Chapter 5

GENERAL DISCUSSION

Proteins can act as nitrogen, carbon and/or sulphur sources in *A. nidulans* when preferred low-molecular weight nutrients are not present in the medium. The presence of exogenous proteins does not induce the production and subsequent release of extracellular proteases. Indeed, the production of these enzymes is controlled by nitrogen, carbon and sulphur repressions and starvation for only one of these nutrients triggers the production of proteases (Cohen, 1973b). This implies the existence of a multiple control circuit which regulates the synthesis of the enzyme in response to different nutritional conditions. The existence of such systems for the control of metabolism of those nutrients which, like proteins, can perform as carbon and nitrogen sources supports this idea [*e.g.*, utilisation of L-proline (reviewed in Marzluf, 1981) and acetamide (Hynes, 1978)].

In agreement with these speculations, extracellular protease assays showed that under non-limiting nitrogen, carbon and sulphur supply, production of extracellular proteases in wild-type strains is kept at low levels. These results were almost similar for *xprF1* strains as well. Test of both wild-type and *xprF1* strains on -N milk + ammonium, showed a similar result.

5.1. *xprF1*

Strains expressing the *xprF1* phenotype show high levels of milk clearing. In addition, they show higher-than-wild-type levels of extracellular protease activities particularly under short term (4 hours) starvation for carbon and starvation for both carbon and nitrogen nutrients. Genetic data (Chapter 3) show that the phenotype could be due to either a single mutated gene or a number of tightly linked genes. The latter possibility is not likely as pleiotropic effects caused by the *xprF1* mutation are similar to those caused by the *xprG1* mutation (discussed later in this chapter). The *xprG* gene is not linked to the *xprF* gene. If various effects of the *xprF1* were due to mutations in different genes, it would be unlikely to have similar effects by a mutation in another gene in another part of the chromosome. The possibility of presence of two sets of these genes (including *xprF*) due to duplication events is not relevant as in genetic crosses, *xprF1* appears to segregate as a single copy (and not two copies). The *xprF* locus is located 25 map units left of the *gatA* gene on chromosome VII (map of the right-hand end of chromosome VII from Clutterbuck, 1993).

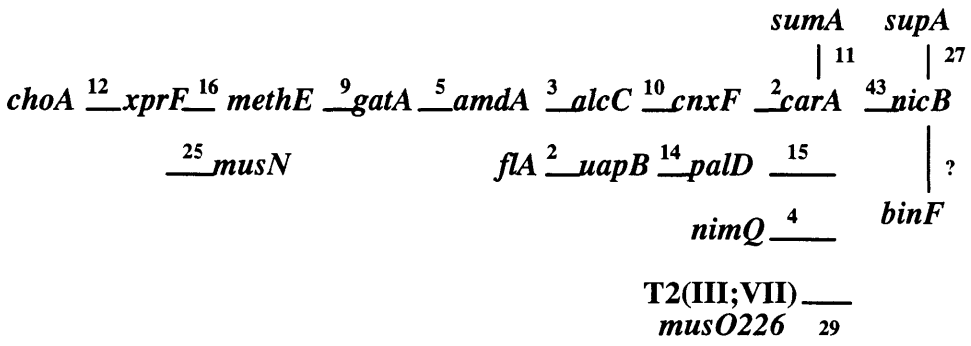

Figure 5.1. Proposed location of the *xprF* gene on the chromosome VII.

Diffrent levels of extracellular protease activity in *xprF1* strains in response to different nutritional conditions and the pleiotropic effects of *xprF1* on utilisation of a variety of nitrogen sources (and the morphology of colonies on these media) suggest the regulatory nature of the *xprF* gene. The pleiotropic

effects of *xprF1* also suggest the *trans*-acting character of the gene. See Section 5.3 below for speculation about the possible nature of the *xprF* gene.

The *xprF1* mutation is completely recessive to the wild-type in respect of its effect on protease production. Since the level of protease production in *xprF1* strains is elevated, one possibility is the negatively-acting nature of the $xprF^+$ and the *xprF1* mutation being a loss-of-function mutation. The *xprF1* is, however, incompletely dominant to $xprF^+$ with respect to the utilisation of nitrogen sources.

5.2. Isolation of the *xprF* Gene

Co-transformation studies on an *xprF1* strain showed that the effect of *xprF1* on the milk clearing is relieved in transformants due to complementation by wild-type copy (copies) of *xprF*. Transformants expressed a range of growth rates and morphologies on hypoxanthine. Although wild-type levels of extracellular protease activities were rescued in all transformed strains, one transformant expressed lower-than-wild-type levels of activities with very strong growth on hypoxanthine. Despite the fact that no direct evidence is available to show the number of integrated copies, these observations indicate that the variability among transformants could be due to the level of expression of wild-type *xprF* [*i.e.*, dose effect (Arst and Scazzocchio, 1985) for utilisation of different nitrogen sources]. This might be of help in speculation of the function of *xprF*.

In comparison to works of Tilburn *et al.* (1983) and Yelton *et al.* (1985) indicating the occurrence of both homologous-site and non-homologous-site integration events, genetic data suggests that homologous-site integration of transforming *xprF* [L32F12 (Brody *et al.*, 1991)] seldom occurs. Also when compared with integration of pAN222, L32F12 integrated copies appear to be more stable during meiosis.

5.3. The Function of the *xprF* Gene

Different repressing systems are speculated to control protease production in *A. nidulans* (Cohen, 1973b). The nitrogen metabolite repression is mediated by the positively-acting *areA* gene. An analogous gene in *Neurospora crassa*, *nit2*, mediates the nitrogen catabolite repression (Hanson and Marzluf, 1975, and Reinert and Marzluf, 1975, cited in Hanson and Marzluf, 1975) which once introduced to *areA*$^-$ strains of *A. nidulans*, is able to complement *areA*$^-$ mutation and rescue normal nitrogen metabolism in many cases (Davis and Hynes, 1987). The mechanism for control of protease production in *A. nidulans*, nevertheless, differs from that of *Neurospora* as, according to Cohen *et al.* (1975), in *N. crassa* it is controlled by induction and repression systems.

