Pigments and Microbes

A number of different kinds of pigments are produced by microorganisms. The pigments vary in color from red, yellow, orange, purple etc. These pigments have been subjected to a lot of research not only for their colorful nature, but also because they play important roles in photosynthesis, photoprotection, pathogenesis etc. Yet other pigments are interesting because their functional role is unclear. One of this latter class of pigments, the xanthomonadins, is the topic of this thesis. This chapter is a review of the structure and function of the xanthomonadins and two other groups of microbial pigments, the carotenoids and melanins. The emphasis on these pigments is due to the possibility that they might be involved in photoprotection.

Xanthomonas

The genus Xanthomonas is comprised almost entirely of plant pathogens. The affected plants include representatives from practically all major groups of higher plants. Xanthomonads are gram-negative, aerobic, rod-shaped bacteria with a single polar flagellum. A characteristic feature of this genus is the production of copious amounts of extracellular polysaccharide. Another distinguishing feature is that most xanthomonads produce yellow pigments called xanthomonadins which are distinct from the yellow
(carotenoid) pigments produced by Erwinia, Pseudomonas, Flavobacterium and Corynebacterium strains (see Carotenoids). The characteristic presence of xanthomonadin in members of the genus Xanthomonas has led them to be used as chemo-taxonomic and diagnostic markers (Starr, 1981). A few non-pigmented xanthomonads have been reported viz. Xanthomonas campestris pathovar (pv.) manihotis (causal agent of bacterial blight in Cassava; Dye and Lelliott, 1974).

Xanthomonadin Pigment

Xanthomonadin (from Xanthomonas juglandis, a walnut pathogen) was reported to be localized at the cytoplasmic membrane by Stephens and Starr (1963). The membrane-bound nature of the pigment was determined based on the kinetics of release of the pigment from cells of X. juglandis upon sonic treatment. It was observed that the cells subjected to sonic or ballistic disintegration released xanthomonadin at a rate identical to that of a membrane component like reduced nicotinamide adenine dinucleotide (NADH) oxidase. The structure of a xanthomonadin, isolated from X. juglandis, was solved by Andrews et al. (1976) using heavy atom Patterson and Fourier crystallographic methods (Figure 1.1). Xanthomonadins were then deduced as brominated aryl polyene pigments. The presence of bromine in this molecule is unique since xanthomonads are terrestrial microorganisms which as a group rarely incorporate bromine in organic molecules. Very little information is available about the biosynthesis of this pigment. It has been postulated that the aromatic ring may be derived from the
Figure 1.1: Structure of Xanthomonadin I

Xanthomonadins are membrane bound, brominated aryl polyene pigments that are characteristic of the genus *Xanthomonas*. The structure of xanthomonadin I was described from *X. juglandis* (a walnut pathogen; Andrews *et al*. 1976).
Figure 1.2: Colonies of *Xanthomonas oryzae* pv. *oryzae*

The above photograph shows yellow colonies of *Xanthomonas oryzae* pv. *oryzae* as seen under a stereo microscope. Colonies were photographed after 4 days of incubation on Peptone Sucrose agar medium (see Materials and Methods in Chapter 2) at 28°C.
shikimate pathway and the polyene chain from the polyketide pathway (Jenkins and Starr, 1982a).

Recently, a 23 Kb gene cluster required for xanthomonadin biosynthesis in *Xanthomonas campestris* pv. *campestris* (a pathogen of crucifers like cabbage, radish etc.) was cloned by Poplawsky et al. 1993. Complementation analysis suggests that this region includes seven complementation groups. RFLP and dot blot analysis, using the xanthomonadin clone as a probe, suggested that these genes are present in all xanthomonads irrespective of whether they produce pigment or not. Sequences homologous to the xanthomonadin gene cluster were not detected in non-xanthomonads and therefore these genes could be used as diagnostic probes for the identification of xanthomonads. A subsequent report indicated that one of the genes (*pig B*) in this cluster encodes a diffusible factor required for both pigment and extracellular polysaccharide production (Poplawsky and Chun, 1997).

