the *xprF* gene.

The efficiency of transformation was not consistent, with several variables affecting the results. As a result, parameters varied among individual transformation experiments. Table 4.1 represents averaged values for these parameters from all transformations at both stages A and B.

4.2. Analysis of Transformants

Transformants were subjected to different tests to show they were genuine transformants. To discriminate them from possible contaminants, their

genetic make-up was compared with that of AM5, the strain used for transformation. Then, integration of transforming DNA was examined by DNA dot blotting and genetic crosses.

Table 4.1. Averaged variables in transformations.

(per treatment↓)	**stage A**	**stage B**
no. of non-protoplasts[a]	5.40×10^3	8.36×10^2
no. of protoplasts[b]	1.93×10^6	2.10×10^6
amount of pAN222 (co-transforming)	4 µg	4 µg
amount of cosmid DNA	3 µg	4 µg
no. of contaminants[c]	0.60	0
no. of pAN222 transformants[d]	140.33	67.20
no. of pAN222+cosmid transformants	N.A.†	N.A.‡
no. of transformants per µg of pAN222	35.08	16.80
no. of transformants per µg of cosmid	N.A.	N.A.‡

a. The number of colonies on "H2" plates with taking the dilution factor in account. *b.* The number of colonies on "S4" plates taking the dilution factor in account and then subtracting the number of non-protoplasts. *c.* The number of colonies on "-DNA" plates. *d.* The number of colonies on pAN222 plates.

† Because cosmid clones were not selectable, it was not possible to distinguish pAN222/cosmid transformants. However, the average number of *prn*$^+$ transformants for pAN222+cosmid treatments was 168.00.

‡ Similar comments as "†", but the average number of *prn*$^+$ transformants was 31.00. However for the cosmid carrying the *xprF*$^+$, there were 4 transformants out of 35 transformed *prn*$^+$ colonies which definitely carried both pAN222 and cosmid clones.

4.2.1. Confirmation of Genetic Markers

Markers on all genetically-purified transformants were compared with those of AM5 (table 4.2).

Table 4.2. Comparison of genetic markers between transformants and AM5.

strain	proline	acetate (C source)	nitrate	-paba	-ribo
niiA4	++	++	-	++	++
y paba mas	++	-	++	-	++
AM5	-	-	-	++	-
AM5-1	++	-	-	++	-
AM5-2	++	-	-	++	-
AM5-3	++	-	-	++	-
AM5-4	++	-	-	++	-
AM5-5	++	-	-	++	-
AM5-6	++	-	-	++	-

Data showed that all transformants carried exactly the same markers as AM5. The only exception was the growth on proline plate which was due to transformation of AM5 with pAN222.

4.2.2. Confirmation of Integration

A set of dilutions of genomic DNAs from AM5-1 and AM5-2 were probed with labelled pBR322 and λ DNA and compared with a negative control (*niiA4*).

Both AM5-1 and AM5-2 genomic DNAs showed strong signals when probed with labelled pBR322 (fig. 4.1). Since pAN222 contains pBR322 sequences, this indicates the presence of pAN222 in bothAM5-1 and AM5-2 transformants.

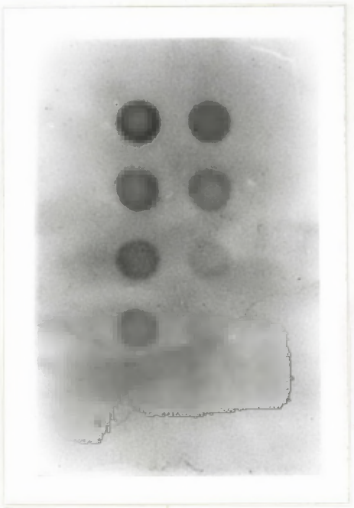

Figure 4.1. DNA dot blot on nitrocellulose membrane probed with pBR322. From left: wild-type (not visible due to no hybridisation), Am5-1, AM5-2
From top: 1 μg, 500 ng, 250 ng, and 125 ng of each corresponding uncut genomic DNA in each lane.

