THE BACTERIAL PIGMENT XANTHOMONADIN PROVIDES PROTECTION AGAINST PHOTODAMAGE

Abstract

_Xanthomonas oryzae_ pv. _oryzae_ is a bacterial pathogen that causes leaf blight, a serious disease of rice. Most members of the genus _Xanthomonas_ produce yellow, membrane-bound, brominated aryl polyene pigments called xanthomonadins whose functional role is unclear. We find that pigment deficient mutants of _X. oryzae_ pv. _oryzae_ exhibit hypersensitivity to photobiological damage. A clone containing the xanthomonadin biosynthetic gene cluster alleviates the hypersensitivity of pigment deficient mutant. Extracts containing xanthomonadin provide protection against photodynamic lipid peroxidation in liposomes. These results lead us to suggest a role for the pigment, namely protection against photodamage.

Introduction

_Xanthomonas oryzae_ pv. _oryzae_ is the causal agent of a serious disease of the rice plant called bacterial leaf blight (Starr, 1981). Most members of the genus xanthomonas produce yellow membrane-bound, brominated aryl-polyene pigments called xanthomonadins (Starr, 1981; Andrews et al. 1976). The chemical structure of such a pigment is given in Figure 4.1. The presence of these pigments exclusively in the genus _Xanthomonas_ has led them to be used as diagnostic and taxonomic markers (Starr, 1981). The functional role
of the pigment in xanthomonads is yet to be delineated. The long polyene moiety that occurs in this molecule is similar to what is seen in carotenoids. Carotenoid pigments that are found in photosynthetic and non-photosynthetic bacteria have been observed to provide protection against damaging photolytic and photodynamic reactions (Cohen-Bazire and Stanier, 1958; Mathews and Sistrom, 1959; Turveson et al. 1988). The polyene moiety in carotenoids has been implied to be necessary for photoprotective action (Mathews-Roth et al. 1974).

This suggestion leads to the possibility of whether xanthomonadin might function as a protecting agent against light-induced damage to the organism. Indeed, a pigment deficient mutant of *Xanthomonas juglandis* was seen to be more vulnerable to photokilling than the pigmented wild type (Jenkins and Starr, 1982), hinting at such a role for xanthomonadin. We have investigated this issue deeper, using wild type and pigment deficient mutants from two different pathotypes of the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. Both these mutants are hypersensitive to photodamage in comparison to the wild type. We have been able to offer protection to a pigment deficient strain by introducing into it a plasmid which contains xanthomonadin biosynthetic genes (see Chapter 5). We have also conducted *in vitro* experiments on lipid peroxidation of liposomes in the presence of methanolic extracts of xanthomonadin from wild type strains and find that it inhibits the extent of lipid peroxidation, while a similar extract from the pigment deficient strain does not. These results suggest a functional role for the xanthomonadin pigments in these organisms.
Figure 4.1: 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).
Bacterial strains, media etc.

The bacterial strains, relevant characteristics and their references are listed in Table 4.1. The \textit{X. oryzae pv. oryzae} strains used in this work were grown in Peptone Sucrose (PS; Tsuchiya et al. 1982) medium at 28°C. Rifampicin was added at a final concentration of 50 µg/ml (to prevent contamination). Spontaneous Rif derivatives of wild type \textit{X. oryzae pv. oryzae} strains were obtained. Pigment deficient mutants were isolated, from either the wild type strain or the Rif derivatives, by visual inspection after ethylmethane sulphonate (EMS) mutagenesis. Toluidine blue O, and EMS were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of pigment deficient mutants

Pigment deficient mutants of \textit{X. oryzae pv. oryzae} were obtained by EMS mutagenesis at a frequency of 1% following a mutagenesis protocol (Miller, 1992) that resulted in 99% lethality. BX044 is a pigment deficient mutant derived from the wild type \textit{X. oryzae pv. oryzae} strain BX01 (a Rif derivative of BX044 i.e. BX047 was used; see Table 4.1). Similarly, BX0712 is a pigment deficient mutant derived after EMS mutagenesis of the \textit{X. oryzae pv. oryzae} strain BX0711 (Table 4.1). The two strains BX01 and BX0711 belong to different pathotypes of \textit{X. oryzae pv. oryzae} (Yashitola et al. 1997). A 16.5 Kb region containing genes required for xanthomonadin biosynthesis was isolated (described in Chapter 5) by screening a
Table 4.1. Strain List