Pigment deficient mutants of many Xanthomonads viz. *X. campestris* pv. *campestris* and *Xanthomonas oryzae* pv. *oryzae* (causal agent of bacterial blight of rice) are fully proficient for virulence when wound inoculated onto their respective hosts suggesting that pigment is not essential for virulence of the pathogen (Poplawsky et al. 1993; Tsuchiya et al. 1982; Durgapal, 1996). A pigment deficient mutant of *X. juglandis* was observed to be more sensitive to photobiological damage when compared to the pigmented wild type strain suggesting that xanthomonadins might serve to protect the bacterium from photodamage (Jenkins and Starr, 1982b).
Xanthomonas oryzae pathovar oryzae

This organism is the subject of study in this thesis. It causes a serious disease of rice called bacterial leaf blight. The symptoms are characterized by yellowing and drying which begin at the tips of rice leaves and spread down either one or both sides of the leaf or through the mid vein (Figure 1.3). The pathogen gains entry into the rice leaf either through wounds or natural openings called 'hydathodes' that are present on the edges of the rice leaf. The bacterium multiplies inside the xylem vessels and travels through them to more distant parts of the leaf and occasionally to other parts of the plant. It also exits from the leaf in the form of ooze droplets that collect on the surface of the leaf. Dispersal of the pathogen is achieved when these ooze droplets are splashed onto neighboring uninfected plants by wind or rain. Dispersal of the pathogen from one infected leaf to other leaves on the same adult rice plant might also occur through ooze (Sridhar Dharmapuri and Ramesh Sonti, unpublished data). During the off-season, the pathogen is presumed to survive either on seeds, dried leaves and stubble or on neighboring alternate host plants (like weeds) till the next season (Alvarez et al. 1989; Figure 1.4).

The objective of this study is to determine the role of the xanthomonadin pigment in the life cycle of X oryzae pv. oryzae. One of the questions addressed is whether the pigment is required for virulence following epiphytic entry of the pathogen into the plant i.e. entry through the hydathodes after residence on the leaf surface. This is more akin to the natural mode of entry into the plant and is
Figure 1.3: Disease symptoms of bacterial leaf blight of rice

*Xanthomonas oryzae pv. oryzae* is a vascular pathogen that traverses down the xylem vessels of the leaf. A, a healthy rice leaf. B, leaf blight symptoms caused by *Xanthomonas oryzae pv. oryzae*. Infection symptoms begin at the leaf tips and advance through the veins/mid rib of the infected leaves.
The pathogen exits from infected leaves in the form of bacterial ooze. Wind and rain contribute to splash dispersal of the pathogen onto neighboring uninfected plants. During the off season the pathogen is presumed to survive in the straw, stubble or on alternate host plants. It has also been suggested that the pathogen might survive the off season in seeds.
likely to result in exposure of the pathogen to air and light. These are conditions under which pigment may enhance viability of the pathogen (by providing photoprotection). Pigment deficient mutants that were isolated after either EMS mutagenesis or marker exchange of transposon insertions obtained in cloned pigment biosynthetic genes were used in this study. Reporter gene fusions to putative pigment promoters were obtained and their expression was studied; specifically to determine whether these genes are expressed when the pathogen is present within the rice plant. During the process of cloning the \textit{X. oryzae pv. oryzae} genes involved in pigment biosynthesis, a number of methods for conducting molecular genetics on an Indian isolate of \textit{X. oryzae pv. oryzae} were standardized and these are detailed in the following chapter.

**Carotenoid Pigments**

Carotenoids are isoprenoids containing a characteristic polyene chain of conjugated double bonds and are located in chromatophores (vesicular, tubular or lamellar membranous structures) which are continuous with the cytoplasmic membrane. The two groups of carotenoids known are the 'carotenes' or hydrocarbons and their oxygenated derivatives called 'xanthophylls'. Unlike xanthomonadins that are found exclusively in xanthomonads, carotenoids are present not only in bacteria but also in photosynthetic algae, plants and some fungi. About 600 carotenoids are known to occur naturally with most of these being considerably similar to each other in structure. Carotenoids usually contain 40 carbon atoms (C40) with
acyclic 'lycopene' (Figure 1.5A) considered as the basic compound from which most, if not all, carotenoids are derived (Goodwin, 1973). The presence of carotenoids in bacteria and fungi is discussed here.