AM5-1 showed weak signals (hardly notable for AM5-2) when probed with labelled λ DNA (figure not shown since it was not possible to reproduce signals on photographs). There could be two reasons to explain this: first, although most of the Lorist2 vector consists of λ DNA sequences, the *Aspergillus* genomic DNA insert in L32F12 is at least 35 kb larger than the vector. This might increase the chance for loosing the hybridised probe during the stringency washes as the amount of homology between two sequences could not be more than 10-12%; second, since the whole λ genome was labelled, it is possible that the amount of DIG-labelled nucleotides inserted in the probe was not high enough (the probe was not hot).

This test was rather qualitative and just gave enough proof to carry on transformations with individual cosmid clones in 73(1) pool. Southern blot analysis was carried out later so that the presence of *xprF*+ integrated copies were examined. *Eco*RI-digested genomic DNAs from wild-type, AM5-1 and AM5-3 strains were probed with labelled *Eco*RI fragments from L32F12. Six distinct bands were observed for wild-type lane showing the presence of *A. nidulans* genomic DNA in L32F12. In addition to these bands, transformants exhibited extra bands which implies the presence of other sequences in transformants (and not in wild-type strain) homologous to some of labelled L32F12 fragments (figure 4.2). These bands were unlikely to be of vector origin since the wild-type *xprF* phenotype were retained in transformants. Therefore, extra bands could be due to integration of L32F12 bearing an intact *xprF*+ copy which resulted in novel restriction fragments. This observation

when compared with results from the DNA dot blot (mentioned above) strongly indicates the successful co-transformation of transformants with both L32F12 and pAN222.

Experiments were performed in order to obtain direct evidence for the number of L32F12 integrated copies using genomic DNAs from all transformants in Southern blot and DNA dot blot experiments. However, no conclusive results were obtained from these approaches probably because of two reasons: firstly, the genomic DNAs from some of transformants were degraded and did not produce detectable signals. Secondly, signals from DIG-labelled probes did not give objective results even in dot blot analysis. It seems that the application of probes labelled with radio-isotope ^{35}P may have given better signals.

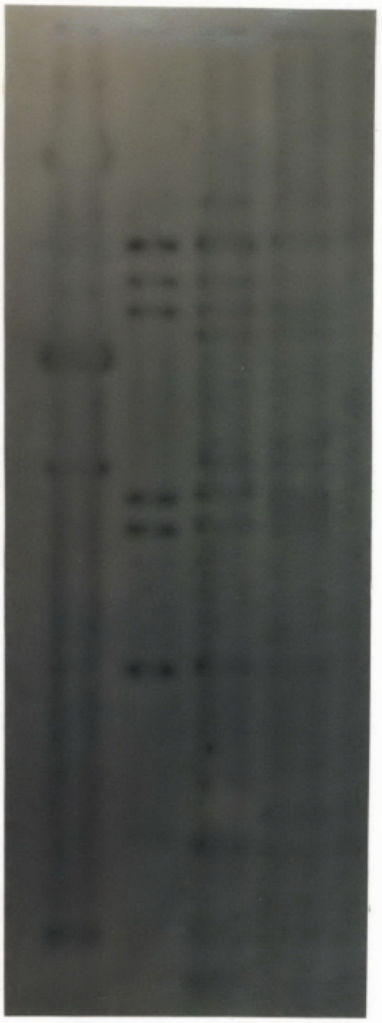

Figure 4.2. Southern blot analysis of genomic DNAs from AM5-1 and AM5-3 transformants probed with DIG-labelled *Eco*RI fragments from L32F12. 1 μg of genomic DNA was resolved in each lane. From left: λ/*Hind*III standard, wild-type, AM5-1, AM5-3.

4.3. The Morphology of Transformants

xprF1 individuals show a particular morphology on solid media containing different nitrogen sources in the presence of D-glucose as the carbon source. As previously explained, milk plates and minimal plates containing 10 mM hypoxanthine were used to characterise the morphology of these individuals which allowed easy identification of *xprF1* individuals from wild-types.

Transformants obtained from co-transformation experiments were grown on hypoxanthine and their morphology was compared with that of *xprF*$^+$, *xprF1* and *xprF1/xprF*$^+$ (diploid) strains (fig. 4.3). The level of milk clearing is also shown in figure 4.4.