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUFR034</td>
<td>IncW, km&lt;sup&gt;r&lt;/sup&gt;, Mob&lt;sup&gt;+&lt;/sup&gt;, mob(P), lacZα&lt;sup&gt;+&lt;/sup&gt;, Par&lt;sup&gt;+&lt;/sup&gt;, cos</td>
<td>Defeyter et al. 1990</td>
</tr>
<tr>
<td>pLR9</td>
<td>pUFR034 + 16.5 Kb insert containing genes required for xanthomonadin biosynthesis</td>
<td>Chapter 5</td>
</tr>
<tr>
<td></td>
<td>from <em>X. oryzae pv. oryzae</em></td>
<td></td>
</tr>
<tr>
<td>BX01</td>
<td>Laboratory wild type; an Indian isolate</td>
<td>Lab collection</td>
</tr>
<tr>
<td>BX043</td>
<td>rif-2; derivative of BX01</td>
<td></td>
</tr>
<tr>
<td>BX044</td>
<td>pig-1</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>BX047</td>
<td>pig-1 rif-3</td>
<td></td>
</tr>
<tr>
<td>BX08</td>
<td>a natural isolate from India</td>
<td>Lab collection</td>
</tr>
<tr>
<td>BX0711</td>
<td>rif-8, derivative of BX08</td>
<td></td>
</tr>
<tr>
<td>BX0712</td>
<td>pig-21 rif-8, derivative of BX0711</td>
<td>This study</td>
</tr>
<tr>
<td>BX0713</td>
<td>BX0712 + pLR9</td>
<td></td>
</tr>
<tr>
<td>BX0714</td>
<td>BX0712 + pUFR034</td>
<td></td>
</tr>
</tbody>
</table>

* pig indicates a mutation that confers pigment deficiency; rif indicates a mutation that confers resistance to rifampicin.
see Chapter 2) using the xanthomonadin clone from *X. campestris* pv. *campestris* (Poplawsky et al. 1993) as a probe. This clone was mobilized into BX0712 using methods as described (Hopkins et al. 1992). The resulting strain BX0713 was restored for pigment production (see Chapter 5). The vector, pUFR034 (Defeyter et al. 1990) used in the construction of this library was also mobilized separately into BX0712. The resulting strain BX0714 (Table 4.1) remains pigment deficient and serves as a control.

Xanthomonadin extraction

Approximately equal cell numbers (1 X 10^8 CFU [colony forming units] / ml) of each *X. oryzae* pv. *oryzae* strain were extracted with methanol as previously described (Starr and Stephens, 1964) with the modification that extractions were conducted in the dark, at room temperature. These extracts were concentrated to half the original volume by flash evaporation. Absorption spectra were recorded on a Hitachi U2000 spectrophotometer.

_in vivo_ photokilling experiments

A single colony of the desired *X. oryzae* pv. *oryzae* strain was inoculated into 2 ml PS broth containing rifampicin and grown overnight at 150 rpm at 28° C. The cells were centrifuged at 6000 rpm at 4°C, washed twice with equal volumes of 1x Minimal A (Miller, 1992) and resuspended to the original volume with 1x Minimal A. A 1.5 ml cell suspension containing approximately 1 X 10^8 CFU/ml was added in a presterilized quartz cuvette and toluidine blue was added at a final concentration of 5 μM, as
described previously (Jenkins and Starr, 1982). A small magnetic bead was introduced into the cuvette for gentle stirring. The cuvette was placed in a fluorimeter (Hitachi: F 4000 with a 150 W Xenon lamp) and illuminated with light of wavelength 640 nm (absorption maximum for toluidine blue). No external gases were introduced. Irradiation was done using the excitation monochromator open (20 nm); the photon flux is estimated, based on earlier experiments using ferrioxalate actinometry and also a light meter, to be $10^{14}$ photons/s or 200 μW/cm² (Guptasarma and Balasubramanian, 1992). Ten μl aliquots were removed at different time points, i.e. 0, 30, 60, 90, 120 minutes, diluted in 1x Minimal A and plated on PS plates containing rifampicin. These plates were covered with aluminium foil and incubated at 28° C for four to five days. Percentage (%) survival was calculated for each strain at various time points.