There are a number of observations which suggest that *areA* is not the only positively-acting factor in transcription of extracellular proteases structural genes:

- An *areA*$^-$ (*areA217*) strain appeared to lack both ability to grow and produce extracellular protease activity on -N milk plates. This is consistent with what is expected, since the activator function of the *areA* product is essential in response to nitrogen limiting conditions. This deficiency was not suppressed by *xprF1* in the *areA217 xprF1* double-mutant. On -C milk plate, however, *areA217 xprF1* double-mutants showed milk clearing activities similar to those of *xprF1* individuals. On -CN milk plate, *areA217 xprF1* individuals also expressed milk clearing activities. The milk clearing observed in the presence of *areA217* in double-mutants on -C and -CN plates suggests that other activating factors, possibly involved in carbon regulation, are also involved in extracellular protease production.

- In extracellular protease assays, high levels of protease activities under limiting sulphur conditions (where preferred repressing nitrogen and carbon sources are present) similarly suggests that other regulatory factors are involved in protease production.

- Extracellular protease activities in assays under -CN conditions are close to those of -C rather than -N. This also suggests the presence of other factors that can even override the effect of *areA* under -CN conditions. It is noteworthy that this effect is not seen on -CN milk plates. As a matter of fact, wild-type strains show no milk clearing activity on -C plates and therefore, no comparison can be made. These observation can not be directly compared

since, in milk media, milk proteins are utilised as the nitrogen and/or carbon source and real starvation does not exist. In addition, low-molecular weight carbon and nitrogen sources are available in milk.

Intracellular status of ammonium and L-glutamine/L-glutamate are reported to effectively influence the expression of the *areA* gene (Kinghorn and Pateman, 1972, Hynes, 1974, Arst, 1981 and Kusnan *et al.*, 1987). There are, in contrast, nutrients which while serving as nitrogen sources, do not activate nitrogen repression (*e.g.*, L-alanine). Extracellular assays investigating the effect of L-alanine did not show a difference between *xprF1* and wild-type strains. Figures were not noticeably different from -N conditions.

In the current study another *areA* mutant was used. The *areA102* mutation changes the specificity of the *areA* protein for its different receptors and hence, causes a variety of consequences. Although according to Hynes (1974), this mutation elevates the level of extracellular protease activities in extracellular protease assays under limited nitrogen conditions, this mutation appeared to affect the milk clearing on -C milk plate neither in *areA102* nor in *areA102 xprF1* strains. This, consistent with the milk clearing activity of *areA⁻ xprF1* double-mutant on -C milk plate, supports that the *areA* gene is not involved in production of extracellular proteases in response to carbon limitation. Observations made on *areA102 xprF1* and *areA217 xprF1* suggest that the *xprF* gene does not directly interact with *areA*. However, data from extracellular protease assays under limited nitrogen conditions show that protease activities are elevated in *xprF1* strains. So although the *xprF* gene product may not be involved in the *areA* function, it seems to affect the nitrogen regulation of extracellular protease production.

- In addition to nitrogen repression, carbon repression seems to operate on the metabolism of nutrients that can be used as nitrogen and /or carbon sources. For instance, *creA204*, *creB15* and *creC27* mutations are able to suppress *areA⁻* (*areA217*) and allow growth on acetamide or L-proline as carbon sources (Hynes and Kelly, 1977). This implies the activation of related structural genes without the function of *areA* gene product. Given proteins, similar to above-mentioned nutrients, can be utilised as both carbon and nitrogen source, activation of transcription of extracellular protease structural genes can be also activated by activators other than *areA* in response to different nutritional limitations.

Consistent with Hynes and Kelly (1977), this study suggested that the *creA* gene has no effect on the regulation of extracellular protease production since *creA204* mutation which increases all activities under *creA* control, did not affect the milk clearing. *creB15* and *creC27* appeared to elevate the level of milk clearing particularly on -C milk plate. While *creA204* showed no genetic interaction with *xprF1*, both *creB15* and *creC27* appeared to have additive effects in *creB15 xprF1* and *creC27 xprF1* double-mutants. Surprisingly, *creB15* and *creC27* expressed negative effects on the utilisation of hypoxanthine similar to that of *xprF1*. This effect was more extreme in double mutants than in any of the *xprF1*, *creB15* or *creC27* parents. It is hard to explain how these mutations can affect the utilisation of nitrogen sources while being involved in carbon regulation.

Neither *xprF1* was subjected to tests performed on *creB* and *creC* mutations (by Hynes and Kelly, 1977) nor *creB15* and *creC27* were tested on other situations which *xprF1* was checked. It would be of interest to compare these mutations in more detail. Despite the similarities between *xprF1* and *creB* and *creC* mutations, *xprF1* does not seem to have as broad effects as *creB15* and *creC27* do. Furthermore, there are substantial differences between *xprF1* and *creB15* and *creC27* mutations, such as the negative effect of *xprF1* on utilisation of acetamide in the presence of sucrose (*creB15* and *creC27* cause vigorous growth) and the insensitivity of *xprF1* to allyl alcohol in the presence of sucrose. *xprF1* does not have any noticeable affect on the utilisation of various carbon sources.

The *xprF1* mutation appeared to have a substantial impact on the production of extracellular protease under limiting carbon conditions. *xprF1* strains expressed elevated milk clearing on -C and -CN milk plates. On -C plates, the *xprF1* completely changed the wild-type phenotype, *i.e.*, while wild-type strains show no milk clearing, *xprF1* strains had large halos. In extracellular protease assays, the *xprF1* caused highly elevated levels of activities with a rapid response to short-term carbon starvation. These observations suggest the involvement of the *xprF* gene in carbon regulation of extracellular protease production.

Four regulatory loci (*scon* genes) have been reported to be involved in sulphur repression (Natorff *et al.*, 1993). This study did not investigate the relationship between *xprF1* and mutations in these loci. However, according to

M.E. Katz *et al.* (unpublished data), none of *scon* mutations appeared to affect the production of extracellular proteases significantly. Results of this study obtained from extracellular protease assays suggest that *xprF* gene is not involved in regulation of extracellular proteases in response to sulphur limitation.