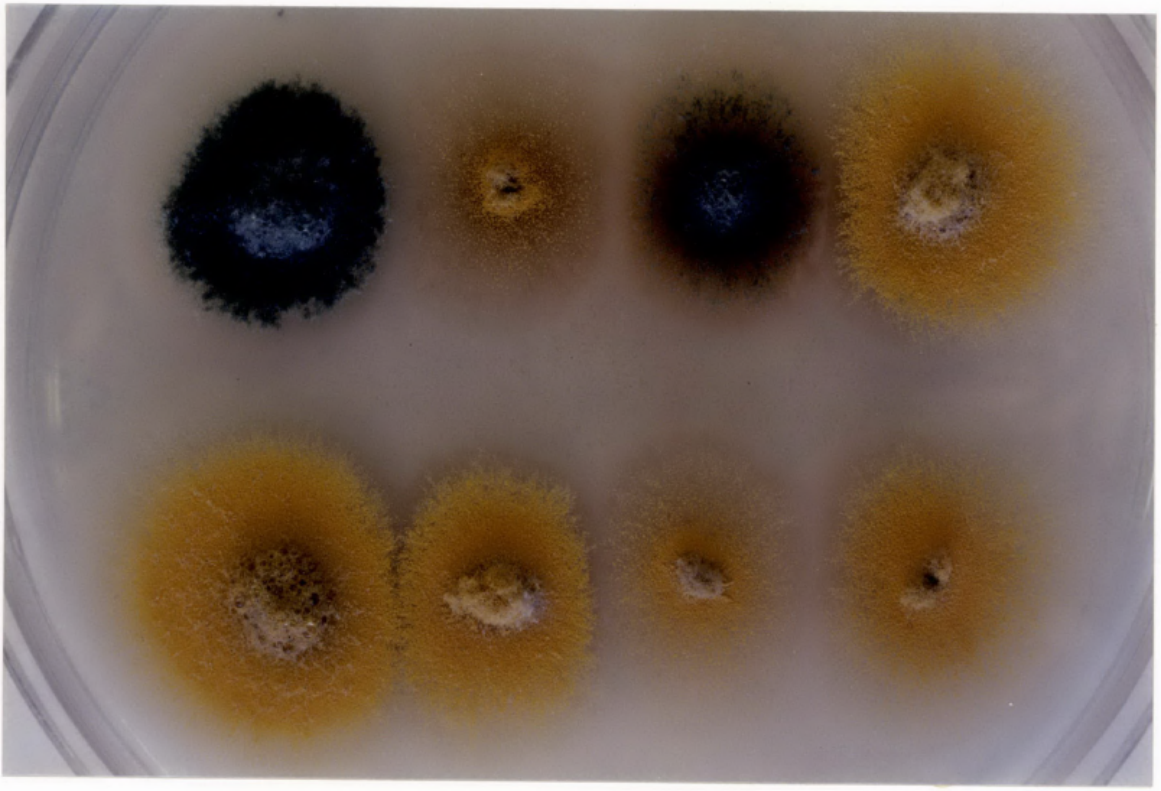

Figure 4.3. Growth and morphology of different transformants on hypoxanthine (after 3 days). Strains: top from left: wild type, AM5, diploid, AM5-1
bottom from left: AM5-3, AM5-4, AM5-5, AM5-6