\[
\% \text{ Survival} = \left( \frac{\text{Total No. of CFU at one time interval}}{\text{Total No. of CFU at '0' min.}} \right) \times 100.
\]

Wild type and corresponding pigment deficient mutants were assayed on the same day. Dye-free controls (% survival in the presence of light and absence of toluidine blue) and light-free controls (% survival in the presence of toluidine blue and absence of light) for both wild type and pigment deficient mutants were also performed on the same day.

**Irradiation of egg PC liposomes.**

A 1 mg/ml solution of egg PC liposomes (small unilamellar vesicles or SUV) was prepared as described previously (Papahadjopoulous and Miller, 1967). To 300 μl of this solution,
toluidine blue was added at the final concentration of 100 μM and irradiated at 640 nm for a period of 90 min. This served as the blank. Subsequently, increasing amounts of concentrated xanthomonadin extracts (0, 12.5 and 25 μl) from either the wild type or pigment deficient mutant were added to individual liposome solutions (containing toluidine blue) prior to irradiation. Unirradiated controls for each sample were also included.

Lipid peroxidation assay

The thiobarbituric acid assay (TBA; Barber and Bernheim, 1967; Gutteridge, 1982) was used as an indicator of lipid peroxidation in the above experiment. At the end of all irradiations, the 300 μl sample was aliquoted into two equal halves. Trichloroacetic acid (TCA) was added at a final concentration of 1% and the solution was heated at 100° C for 20 min. following which TBA was added at a final concentration of 3.6 mg/ml in 50 mM NaOH and again heated at 100° C for further 20-30 min. The solution was then cooled and the intensity of fluorescence measured using an excitation wavelength of 532 nm and emission at 553 nm. Unirradiated (liposomes + toluidine blue + xanthomonadin but no irradiation) and xanthomonadin-free (liposomes + toluidine blue + irradiation but no xanthomonadin) controls were also processed in the same manner. The level of lipid peroxidation for each set was calculated as:

Irradiated blank - Unirradiated blank = T (Total peroxidation)
Irradiated X_wt - Unirradiated X_wt = x(wt)
Irradiated X_mut - Unirradiated X_mut = x(mut)
where wt and mut refer to wild type and mutant strains respectively.

The percentage lipid peroxidation was calculated as follows:
Taking 'T' as the total level of peroxidation i.e. 100%;
Percentage (%) lipid peroxidation for T_x(wt) = x(wt)/ T X 100,
similarly % lipid peroxidation for T_x(mut) = x(mut)/T X 100.

Results

Isolation of pigment deficient mutants

Pigment deficient mutants of _X. oryzae pv. oryzae_ were obtained by EMS mutagenesis (see Materials and Methods). BX047 and BX0712 are pigment deficient mutants derived from wild type strains BX01 and BX0711 respectively. Methanolic extracts were prepared from both wild type and mutant strains and their absorption spectra recorded. Both wild type strains showed a peak at 440 nm and shoulders at 420 and 460 nm characteristic of xanthomonadin while the pigment deficient mutants showed neither the peak nor the shoulders. The absorption scan for one wild type and one pigment deficient mutant strain are shown in Figure 4.2. It is evident that the pigment deficient strain has little or no xanthomonadin. The residual absorption seen in the 400-500 nm region in these cases could be due to intermediates in pigment biosynthesis that may be present.
Figure 4.2: Absorption spectra of methanolic extracts of *Xanthomonas oryzae* pv. *oryzae* strains.