Summarising all aspects of the *xprF1* phenotype, it can be seen that under certain conditions, the *xprF1* expresses itself independently to the presence/absence of preferred carbon and nitrogen sources. The weak growth on hypoxanthine is seen in the presence of glucose and absence of ammonium whereas elevated milk clearing activities on -C milk medium occurs in the presence of ammonium and absence of glucose. One possibility is that the *xprF* gene is expressed in response to both carbon and nitrogen limitations. But if so, it is hard to understand why when the *xprF* gene is expressed in response to carbon limitation, it also affects the nitrogen metabolism. *xprF1* strains show elevated levels of extracellular protease activities whereas in *xprF1* *xprF*$^+$ transformants, the figures go back to normal levels. One transformant (AM5-3) even shows milk clearing at levels lower than that of wild-type strains. This suggests the possible negative-acting nature of the *xprF* in regulation of extracellular protease production. The weak growth of *xprF1* strains on secondary nitrogen sources and the dose effect seen in *xprF1* *xprF*$^+$ transformants on hypoxanthine medium (incomplete dominance of *xprF1* to *xprF*$^+$) imply the possible positive-acting nature of *xprF* in respect to utilisation of these nitrogen sources and also suggests that the *xprF* gene is transcribed at low levels. It is possible that the *xprF* gene is expressed constitutively (irrespective to carbon and nitrogen repression) at low levels and dictates a positive effect on the utilisation of secondary nitrogen sources while having a negative role in regulation of extracellular protease production. This hypothesis would help to explain the dose effect seen in transformants when grown on hypoxanthine.

It is unlikely that the *xprF* gene product is a positive regulator of the production of extracellular proteases as it can not explain the lower-than-wild-type milk clearing of AM5-3. That is, whatever the effect of *xprF1*, the milk clearing activities in AM5-3 should not fall below the wild-type level. The negative-acting role of *xprF* in utilisation of secondary nitrogen sources could not be the case, either. That is because if *xprF1* is a loss-of-function mutation,

we would expect strong growth in *xprF1* individuals on hypoxanthine (for instance) and if it is a gain-of-function mutation, even weaker growth would be expected in diploid and transformed strains.

Proteins seem to be of one of lowest priorities as carbon or nitrogen nutrients as their catabolism is more costly and lagging in providing anabolic metabolites. Such an observation is made in extracellular protease activities under limiting carbon conditions where it seems that the wild-type organism does not switch to utilisation of exogenous protein until a certain stage. Therefore, there could be a regulatory mechanism which gives the priority to the utilisation of secondary nitrogen sources while repressing the activities needed for utilisation of proteins. If the *xprF* is involved in this regulatory system, its continuous expression at low levels ensures the adequate availability of enzymes required for utilisation of secondary nitrogen sources under normal conditions with protein utilisation being postponed to the stage when the organism is in trouble. When this happens, the production of extracellular proteases or other pathways related to protein utilisation would be controlled by distinct mechanisms such as the availability of the *xprE* gene product and also the nitrogen activator (the *areA* protein) and the putative carbon activator (as yet unidentified) and their specific activating natures. The latter can explain the differences in extracellular protease activity profiles in response to carbon and/or nitrogen starvation.

The *xprG1* mutation shows the same effects as *xprF1*. These mutations, occurred in two unlinked loci, suppress the *xprE1* mutation. According to M.E. Katz (unpublished data), the *xprG1* mutation behaves in a very similar manner to the *xprF1*. This suggests that both genes are probably involved in the same regulatory pathway. It would be incorrect to assume that *xprE1* is a major mutation (*e.g.*, a deletion) in the regulatory region of a major extracellular protease structural gene leading to loss to activation of transcription by carbon and nitrogen activators and *xprF1* and *xprG1* mutations alter the structural genes that let the transcription take place. The reason is that *xprF1* and *xprG1* have occurred in two unlinked genes and the *xprE1* can not be the same mutation in two different structural genes. Besides, *xprF1* and *xprG1* can not be two suppressing mutations accidentally with same characteristics. Instead, the *xprE* gene product could be a factor with a positive-acting nature involved

in the regulation of extracellular protease production. There are three possible explanations:

a) *xprF1* and *xprG1* are mutations in the *cis*-acting regulatory region of two structural genes for extracellular proteases resulting in suppression of *xprE1* mutation and elevated levels of protease production. This seems unlikely because it would not explain the effect of these mutations on the utilisation of nitrogen sources. Nor would it explain the fact that these two mutations are recessive to their wild-type alleles.

b) *xprF* and *xprG* gene products are parts of a regulatory complex or they form a regulatory dimer which is involved in regulation of protease production.

c) both *xprF* and *xprG* genes are involved in production of a metabolite whose intracellular concentration affects regulatory factors involved in extracellular protease production and utilisation of nitrogen sources. If so, the presumed metabolite must either inhibit positively-acting factor(s) involved in protease production or stimulate a negatively-acting factor in the same system. Explanation of the effects on utilisation of secondary nitrogen sources still remains difficult unless we assume that the factor affected by the metabolite has a contrasting wide-domain acting nature. Since it seems likely that different transcription activators are involved in the production of proteases, for the former assumption, the presumed metabolite has to interact with at least two different carbon and nitrogen activators.

Our current knowledge of the *xprF* and *xprG* genes and their interactions is not enough to reject any of the above possibilities. Anyhow, the change in these two genes, leads to a common consequence which affects the utilisation of secondary nitrogen sources and the production of extracellular proteases. The *xprF2* mutated gene is an allele to *xprF1* and results in similar effects on the utilisation of nitrogen sources. Its effect on protease production, however, is not as extreme as that of *xprF1*. It is possible that *xprF2* is a leaky mutation allowing some residual activity of the *xprF* gene product while *xprF1* affects the function of the gene more drastically.

Suppression of the *xprE1* mutation by *xprF1*, *xprF2* and *xprG1* mutations implies that *xprF* and *xprG* products can not be repressors acting directly on protease structural genes. That is, because the loss of the negative effect caused by either *xprF1*, *xprF2* or *xprG1* mutations would not bypass the need for the

presumed positive-acting product of *xprE*. In other words, removing the repressor would not initiate the transcription.