Carotenoids in photosynthetic bacteria

Approximately 60 different carotenoids have been identified from photosynthetic bacteria and these have been tabulated by Goodwin (1973). Photosynthetic bacteria are divided into two classes i.e. the purple photosynthetic bacteria and the green photosynthetic bacteria. All purple photosynthetic bacteria contain acyclic carotenoids e.g. Spirilloxanthin (Figure 1.5B). In contrast, the green photosynthetic bacteria are characterized by the presence of aromatic carotenoids e.g. chlorobactene (Figure 1.5C). In photosynthetic bacteria, two possible functions attributed to carotenoids are that they act as accessory pigments in photosynthesis and also function to provide protection against photodamage.

The first study that provided evidence for photoprotection by carotenoids was with a mutant of the photosynthetic purple bacterium Rhodopseudomonas spheroides (Griffiths et al. 1955). It was observed that the mutant strain (designated as 'blue-green') accumulated a colorless carotenoid precursor called phytoene. Bacteriochlorophyll was synthesized in the mutants but to lower amounts when compared to the wild type. The growth of this mutant strain proceeded normally under anaerobic conditions. However, under aerobic conditions, the cells of 'blue-green' were rapidly
Figure 1.5 A-C: Structures of lycopene (A), spirilloxanthin (B) and chlorobactene (C).

Around 600 carotenoid structures have been reported. Most carotenoids are derived from lycopene. An example of an acyclic carotenoid i.e. spirilloxanthin and a cyclic carotenoid like chlorobactene are shown here.
killed due to exposure to light and oxygen (in this case bacteriochlorophyll acts as the photosensitizer). It was proposed that the mechanism by which photoprotection occurs is that in the presence of excess quanta, chlorophyll gets excited to its triplet state and chlorophyll in its triplet state could mediate abnormal photosensitized reactions. Under these conditions, the carotenoid pigments serve to quench the triplet state of chlorophyll. This hypothesis is supported by the fact that carotenoid pigments can interact with the triplet state of chlorophyll (Fujimori and Livingston, 1957). It was also observed that carotenoids have the ability to quench singlet oxygen ($^{1}$O$_{2}$) and that the ability of a carotenoid to quench singlet oxygen in vitro is dependent on the number of conjugated double bonds. The efficiency of photoprotection by carotenoids was studied by a number of groups and it was concluded that the least unsaturated carotenoid that shows a protective effect was neurosporene with only 9 conjugated double bonds and that with increased desaturation there was a concomitant increase in photoprotection (Mathews-Roth et al. 1974).

Carotenoids in non-photosynthetic bacteria

A number of unique C30, C45 and C50 carotenoids are found in non-photosynthetic bacteria. Photoprotection offered by carotenoids in non-photosynthetic bacteria was first demonstrated by Mathews and Sistrom (1959) in Sarcina lutea. They observed that a carotene-less mutant of S. lutea was rapidly killed upon exposure to light, air and a photosensitizer like toluidine blue whereas the
pigmented strain was unaffected. A photoprotective effect offered by carotenoids was also observed in *Corynebacterium poinsettiae* where unlike the pigmented strain, the colorless mutant succumbed rapidly to light and air in the presence of an exogenous photosensitizer (Kunizawa and Stanier, 1958). This photosensitive effect on the mutant strain was not seen in an atmosphere of nitrogen. Similar experiments without the use of an exogenous photosensitizer were performed with *Mycobacterium marinum*. This bacterium produces carotenoids only in the presence of light; therefore, dark-grown pigmentless bacteria were compared with light grown pigmented bacteria for survival under bright light in the presence of air. The results showed that the non-pigmented bacteria succumbed more rapidly to the photodamage than the pigmented cells (Wright and Rilling, 1963).

**Fungal Carotenoids**

Many fungal species are characterized by the presence of carotenoid pigments. Both carotenes and xanthophylls have been reported. They can be found among Phycomycetes, Ascomycetes, Basidiomycetes and Myxomycetes. Two fungi, *Sporidiobolus johnsonii* and *Dacryopinax spathularia*, showed an increase in sensitivity to light if the pigment was absent indicating a role for the carotenoid pigment in photoprotection (Margalith, 1992).