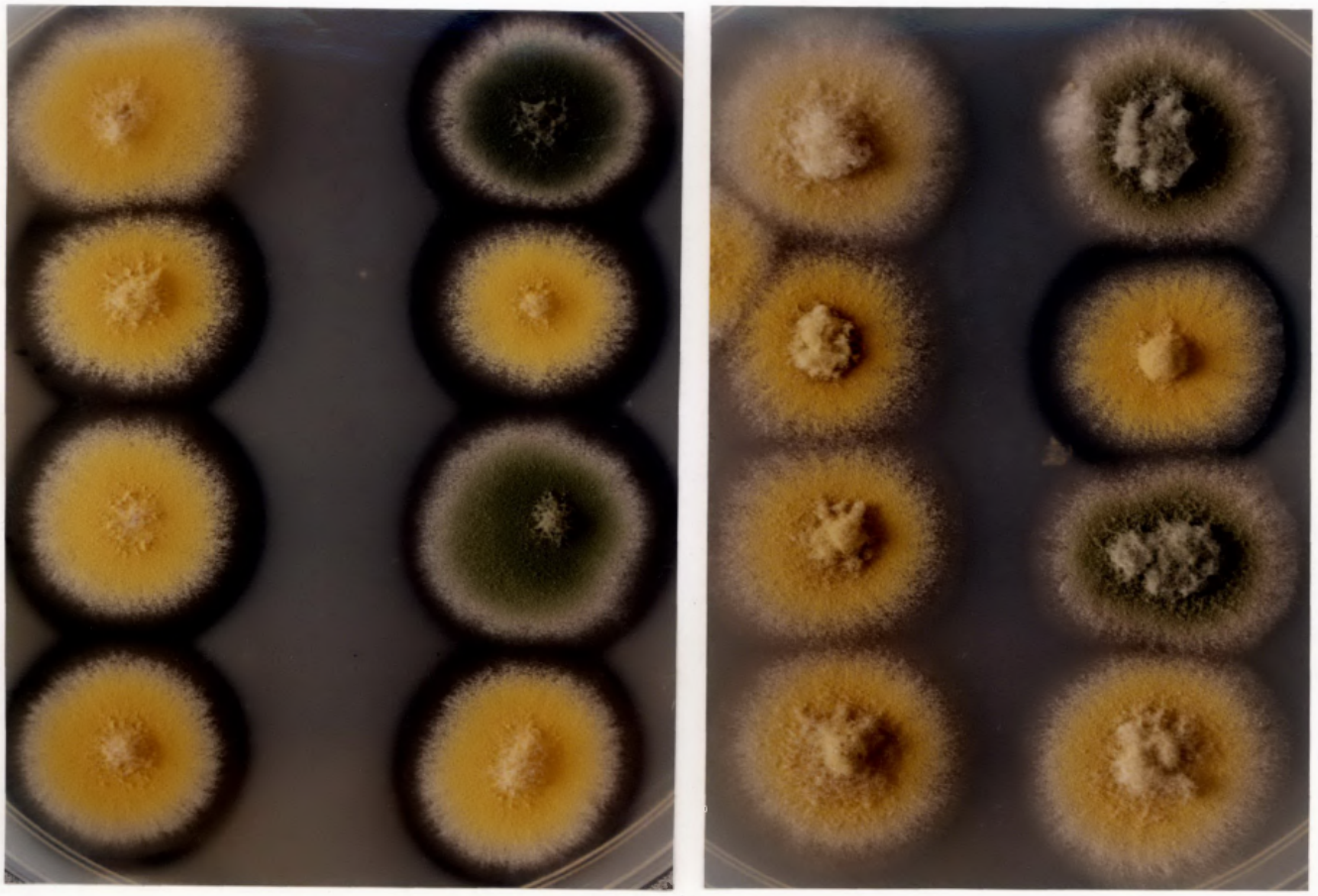

Figure 4.4. Milk clearing activities in different transformants on -N (left) and -C (right) milk plates. Strains in each photograph: left from top: AM5-3, AM5-4, AM5-5, AM5-6
right from top: wild type, AM5, diploid, AM5-1

As is seen in figure 4.3, different transformants showed different morphologies when grown on hypoxanthine. While AM5-1 and AM5-4 had a morphology similar to that of wild-type, AM5-5 and AM5-6 were similar to the diploid. However, the brown background colour in AM5-5 and AM5-6 was not as much as that of the diploid. Interestingly, AM5-3, when compared to the wild-type strain, had a more vigorous growth.

The milk clearing activity in transformants were comparable to the wild-type strain (fig. 4.4). The only exception was the AM5-3 strain which showed no milk clearing, even on -N milk plate.