Young and Marzluf (1991) reported on finding homologous sequences of *N. crassa nmr* gene in *A. nidulans* genomic DNA and suggested the possibility of an *A. nidulans* gene analogous to the *nmr* gene. The *nmr* gene is a negatively-acting regulator which, in addition to positively-acting *nit-2,* is involved in regulation of nitrogen metabolism in *N. crassa* as mutations in *nmr* (unlinked to *nit-2*) results in insensitivity to nitrogen repression (Premakumar *et al.*, 1980 and DeBusk and Ogilvie, 1984). *xprF* could not be analogous to the *N. crassa nmr* gene for at least two reasons. First, loss-of-function mutations in *nmr* cause derepression in all activities under *nit-2* control whereas this is not observed in *xprF1*. *xprF1* shows neither tighter repression nor derepression of activities under *areA* control. Second, *xprF* appears to have effects under limiting carbon conditions whereas no such an effect has been reported for *nmr*.

Proposed Model for the Function of the *xprF* Gene

Because of the complexity of regulatory circuits in eukaryotic systems and considering the limited information available for functional characteristics of *xprE1, xprF1, xprF2* and *xprG1* mutations, proposing a concrete model for the function of the related loci is not yet possible. Therefore, the model proposed here remains mainly tentative and is a first attempt to co-relate all data obtained for the above mutations.

Since the *xprF* gene does not appear to be involved in sulphur regulation, the sulphur control of extracellular protease production is excluded from this model. The model is based largely on what is known about regulation of transcription in eukaryotes, and in particular fungi, as described in Chapter 1. In this model it is assumed that the products of *xprF* and *xprG* genes operate directly in the regulation of either extracellular protease production or utilisation of secondary nitrogen sources.

According to the model (fig. 5.2), the *xprE* product is necessary for activation of extracellular protease production by nitrogen and carbon activators. This might happen either by binding to activators to facilitate their binding to a target site or by binding to activators after they have bound to their

target sites and then facilitate transcriptional activation. Products of *xprF* and *xprG* genes are assumed to be proteins which form a dimer. The dimer (FG) acts directly on the *xprE* product preventing it from activating transcription. The binding of the FG dimer and the *xprE* product is reversible.

Then, *xprF1*, *xprF2* and *xprG1* mutations alter the conformation of the FG dimer or change its affinity for binding to the *xprE* product in a way that the FG dimer no longer bind to the *xprE* product. Therefore, *xprE* would be absolutely free (in respect to negative effect of the FG) to take part in transcription activation. The level of protease production, then, depends on absence/presence of nitrogen and carbon activators as well as their particular regulatory natures. So under non-limiting conditions there would be no elevated levels as there is no activator. The *xprE1* mutation could cause a change in the *xprE* product that leads to a irreversible or highly-stable binding between the FG dimer and the *xprE* product. That is, *xprE* would be locked and *xprE1*, therefore, leads to loss of extracellular protease production under limiting carbon and nitrogen conditions.

It must be remembered that the exact nature of the *xprE1* mutation is not known yet, but what is known indicates that the presumed positive role of the *xprE*$^+$ product is lost in *xprE1* strains and this does not necessarily mean that the *xprE1* product is unfunctional. In *xprE1 xprF1*, *xprE1 xprF2* and *xprE1 xprG1* double mutants, due to the loss of affinity of the FG dimer for the *xprE* product, the latter can still function as the negative effect has been removed. It does not produce as high levels as *xprE*$^+$ *xprF1* , possibly due to the change in its structure which reduces its functional efficiency. The other possibility is that *xprE1* mutation reduces the level of *xprE* transcription without changing its affinity for the FG dimer. Therefore, in the *xprE1* strain, the low amount of produced *xprE* is all blocked by the function of FG dimer and due to inadequate availability of the *xprE* product which is required for the function of carbon and nitrogen activators, extracellular proteases are not produced at high levels under limiting carbon and nitrogen conditions. But in mutated *xprF* or *xprG* strains, due to the removal of the repressing effect, low amounts of available *xprE* is enough to produce extracellular proteases, but not as high as when wild-type *xprE* is operating. The FG dimer could interact directly/indirectly in activation of activities needed for catabolism of secondary nitrogen sources. Its positive effect would be abolished due to mutations in *xprF* and *xprG* genes which change the affinity of the dimer for binding to the corresponding regulator(s).

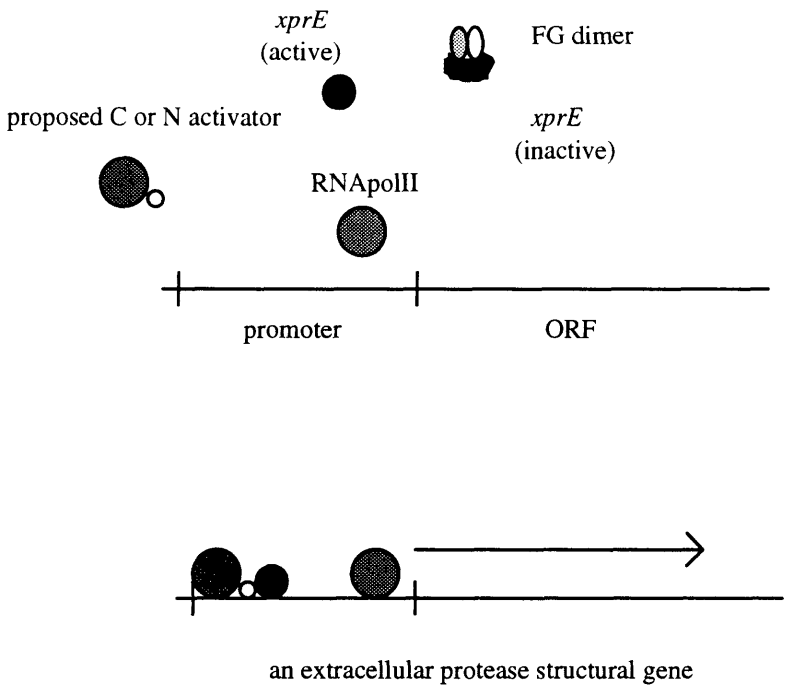

Figure 5.2. Proposed model for the function of *xprE*, *xprF* and *xprG* gene products in regulation of extracellular protease production. Binding of FG dimer (*xprF-xprG* products) inhibits *xprE* product from interacting with activators to elevate transcription levels.