**Melanin Pigments**

The black pigments often observed in microbes are considered to be melanin or melanin-like pigments. Melanins are brownish or
black pigments obtained by the oxidation of aromatic amino acids, principally tyrosine, accompanied by polymerization of the resulting derivatives. Melanins are produced by a number of bacteria for e.g. the marine bacterium *Shewanella colwanella; Proteus mirabilis* as well as Rhizobium spp. Some *Pseudomonas aeruginosa* strains were shown to produce melanin-like pigments under specific growth conditions (Margalith, 1992). A number of other microbes like *Serratia marcescens* and Streptomycetes have been shown to produce melanin or melanin-like pigments in the presence of tyrosine (Trias *et al.* 1989; Margalith, 1992). A few *Xanthomonas* spp. have also been observed to produce a brown diffusible pigment e.g. *Xanthomonas ampelina* (causes canker disease on grapevine) and *Xanthomonas phaseoli* pv. *fuscans* (which causes a disease on bean) {Dye and Lelliott, 1974}. However, it is not known whether this brown pigment is melanin or a melanin-like pigment.

In fungi, melanin production is observed in structures such as hyphae, conidia etc. (Margalith, 1992). A number of phytopathogenic fungi produce melanin (Margalith, 1992; Bell and Wheeler, 1986). One such interesting example is *Magnaporthe grisea* which causes the 'rice blast disease'. The fungus penetrates the host cell wall by increasing the hydrostatic pressure on its hyphae. Howard and Ferrari (1989) showed that melanins in the cell walls of appressoria, or swollen hyphal tips, of this pathogen imparts differential permeability leading to a high internal solute concentration. This creates a turgor pressure which facilitates the penetration into the host tissue. Melanin deficient mutants of *Magnaporthe grisea* were observed to be non-pathogenic on rice
(Chumley and Valent, 1990). Melanin biosynthetic inhibitors like 'tricyclazole' have been useful in prevention of penetration of appressoria into host tissues and are therefore being used to control the rice blast fungus (Woloshuk et al. 1983; Margalith, 1992). The human pathogenic fungus, Cryptococcus neoformans, produces melanin and a polysaccharide coat. Both these phenotypes have been associated with virulence of the pathogen (Nosanchuk and Casadevall, 1997).

In several fungi, melanins have been shown to provide protection to fungal spores against ultraviolet and solar radiation (Margalith, 1992). Survival of conidia and sclerotia over prolonged periods is correlated with the occurrence of melanins or melanin-like pigments. It was observed that melanized sclerotia or cell walls are resistant to attack by microorganisms unlike non-melanized portions of the two. Melanins have also been proposed to trap free radicals (hydroxyl radical, OH\(^{\cdot}\)) and protect cells from oxidizing conditions (Goodchild et al. 1981; Margalith, 1992).

The genes responsible for melanin biosynthesis have been described in Streptomyces lividans, Streptomyces antibioticus and from a number of Rhizobium species. Melanin biosynthetic genes from Rhizobium are reported to be present on a plasmid (Cubo et al. 1988). Melanin may be required for detoxification of polyphenolic compounds which may accumulate in senescing nodules of legumes (Margalith, 1992).

A great many other pigments, not discussed in this chapter, are produced by microbes for e.g. bacteriochlorophyll produced by photosynthetic bacteria, phenazine antibiotic pigments produced by
certain *Pseudomonas aeruginosa* strains, riboflavins produced by fungi, bacterial indigo produced by *Pseudomonas indoloxidans* to name a few. The function of some of these pigments is well established while that of others is largely unknown. The pigments whose functions are unknown are likely to be more than just secondary metabolites. Their physiological functions may become more apparent with further studies.
References


CHAPTER II

DEVELOPMENT OF METHODOLOGY FOR CONDUCTING MOLECULAR GENETIC STUDIES ON AN INDIAN ISOLATE OF XANTHOMONAS ORYZAE PATHOVAR ORYZAE