The phenotypes of transformants, specially that of AM5-3, suggest that the transformation of the AM-5 strain by the *xprF*+ retains the wild-type phenotype. This clearly demonstrates the effect of *xprF1* on milk clearing and the utilisation of hypoxanthine as a secondary nitrogen source. The variation in phenotypes of transformants is likely to be due to the level of exprerssion of introduced *xprF*+ copy/copies.

4.4. Segregation of Integrated DNA

Transformed strains were able to grow on proline plate and behaved like wild-type strain on -C milk plate. This showed that transformants contained intact *prnB*+, *prnD*+ and *xprF*+ genes. DNA dot blot and Southern blot analyses confirmed that the integration had taken place. However, it was of interest to know more about the site of genomic integration.

Tilburn *et al.* (1983) reported on both homologous site and non-homologous site integration events. Findings of Yelton *et al.* (1985) were consistent with this concept. They also found that during homologous events, both additions and replacements take place. Three transformed strains were outcrossed to study integration events here.

AM5-1 × J9

AM5-1 was one of transformants with wild-type phenotype isolated during transformation experiments. It was outcrossed to J9 which carried *xprF*+ and *prnΔ 309*. Neither parents showed milk clearing activity on -C milk. AM5-1 was also able to grow on L-proline as the nitrogen source. This

cross was designed to show whether the *xprF1* mutation was still existing in AM5-1 and to provide information about integration events.

47 segregants were tested on L-proline and different milk plates. Four groups were observed (table 4.3).

Integration of L32F12 could take place either at the *xprF* locus or somewhere else within the genome. Integration could also happen as the addition of *xprF*$^+$ copy (copies) or replacement of *xprF1* (if at homologous site). If integration happens at the homologous site, *i.e.*, the *xprF* locus, whether it is addition or replacement, it would be expected to result in 100% of segregants with *xprF*$^+$ phenotype since the other parent (J9) is wild type. On the other hand, integration of a wild-type copy of *xprF* at a place not linked to *xprF* locus is expected to result in segregation of 25% of individuals as *xprF1*. If integration is brought about on the same chromosome and close to the *xprF* locus (linkage), depending on the distance between two sites, the frequency of *xprF1* is expected to fall in the range between 25% and 0.

Table 4.3. Results from the cross AM5-1 × J9

group*	milk clearing†			growth on L-proline‡	number	frequency
	a	b	c			
1	++	+±	-	++	11	23%
2	+++	+++	+++	++	1	2%
3	++	+±	-	-	19	40%
4	+++	+++	+++	-	16	35%

* No direct prediction could be made about the genotype of *xprF*$^+$ segregants since there could be only one *xprF*$^+$ allele come from J9 or there could be an *xprF1* complemented by one or more *xprF*$^+$ copies after transformation. However, individuals in groups 1 and 3 expressed parental phenotypes of AM5-1 and J9, respectively.

† scores are for the size of halo relative to that of *xprF1* control.

‡ scores for the amount of growth relative to that of AM5-1 control.

a. 1% glucose; 1% skim milk (nitrogen source)

b. 1% skim milk (nitrogen and carbon source)

c. 10 mM ammonium; 1% skim milk (carbon source)

In this case, 37% of segregants expressed the *xprF1* phenotype. This is a direct evidence that AM5-1 is a genuine transformant. It also suggests that

non-homologous integration has occurred and the *xprF1* has not been replaced. Assuming that integration has taken place at a site not linked to the *xprF* locus, the $\chi^2 = 3.13$ value for differences between expected and observed numbers of segregants in each group is not statistically significant (P>0.05).

According to Tilburn *et al.* (1983), integrated copies appear to be meiotically unstable whilst being stable through mitosis. Provided that no replacement of the mutated gene takes place, meiotic instability results in an increase in the frequency of the segregants with the mutated phenotype. In this case, meiotic instability can partly explain why there are more than 25% *xprF1* segregants. The insignificant value of χ^2 , however, suggests that the deviation could be due to chance. If so, L32F12 inserts must be fairly stable through meiosis.