This model for the function of *xprF* and its interaction with *xprE* and *xprG* genes can explain the following observations in this study:

- Suppression of *xprE1* by *xprF1*, *xprF2* and *xprG1*.

- Weaker growth on hypoxanthine as an incomplete dominant trait with dose effects in transformants.

- Higher levels of extracellular protease activities on milk plates. In particular, on -C milk plate, the wild-type strain does not produce halo as the low-molecular weight carbon sources are enough to repress elevation of carbon activator and/or *xprE*. The effect of low amount production of these factors is inhibited by the function of FG dimer. By removing this effect, activators can initiate the extracellular protease production. This possibility is yet to be investigated on -C dialysed milk medium where the low-molecular weight carbon and nitrogen sources are excluded. If the speculation of the *xprF*

function is right, wild-type strains must produce milk clearing at lower amounts than the *xprF1* strain.

- The rescue of the wild-type levels of milk clearing in transformants as the negative acting FG dimer retains the repression. AM5-3 with lower-than-wild-type milk clearing can be explained by super active expression or the multiple copy integration of integrated *xprF*$^+$.

- Rapid response of *xprF1* strains in extracellular protease assays in response to nitrogen and carbon starvations. Difference between figures in -N and -C would be due to different natures of carbon and nitrogen activators and also their activation efficiency. -CN is close to -C probably due to unknown interactions between carbon and nitrogen regulatory systems.

- Absence of genetic interaction between *xprF1* and *areA*$^-$ on -N milk as with removal of the active nitrogen activator in *areA*$^-$, under N limiting conditions, extracellular proteases are not produced.

- Milk clearing activity of *areA217 xprF1* double-mutants on -C milk as the extracellular protease production can be started by only the carbon activator and removal of the negative effect of FG dimer facilitates the protease production by the available amounts of carbon activator and *xprE*.

- Additive effects of *creB15*, *creC27* and *xprF1* on milk clearing. The mode of action of *creB* and *creC* genes is not known. But as a result of *creB15* and *creC27* mutations, extracellular protease activities on milk plates is elevated (particularly on -C milk medium). Since these mutations are reported to be involved in the carbon regulation, they probably dictate their effect through the carbon activator. Therefore, higher activity of the carbon activator together with the absence of negative acting FG dimer is expected to elevate the milk clearing activities even higher than *xprF1*, *creB15* and *creC27* strains. The slight derepression (not a very big halo after 48 hours) of extracellular proteases on the -N milk plate supplemented with ammonium in *xprF1 creB15* and *xprF1 creC27* double mutants can be due to more-active-than-wild-type carbon activator which is not stopped by the effect of FG dimer.

Further Studies

Further investigations are required to clarify the mechanism of action of the *xprF* gene. These include performing studies to test the proposed model as well as exploring discussed possibilities about the nature of the gene.

- M.E. Katz *et al.* (unpublished data) showed that the regulation of the only isolated structural gene for one of extracellular protease in *A. nidulans*, *prtA*, takes place at the transcriptional level. This suggests that the regulation of production for other extracellular proteases in this organism could probably follow the same trend. However, after isolation and molecular characterisation of other structural genes for proteases the level of mRNA transcripts from these genes can be examined by Northern blot analysis of RNA preparation from mycelia grown under different nutritional conditions. The results from the wild-type strain could show whether the regulation takes place at transcription level. That is, if the elevation of extracellular protease production is due to the increase in transcription rate for the protease structural genes, more mRNA transcripts must be detected (stronger signals for corresponding mRNAs) in mycelia subjected to carbon and/or nitrogen limitation. If so, similar experiment can be done for *xprF1*, *xprF2* and *xprG1* strains could indicate the effect of these mutations on the level of transcription. If these mutations increase the transcription of protease structural genes, levels of mRNA transcripts in mycelia from strains carrying any of these mutations grown under limiting conditions are expected to be higher than wild-type figures under the same conditions. The structural gene for the xanthine dehydrogenase I, *hxA*, has been isolated (Davis and Hynes, 1989). In a similar approach, Northern blot analysis of mycelia grown with hypoxanthine as the sole nitrogen source could show if the regulation of xanthine dehydrogenase I production is also at transcription level. The results from wild-type and *xprF* and *xprG* mutated strains could show the effect of these mutations.

- A cosmid clone carrying the *xprF* gene has been isolated. After isolation and sequencing the gene, using an appropriate RNA probe, Northern blot analysis can be carried out in order to investigate the transcription rate of the gene. Comparison between results from wild-type and *xprF1* and *xprF2* strains could provide direct evidence if the *xprF* is transcribed continuously at a fixed level or its transcription is regulated in response to different conditions. That is, if *xprF* is transcribed continuously, signal strength detected for its mRNA transcript must be consistent in preparations from limiting and non-

limiting carbon and/or nitrogen conditions. According to the model, *xprF1* and *xprF2* do not change the transcription rate of the *xprF* gene. Therefore, the pattern of signal strength in Northern blots derived from wild-type, *xprF1* and *xprF2* strains under various conditions must remain similar.

- A similar approach could be taken for *xprE*$^+$ and *xprE1* strains (after molecular characterisation of the *xprE* gene). If the amount of *xprE* mRNA transcript in wild-type strain is constant under different nutritional conditions, it will be shown that the gene is transcribed continuously. Comparing the figures with *xprE1* transcript under various conditions then, might show whether *xprE1* affects the transcription rate of *xprE* or not.