Abstract

Xanthomonas oryzae pv. oryzae causes one of the serious diseases of rice. This chapter describes the standardization of methods for conducting molecular genetic studies on our laboratory wild type strain of X. oryzae pv. oryzae, which belongs to the dominant pathotype of this pathogen in India. Conditions for gene transfer by electroporation as well as by conjugation with donor E. coli cells were standardized. A genomic library of this strain, with an average insert size of 30 Kb, was constructed in the broad host range cosmid vector pUFR034. Restriction digestion and Southern hybridization of two randomly selected clones from the library suggests that cloned DNA is not rearranged when reintroduced into X oryzae pv. oryzae. The results obtained from colony hybridizations, using previously identified sequences from a Philippine isolate of X oryzae pv. oryzae as probes, indicate that the genomic library constructed is an adequate representation of the X. oryzae pv. oryzae genome. Conditions were also standardized for performing transposon mutagenesis of cloned X. oryzae pv. oryzae DNA in E. coli using a mini-Tn5gus transposon and marker exchange of these insertions into the X. oryzae pv. oryzae chromosome.
Introduction

*Xanthomonas oryzae* pv. *oryzae* is the causal agent of bacterial leaf blight, a serious disease of rice (Starr, 1981). To date most of the research on this pathogen has been conducted using Philippine isolates. Owing to quarantine reasons, these strains could not be used in this study. This chapter describes the standardization of some methods for conducting molecular genetic studies on our laboratory wild type strain (called BXO1) of *X. oryzae* pv. *oryzae*. This strain belongs to the dominant pathotype of *X. oryzae* pv. *oryzae* in India (Reddy and Reddy, 1989; Yashitola et al. 1997). It was provided to us by Dr. A. P. K. Reddy of the Directorate of Rice Research, Hyderabad where it was isolated from an infected leaf sample collected at Chinsuria, West Bengal.

Conditions for gene transfer into BXO1 by conjugation with donor *E. coli* strains as well as by electroporation were standardized. A genomic library of *X. oryzae* pv. *oryzae* was constructed in the broad host range cosmid vector pUFR034 (DeFeyter et al. 1990) using *Escherichia coli* DH5α, as the host. Plasmids were isolated from ten random clones of the genomic library, to estimate the average insert size. In order to confirm that the library included a complete representation of the *X. oryzae* pv. *oryzae* genome, two previously identified clones from a Philippine isolate of *X. oryzae* pv. *oryzae* (Kelemu and Leach, 1990; Hopkins et al. 1992) were used as probes in colony hybridizations.

A previous report on *Xanthomonas campestris* pv. *malvacearum* (a cotton pathogen) indicated that DNA cloned in *E. coli* can be subjected to substantial rearrangement when reintroduced back into
X. campestris pv. malvacearum (DeFeyter et al. 1990). If this were the case in X. oryzae pv. oryzae, it would interfere with subsequent genetic analysis of the cloned DNA. Therefore, two randomly selected clones from the genomic library were mobilized into X oryzae pv. oryzae. The plasmids were isolated from the transconjugants and subjected to Southern analysis to assess their structure in X. oryzae pv. oryzae.

In addition, procedures were standardized to obtain transposon insertions into cloned X. oryzae pv. oryzae DNA using a mini Tn5 transposon harboring the E. coli gus gene (Wilson et al. 1995). The conditions for marker exchange of these Tn5 insertions into the X oryzae pv. oryzae chromosome were also standardized. This mini Tn5 transposon functions as a promoter-probe transposon where β-glucuronidase activity is observed only if the gus gene is driven by endogenous bacterial promoters. Since both X. oryzae pv. oryzae and rice possess minimal endogenous β-glucuronidase activity, the use of the mini-Tn5gus transposon would facilitate studies on X. oryzae pv. oryzae gene expression in planta.

Materials and Methods

Bacterial strains, media etc.

All bacterial strains used are listed in Table 2.1. BXO1 is the laboratory wild type strain that belongs to pathotype I, the dominant pathotype of X. oryzae pv. oryzae in India (Reddy and Reddy, 1989; Yashitola et al. 1997). All X. oryzae pv. oryzae strains used here were grown on Peptone-Sucrose (PS) medium (Tsuchiya et al. 1982)