Since J9 carried the *prnΔ 309* mutation, no comments could be made on segregation of *prn*$^+$ and *prn*$^-$ individuals. This is because, whether integration is at homologous or non-homologous site or even if it is a replacement and not an addition, 50% of segregants will be expected to be *prn*$^-$. Instead, the *prn*$^-$ genotype of J9 was of a help to see whether L32F12 and pAN222 integrate at the same site or not. If these two transforming DNAs integrate at the same site, firstly, the two inserts will segregate together and no *xprF1 prn*$^+$ individual would be expected, and secondly, 50% of *prn*$^-$ segregants will be expected to be *xprF1*. In contrast, if transforming L32F12 and pAN222 integrate at different sites, 12.5% of segregants of the cross will be expected to be *xprF1 prn*$^+$ and 25% of *prn*$^-$ segregants will be expected to be *xprF1*. Among 47 segregants of this cross one *xprF1 prn*$^+$ segregant (2% of segregants) was observed. This could suggest that crossing-over was possible between L32F12 and pAN222 inserts in AM5-1. The low frequency of recombinants could be, then, explained by the genetic linkage. That is, the integration sites could be close enough to reduce the frequency of crossing-over. The second explanation is that the *xprF1 prn*$^+$ segregant was resulted from the loss of L32F12 in a transformant (transformed by both L32F12 and pAN222) due to meiotic instability and L32F12 and pAN222 had integrated at the same site. In this cross, 46% of *prn*$^-$ segregants were *xprF1* which is closer to 50% rather than 25% ($\chi^2 = 0.26 \Rightarrow$ P>0.05). The latter observation suggests that in AM5-1, L32F12 and pAN222 have integrated at the same site.

AM5-5 x HNA795

HNA795 carried wild-type copies of *xprF* as well as all genes in the *prn* gene cluster. 48 segregants were tested on L-proline, hypoxanthine and different milk plates.

Table 4.4. Results from the cross AM5-5 x HNA795

group*	milk clearing†			growth on L-proline‡	number	frequency
	a	b	c			
1	++	+±	-	++	19	40%
2	++	+±	-	-	14	29%
3	+++	+++	+++	++	12	25%
4	+++	+++	+++	-	3	6%

* No direct prediction could be made about the genotype of *xprF*$^+$ segregants since there could be only one *xprF*$^+$ allele come from HNA795 or there could be an *xprF1* complemented by one or more *xprF*$^+$ copies after transformation. However, parental phenotype (wild type) of both AM5-5 and HNA795 was as is seen in group 1.

† scores are for the size of halo relative to that of *xprF1* strain.

‡ scores are for the amount of growth relative to that of HNA795 control.

a. 1% glucose; 1% skim milk (nitrogen source)

b. 1% skim milk (nitrogen and carbon source)

c. 10 mM ammonium; 1% skim milk (carbon source)

For convenience, data are analysed for *xprF1* and *prnΔ 309* separately. Data for growth on hypoxanthine will be discussed later.

31% of segregants in this cross expressed *xprF1* phenotype. The χ^2 value for the difference between the expected and observed numbers of segregants (if integration is not at the homologous site) is not significant at the 0.05 level (χ^2 = 1.00 $\Rightarrow$ P>0.05). This suggests that the *xprF*$^+$ copy has integrated at a place other than the *xprF* locus and is probably not linked to it.

The question of where pAN222 integrated relative to the *prn* locus was examined in the same way as for L32F12 integration. The differences between expected and observed numbers of *prn*$^-$ and *prn*$^+$ segregants is not significant at 0.05 level (the frequency of *prn*$^-$ segregants is 35%; χ^2 = 4.17). The non-

significant χ^2 value for differences, therefore, suggests that the integration has not been at *prn* locus.

To investigate whether pAN222 and L32F12 integrate at the same site, data from this cross were used to examine two contradictory hypotheses. As discussed before, it is unlikely that either L32F12 or pAN222 has integrated at its corresponding locus. The first hypothesis is that L32F12 and pAN222 inserts are linked (integrated together) and not linked to either *xprF* or *prn* loci. Then, a ratio of 1:1:1:5 would be expected for *xprF1*, *prn*$^-$, *xprF1 prn*$^-$ (double mutant) and wild-type segregants, respectively. Expected and observed numbers and their difference between them were as follows:

	wild type	*xprF1 prn*$^+$	*xprF*$^+$ *prn*$^-$	*xprF1 prn*$^-$
expected number	30	6	6	6
observed number	19	12	14	3
difference	11	5	8	3