- To investigate whether the *xprF1* and *xprF2* mutations have occurred in a similar region, after molecular characterisation of the wild-type *xprF* gene, probes can be used to isolate *xprF1* and *xprF2* mutated genes from λ genomic libraries of *xprF1* and *xprF2* strains. Comparison of DNA sequence in these strains with that of *xprF*$^+$ could show the nature of *xprF1* and *xprF2* mutations. That is, both major mutations such as deletions and inversions and minor mutations like frame-shift mutations or base-substitutions/conversions could be detected. Also, after definition of the open reading frame in the *xprF* gene, it would be possible to determine whether these mutations occur within the coding region (to possibly change the function of the gene) or they alter the upstream regulatory region of the gene which results in alterations in the transcription levels.

- After isolation and characterisation of *xprF* and *xprE* genes, site-directed mutagenesis methods can be applied to obtain more mutations in these loci *in vitro*, which would help improve genetic characterisation of these genes in respect to different possible effects conferred by them. Also, recombinant DNAs carrying different mutated copies of *xprF* (*in vitro* mutagenesis) can be introduced to a strain carrying a non-functional *xprF*. The latter would depend on being able to isolate such a strain either by site-directed mutagenesis or by gene replacement techniques. By studying the effect of mutations in different parts of the gene, essential regions and their roles could be identified.

- Molecular characterisation and sequencing the *xprF* gene will indicate whether the *xprF* codes for a polypeptide (protein) or not. If the product is a protein, the possible amino acid sequence can be analysed. this will help to explore the presence of DNA-binding motifs (*e.g.*, zinc fingers) or protein dimerisation motifs (*e.g.*, leucine zippers). Furthermore, isolation of the *xprF*

protein would allow it to be tested for specific DNA-binding *hxA* and *prtA* sequences in gel retardation assays. Retardation of these DNAs in the presence of the protein would suggest the DNA-binding capacity of the *xprF* product. A similar approach for the *xprE* gene product would show whether it has a DNA-binding nature.

According to the model, the *xprE* product has to interact with the presumed FG dimer as well as either target DNA site for activation of transcription or activator proteins themselves. This implies that the *xprE* product has to carry protein-protein binding motifs for the FG dimer and activator proteins or a protein-protein motif for the FG dimer and a specific DNA-binding motif for the target site. After molecular isolation and characterisation of the *xprG* gene, its sequence can be compared to that of the *xprF* gene. If data show that the *xprF* protein has any protein-protein interaction motif, a compatible motif would be expected to be present in the *xprG* product.

5.4. Concluding Remarks

Initial aims of this study consisted of three main approaches (section 1.6):

- **Genetic Characterisation**. Information from this work is enough to cover all related aims. *xprF1* is a single mutated gene located on chromosome VII, 25 and 30 map units left to *gatA* and *amdA* genes, respectively. It expresses pleiotropic effects on production of extracellular proteases and utilisation of secondary nitrogen sources. *xprF1* is recessive to *xprF*$^+$ for the former effect and semi-dominant for the latter one.

- **Molecular Isolation**. This goal was partially achieved by isolation of L32F12 and providing direct molecular and genetic evidence proving its integration which causes wild-type effects the *xprF* gene on milk clearing and to some extents, on utilisation of secondary nitrogen sources be resumed in transformed strains. Of course, the insert in L32F12 is very big and therefore, it has to be subclones to have the intact *xprF* gene in a clone with shortest possible fragment

- **Functional Characterisation**. In this approach, due to complicated regulatory interactions present in eukaryotic systems and regarding the information available, no direct conclusion could be made. Data suggest that the *xprF* gene may be a regulatory gene. However, an objective analysis of its nature demands more information about the *xprF* gene at molecular level (as discussed above). More mutations in this locus also would permit us to characterise the function of the gene more extensively.

Data collected in this research has contributed to understanding the scope of the regulation of extracellular protease production in *A. nidulans*. In a broader sense, it is hoped that this work helps to clarify one more corner, out of many, in regulatory systems in eukaryotes.

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APPENDICES

Appendix 1

Growth Media and Related Solutions

The following recipes are for preparation of 100 ml of each medium. For preparation of solid media 1.2% (w/v) of Oxoid agar No.1 was added prior to autoclave. For preparation of Protoplast Regeneration Medium and 2× YT agar medium 1% agar was used. For preparation of Sulphur-free media, -S Stock Salt Solution was used instead of normal Salt Solution. pH was adjusted with Sodium Hydroxide unless otherwise specified.

- **Standard -N Glucose Minimal Medium**

ingredients	amounts	pH	autoclave
D-glucose	1.000 gm		
Salt Solution	2.0 ml		
		6.5	yes

- **Carbon-free (-CN) Medium**

ingredients	amounts	pH	autoclave
Salt Solution	2.0 ml		
		6.5	yes

- **Complete Medium**

ingredients	amounts	pH	autoclave
D-glucose	1.000 gm		
Peptone	0.200 gm		
Casein,enzyme hydrolysate	0.150 gm		
Yeast Extract	0.100 gm		
Ammonium tartrate (crystals)	0.184 gm		
Salt Solution	2.0 ml		
Vitamin Solution	1.0 ml		
Riboflavin Solution	1.0 ml		
1 M Sodium thiosolphate Solution	1.0		
		6.5	yes

Whenever conodia of any strain were required, conidia were grown on complete plates by streaking them on several diagonal lines crossing in the middle of the plate. To reinforce the solid medium when scraping conidia off the plate, 2.2% agar was used.

- **Milk Plates**

Milk media were based on either -N minimal medium or -CN (carbon-free). Therefore, additives were added to the corresponding molten sterile -N or -CN media. Except, vitamin and Deoxycholate solutions, all other additives were sterilised by autoclave. Deoxycholate sodium salt was also added to different milk media. The latter is a detergent and inhibits the mycelia to spread freely. This helps to score the cleared zone around colonies. In -N, -C and -CN milk media, milk serves as the sole nitrogen source, the sole carbon source and both carbon and nitrogen sources, respectively.

-N Milk

ingredients	amounts
-N minimal medium	100.0 ml
5% Sterile Skim Milk Stock Solution	20.0 ml
10% Deoxycholate (w/v) Solution	0.8 ml
Vitamin Solution	1.0 ml

-N Dialysed Milk Medium

In order to exclude any effect of low-molecular weight carbon sources, another type of milk plate containing 1% dialysed skim milk was used in some cases. Dialysis tubes were first boiled for 10 minutes in 2% Sodium Hydrogen Carbonate ($NaHCO_3$); 1 mM EDTA. Then, they were rinsed in distilled water follwed with boiling in distilled water for 10 minutes. Cool tubes were stored at 4 °C in 2 mM Tris; 1 mM EDTA; 2 mM NaCl. Tubes were filled with 10% skim milk solution and put in distilled water while being stirred. For the first few hours the water was regularly changed. Dialysis was carried on overnight. Dialysed milk was added to -N minimal medium plus 1.2% agar to obtain 1% milk concentration. After autoclave, 1% vitamin solution was added and plated were poured.

-C Milk

ingredients	amounts
-CN minimal medium	100.0 ml
1 M Ammonium Tartrate	1.0 ml
5% Skim Milk Stock Solution	20.0 ml
10% Deoxycholate (w/v) Solution	0.8 ml
Vitamin Solution	1.0 ml

-CN Milk

ingredients	amounts
-CN minimal medium	100.0 ml
5% Skim Milk Stock Solution	20.0 ml
10% Deoxycholate (w/v) Solution	0.8 ml
Vitamin Solution	1.0 ml

- -N Sucrose Minimal Medium

This medium is prepared in the same way as -N glucose minimal medium except 1% sucrose is used instead of 1% glucose. That is 1.000 gm of sucrose is used to prepare 100 ml of medium.

- -N Sorbitol Minimal Medium

This medium is prepared in the same way as -N glucose and -N sucrose media. 1% sorbitol is used as the sole carbon source.

- Acriflavine Medium

ingredients	amounts
Complete Medium	100 ml
Acriflavine (1%) Stock Solution	0.1 ml

- Allyl Alcohol Medium

ingredients	amounts
-N Sucrose Minimal medium	100 ml
Allyl Alcohol (250 mM) Stock Solution	1.0 ml
1 M Ammonium Tartrate	1.0 ml
Vitamin Solution	1.0 ml

- Benomyl Medium

ingredients	amounts
Complete Medium	100 ml
Benomyl Stock Solution	0.1 ml

- Protoplast Regeneration Medium

ingredients	amounts	pH	autoclave
D-glucose	1.000 gm		
Sucrose	41.077 gm (1.2 M)		
Salt Solution	2.0 ml		
		7.0	yes

- **L Broth**

ingredients	amounts	pH	autoclave
Bactopeptone	1.000 gm		
Yeast Extract	0.500 gm		
Sodium Chloride	0.500 gm		
		7.0	yes

- **2× YT**

ingredients	amounts	pH	autoclave
Tryptone	1.600 gm		
Yeast Extract	1.000 gm		
Sodium Chloride	0.500 gm		
		7.0	yes

- **Selective Media**

Different selective media were used to test markers. The following is the general approach for preparation of these media:

selective media	ingredients
different Nitrogen Sources	-N minimal media + 10 mM desired nitrogen source + 1% vitamin solution
different Carbon Sources	-CN medium + 50 mM desired carbon source + 1% vitamin solution
different Vitamins	-N glucose minimal medium + 10 mM ammonium tartrate + 1% vitamin solution lacking the desired vitamin

- **Trace-Element Stock Solution**

ingredients	amounts (for 100 ml)
Sodium Tetra-borate	0.008 gm
Copper Sulphate	0.080 gm
Ferric Orthophosphate	0.200 gm
Manganese Sulphate	0.120 gm
Sodium Molybdate	0.160 gm
Zinc Sulphate	1.600 gm

- **Salt Stock Solution**

ingredients	amounts (for 100 ml)
Potassium Chloride	2.600 gm
Magnesium Sulphate*	2.600 gm
Potassium di-Hydrogen Orthophosphate	7.600 gm
Trace Element Stock Solution	5.0 ml

* For preparation of -S Salt Solution, Magnesium Sulphate was not used.

- **Vitamin Stock Solution**

ingredients	amounts (for 100 ml)
p-Amino-Butyric-Acid	0.004 gm
Thiamine HCl	0.005 gm
10% (w/v) Biotin Solution	10.0 µl
Nicotinic Acid	0.010 gm
Pantothenic Acid	0.020 gm
Riboflavin	0.010 gm
Pyridoxine	0.005 gm

Other forms of vitamin solutions were also used which lacked only one of above vitamins. Addition of any of these vitamin solution resulted in media lacking only one of vitamins (e.g., -paba or -riboflavin)

Appendix 2

Solutions

When handling star items (*), fume hood and gloves must be used.

- Acriflavine Stock Solution*

0.1 gm of acriflavine was added to 10 ml sterile water and kept refrigerated.

- Allyl Alcohol Stock Solution*

170 µl of allyl alcohol was added to 10.0 ml distilled water and kept in the refrigerator. The concentration is 250 mM.

- Benomyl Stock Solution

0.010 gm of benomyl was dissolved in 10 ml methanol.

- Chloroform/iso-Amyl Alcohol*

Equilibrated phenol and iso-amyl alcohol were mixed at 24:1 ratio and kept under fume hood.

- Gel Loading Dye

ingredients	amounts
Bromophenol Blue	0.25%
Xylene Cyanol FF	0.25%
Glycerol (in water)	30%

2× GET Solution

ingredients	concentration	amounts (for 100 ml)
D-glucose	100 mM	1.800 gm
1M Tris; pH8.0	50 mM	5.0 ml
0.5 M EDTA; pH8.0	20 mM	4.0 ml

Autoclaved.

- Equilibrated Phenol*

Frozen phenol was thawed at 65 C. 100 ml of 50% phenol; 50% TE Buffer was prepared. Aliquot of 8-hydroxyquinolone was added and after a good shake, the mixture was left to settle. The upper phase was removed and another 50 ml TE Buffer was applied and shaking was repeated. About 40 ml of the upper phase was removed. The stock solution was kept refrigerated.

- Phenol/Cloroform/iso-Amyl Alcohol*

Ingredients were mixed at 50:49:1 ratio and kept in the refrigerator. Equilibrated phenol was used for preparation.