$\chi^2 = 22.22$

The opposite hypothesis that non-homologous integration of L32F12 and pAN222 have occurred at separate independent sites would lead to a predicted ratio of 3:3:1:9 for *xprF1*, *prn*$^-$, *xprF1 prn*$^-$ (double mutant) and wild-type segregants, respectively. Expected and observed numbers for each group are mentioned in table next page.

	wild type	*xprF1 prn*$^+$	*xprF*$^+$ *prn*$^-$	*xprF1 prn*$^-$
expected number	27	9	9	3
observed number	19	12	14	3
difference	8	3	5	0

$\chi^2 = 6.15$

As the χ^2 values show, differences are significant for the first hypothesis ($P<0.001$) whereas for the second hypothesis, differences are not significant at

0.05 level. This would suggest that in AM5-5, L32F12 and pAN222 have not integrated together.

As Tilburn *et al.* (1983) and Yelton *et al.* (1985) described, multiple integrations of transforming DNA are likely to take place. Multiple integration usually leads to a chain of DNA inserts at the same place (tandem integration) or it may involve random independent integration events spread throughout the genome. The effect caused by *xprF1* on milk clearing is completely recessive to the wild-type allele whereas the effect on the utilisation of hypoxanthine is incompletely dominant. The latter means that the integrated copies of *xprF*+ might express additive effects on hypoxanthine plate. AM5-5 showed a phenotype close to the diploid strain. The diploid strain carries one copy of each *xprF1* and *xprF*+ and therefore, it might be suggested that the AM5-5 carries one active copy of *xprF*+. By assuming only one *xprF*+ copy is present in AM5-5, three phenotypic groups would be expected. Segregants were tested on hypoxanthine plate and three phenotypic groups were observed. What follows compares observed and expected numbers for each group.

presumed genotype	growth on hypoxanthine	milk clearing on -C milk plate	expected number	observed number
xprF+ or *xprF*+ *xprF*+	+++	-	24	24
xprF1 xprF+	+±	-	12	9
xprF1	±	++±	12	15

The differences for expected and observed numbers are not significant at the 0.05 level ($\chi^2 = 1.50$). Results from this cross suggest that if there is more than one integrated *xprF*+ copy in AM5-5, the multiple copies have probably integrated at the same site (tandem integration). On the other hand, comparison between the phenotypes of segregants in the second group (similar to AM5-5) with that of diploid strain suggests that AM5-5 is likely to have only one active copy of *xprF*+.

AM5-3 x HNA795

The third *xprF1 xprF+* transformant outcrossed was AM5-3. 48 segregants of this cross were tested on hypoxanthine and different milk plates.

Table 4.5. Results from the cross AM5-3 x HNA795

group	presumed genotype	milk clearing* a	b	c	number	frequency
1	*xprF+* $(xprF^+)_n$	±	±	-	9	19%
2	*xprF1* $(xprF^+)_n$	++	+	-	11	23%
3	*xprF+*	++	++	-	13	27%
4	*xprF1*	+++	+++	+++	15	31%

* scores are for the size of halo relative to that of AM5-3 control.
a. 1% glucose; 1% skim milk (nitrogen source)
b. 1% skim milk (nitrogen and carbon source)
c. 10 mM ammonium; 1% skim milk (carbon source)

For integration of *xprF1* it was assumed that the place of integration is not linked to the *xprF* locus and also *xprF+* inserts are integrated stably in the recipient genome. The observed numbers were not significantly different from expected numbers (χ^2 = 1.00) at 0.05 level (P>0.05). Therefore, comments discussed for previous crosses are valid and are not repeated here.

presumed genotype	growth on hypoxanthine	milk clearing on -C milk plate	observed number
$(xprF^+)_n$ or *xprF1* $(xprF^+)_n$	+++	-	33
xprF1	±	++±	15

AM5-3 showed strong growth on hypoxanthine which was different from both AM5-5 and diploid strains. Also it expressed milk clearing activities at

levels lower than wild-type strains. The qualitative comparison between the number of integrated *xprF+* copies in AM5-5 and AM5-3 was made by testing the segregants of this cross on hypoxanthine plate. Only two phenotypic groups were identified:

The strong growth of AM5-3 on hypoxanthine when compared to that of AM5-5, suggests that either more copies of *xprF+* are present in AM5-3 or if there is only one inserted *xprF+*, it is expressed at high levels. Considering the additive effect of *xprF+* on the utilisation of hypoxanthine (in the presence of *xprF1*), if there are several *xprF+* copies in AM5-3 which have been integrated at different sites, a range of growth rates among segregants would be expected as different combinations of integrated copies are possible during the meiosis. This is not the case here and all segregants without milk clearing on -C milk show equally strong growth on hypoxanthine. This implies that all presumed *xprF+* copies are present at the same site and segregate together.

Results from the last two crosses are consistent and suggest an additive effect of *xprF+* with respect to the utilisation of hypoxanthine. They also indicate the negative effect of *xprF+* on milk clearing as the strong expression of the *xprF+* in AM5-3 causes more extreme effects than that of one *xprF+* copy in a wild-type strain (table 4.5).

4.5. Extracellular Protease Activities in Transformants

Transformants varied in their morphology on hypoxanthine and the level of milk clearing for one of them (AM5-3) was considerably lower than for the wild-type strain (fig 4.4). Mycelia from a number of wild-type transformants were subjected to extracellular protease assays (4-hour starvations) and results were compared to those of wild-type and *xprF1* strains (table 4.6). Due to the variability of data normally seen in protease assays and because the assay was performed only once, data in table 4.6 are intended only to show the general difference caused by the presence of *xprF+* copy (copies) after transformation events.

Levels of activities in transformants were generally lower than those of *xprF1* and very close to wild-type levels. The drop in levels was considerable particularly for -C and -C-N conditions when compared with activity levels in AM5. This included drops above 3 folds for -C; above 4 folds in -C-N and below 1.7 folds in -N. These drops can be due only to addition of wild-type copy (copies) of *xprF* in transformed strains. Except from figures for AM5-3 under -N conditions, these findings are consistent with results from milk clearing activity tests on different milk plates. That is, firstly *xprF1* is recessive to the wild-type allele (for extracellular protease production), secondly, the *xprF* gene is involved in the regulation of extracellular protease production and thirdly, the *xprF*$^+$ has a negative effect on the production of extracellular proteases.

Milk clearing of AM5-3 on -N milk plate is lower than that of wild-type strain. This effect is repeatable and has been observed several times in different tests. According to this observation, activities in table 4.6 under nitrogen limited supply are expected to be lower than wild-type. It is advisable to repeat extracellular protease assays under the same conditions with AM5-3 and wild-type to see whether these figures are repeatable. A general point to consider is the difference between -N conditions in assays and -N milk. While in the former there is no nitrogen source, in the latter, milk is present as the nitrogen source. In addition, in -N milk medium non-protein low-molecular weight nitrogen sources are present. This might explain slight differences between behaviours under these two conditions, but for considerable differences, at this stage, no reason could be suggested by the author.

Table 4.6. Extracellular protease activities under limited/non-limited carbon and/or nitrogen supply

nitrogen/carbon source	**activities in different strains**									
	wild type		*xprF1*		*xprF1,xprF*$^+$ (AM5-1)		*xprF1,xprF*$^+$ (AM5-3)		*xprF1,xprF*$^+$ (AM5-5)	
	absolute	relative	absolute	relative	absolute	relative	absolute	relative	absolute	relative
10 mM ammonium; 1% D-glucose	3.11	1.00*	2.94	0.95	3.84	1.23	1.21	0.39	3.57	1.15
10 mM ammonium	10.19	3.28	28.34	9.11	9.93	3.19	9.06	2.91	10.97	3.53
1% D-glucose	55.04	17.70	105.84	34.03	82.22	26.44	65.14	20.95	86.40	27.78
none	8.17	2.63	54.46	17.51	10.90	3.50	8.76	2.82	12.93	4.16

* All relative values are based on the activity of the wild-type strain under non-limiting conditions.