- RNase Stock Solution

10 mg of pancreatic RNase (RNase A) was added to 10 µl of 1 M Tris-HCl; pH7.5, 15 µl of 1 M Sodium Chloride and 970 µl of sterile distilled water. The mixture was boiled for 15 minutes to remove DNase. The stock solution was kept at -20 °C.

- Sodium Orthophosphate Buffer

1 M solutions of di-Sodium Orthophosphate and Sodium di-hydrogen Orthoposphate were prepared. For this purpose 14.196 gm of di-Sodium Orthophosphate and 15.601 gm of Sodium di-Hydrogen Orthophosphate were used to prepare 100 ml of solutions, respectively. The latter has acidic nature wheras the former is basic. The acidic solution was added to the di-Sodium Orthophosphate until the desired pH (e.g., 7.2) was obtained.

- **20× SSC**

ingredients	concentration	amounts (for 100 ml)
Sodium Chloride	3 M	17.532 gm
tri-Sodium Citrate	0.3 M	8.823 gm

pH7.0.

- **Substrate for Extracellular Protease Assays**

ingredients	concentration	amounts (for 100 ml)
Azocasein	0.5%	0.500 gm
Sodium Orthophosphate Buffer; pH7.2	100 mM	10.0 ml

- **TE Buffer**

ingredients	concentration	amounts (for 100 ml)
1 M Tris; pH8.0	10 mM	1.0 ml
0.5 M EDTA; pH8.0	1 mM	0.2 ml

- **Solutions Used in Chemiluminescence Detection**

Buffer 1

ingredients	concentration	amounts (for 100 ml)
Maleic Acid	0.1 M	1.161 gm
Sodium Chloride	0.15 M	0.877 gm

pH7.0. Autoclaved.

Blocking Stock solution

10.000 gm of Blocking Reagent (appendix 3) was used to make up a 100-ml stock solution. Buffer 1 was used as the solvent. After autoclave the bottle was shaked vigorously to dissolve the blocking reagent.

Buffer 2

10 ml of blocking stock solution was diluted in 90 ml of buffer 1.

Buffer 3

ingredients	concentration	amounts (for 100 ml)
Tris	0.1 M	1.211 gm
Sodium Chloride	0.1 M	0.584 gm
Magnesium Chloride	0.05 M	1.017

pH9.5. Autoclaved.

- **Solution Used in Large-Scale Alkaline Lysis for DNA Preparations**

Solution I

ingredients	concentration	amounts (for 100 ml)
D-glucose	50 mM	0.900 gm
1 M Tris; pH8.0	25 mM	2.5 ml
0.5 M EDTA; pH8.0	10 mM	2.0 ml

Autoclaved.

Solution II

ingredients	concentration	amounts (for 100 ml)
Sodium Hydroxide	0.2 M	0.800 gm
10% SDS	1%	10.0 ml

Autoclaved

Solution III

ingredients	concentration	amounts (for 100 ml)
Potassium Acetate	5 M	29.442 gm

In fact, 29.42 gm potassium acetate makes 60 ml of 5 M solution. In addition to this 60 ml, 11.5 ml of glacial acetic acid and 10.0 ml of distilled water was added. The pH was adjusted at 4.8 using concentrated HCl. The volume was brought to 100.0 ml and then autoclaved.

- **Solutions Used in Modified Birnboim & Doly DNA preparation**

Lysozyme/RNase

ingredients	concentration	amounts (for 10 ml)
2× GET Solution:		5.0 ml
100 mM D-glucose	50 mM	
50 mM Tris; pH8.0	25 mM	
20 mM EDTA; pH8.0	10 mM	
50 mg/ml Lysozyme	2 mg/ml	400 µl
10 mg/ml RNase	40 mg/ml	40 µl

Alkaline SDS

This solution was similar to Solution II of the large-sclae DNA-preps.. Therefore, solution II was used.

Potassium Acetate

This Solution was similar to Solution III of the large-scale DNA-preps. and the latter was used, instead.

- **Solutions Used in Transformation Experiments**

Osmotic Medium

ingredients	concentration	amounts (for 100 ml)
Magnesium Sulphate	1.2 M	29.576 gm
1 M Sodium Orthophosphate Buffer; pH7.0	10 mM	1.0 ml

The final pH was adjusted to 5.8 using 0.2 M di-Sodium Hydrogen Orthophosphate solution. This solution was filter-steriled using 0.2 µm filters in Schleider and Schnell FP030/3 filter holders.

1× STC

ingredients	concentration	amounts (for 100 ml)
Sorbitol	1.2 M	21.864 gm
1 M Tris; pH7.5	10 mM	1.0 ml
1M Calcium Chloride	10 mM	1.0 ml

pH adjusted to 7.5. Autoclaved.

2× STC

ingredients	concentration	amounts (for 100 ml)
Sorbitol	2.4 M	43.728 gm
1 M Tris; pH7.5	20 mM	2.0 ml
1M Calcium Chloride	20 mM	2.0 ml

pH adjusted to 7.5. Autoclaved.

Trapping Buffer

ingredients	concentration	amounts (for 100 ml)
Sorbitol	0.6 M	10.932 gm
1 M Tris; pH 7.0	100 mM	10.0 ml

pH was adjusted to 7.0. Autoclaved.

PEG

ingredients	concentration	amounts (for 100 ml)
Polyethylene Glycol 4000	60%	60.000 gm
1 M Tris;pH7.5	10 mM	1.0 ml
1 M Calcium Chloride	10 mM	1.0 ml

pH was checked to be at 7.5 with paper pH indicator. Autoclaved.

Appendix 3

Kits

The following Kits were used in molecular isolation of the *xprF* gene. Kit components and ordering information are provided in relevant catalogues.

application	kit	producer	cat.#
DNA purification	Progenius DNA Purification Kit	Progen Industries Pty. Ltd.	600 0010
DNA labelling	Nonradioactive DNA - Labelling Kit	Boehringer Mannheim	1093 657
chemiluminescent - detection	AMPPD (Lumigen)	Boehringer Mannhein	1357 328
plasmid purification	Magic Maxipreps - Purification Kit	Promega	A7270