Methanolic extracts of xanthomonadin were prepared and absorption spectra recorded as described in Materials and Methods. BX0711 is a pigment proficient strain and BX0712 is a pigment deficient mutant derived from BX0711.
Pigment deficient mutants show hypersensitivity to photobiological damage *in vivo*

In both the *in vivo* and *in vitro* assays, toluidine blue was used as the exogenous photosensitizer that liberates reactive oxygen species upon irradiation at 640 nm. The *in vivo* assay measures the kinetics of survival of wild type and mutant strains when exposed to light and air in the presence of the photosensitizer. Percentage survival values for each strain at different time points were calculated as described in Materials and Methods. Figure 4.3 shows that the pigment deficient mutant BX047 shows 10-100 fold greater sensitivity to photokilling than the pigmented strain BX043 (a Rif derivative of BX01). Percentage survivals in light-free and dye-free controls were also determined after a period of 120 min. for both strains, which established that the dye by itself and irradiation by itself is not cytotoxic *per se* (data not shown). Thus in the presence of both the photosensitizer and light, the wild type is able to survive better than the pigment deficient mutant. As further confirmation, we included another wild type *X. oryzae* pv. *oryzae* strain BX0711 and a pigment deficient mutant derived from it i.e. BX0712. The results plotted in Figure 4.4 indicate that the mutant strain BX0712 shows 10-100 fold greater sensitivity to photokilling when compared to the pigmented strain BX0711. Percentage survivals in dye-free and light-free controls in these cases too gave similar values as with the BX043-BX047 pair. This experiment was also repeated thrice. These results therefore indicate that pigment deficient mutants of *X. oryzae* pv. *oryzae* show hypersensitivity to
Figure 4.3: Kinetics of survival of *Xanthomonas oryzae* pv. *oryzae* strains after exposure to light and air in the presence of toluidine blue. Percentage survival was calculated for each strain as described in Materials and methods. The datum shown at each time point represents an average of two independent experiments. Note the logarithmic scale of the Y axis. BX043 is a pigment proficient strain and BX047 is a pigment deficient mutant derived from BX043. In Figure 4.4: BX0711 is a pigment proficient strain and BX0712 is a pigment deficient mutant derived from BX0711.
photodamage thereby confirming the earlier studies with X. juglandis (Jenkins and Starr, 1982).

Clone of xanthomonadin biosynthetic genes restores photoprotection to pigment deficient strains

In the next set of experiments we worked with strains BX0713 and BX0714 that are both derived from the pigment deficient strain BX0712. The strain BX0713 was constructed by introducing into BX0712 a plasmid containing the genes for xanthomonadin biosynthesis, while BX0714 was also constructed from BX0712 with a plasmid that did not include the xanthomonadin genes (see Materials and Methods and Table 4.1). BX0713 is thus a complemented strain which is pigment proficient, while the pigment deficient BX0714 serves as a control.

Figure 4.5 shows that BX0713 has 10-fold greater number of cells that survive the photodynamic assault of toluidine blue, while the response of BX0714 after 120 min. is comparable to that of BX0712. The protection offered in this instance is modest in comparison to what is seen in the pairs in Figures 4.3 and 4.4, and we wonder whether this could be because of the level of expression of the pigment in the clone under study. Nevertheless, the point gains ground that the pigment offers protection against photodamage.
Figure 4.5: A clone containing the xanthomonadin biosynthetic genes alleviates the hypersensitivity of the pigment deficient mutant.
Percentage survival was calculated for each strain as described in Materials and methods. The datum shown at each time point represents an average of two independent experiments. Note the logarithmic scale of the Y axis. BX0713 is the pigment deficient mutant that is complemented for pigment production and is therefore pigment proficient; BX0714 is the pigment deficient mutant into which only the plasmid pUFR034 has been introduced and is thus pigment deficient.
Xanthomonadin provides protection to lipids from peroxidation

In order to determine the nature of the antioxidant properties of xanthomonadin, we conducted lipid peroxidation studies in the presence of this pigment. Methanolic extracts were prepared from both wild type strains BX043 and BX0711 as well as from the pigment deficient mutants BX047 and BX0712. Liposomes (SUV) were prepared and the methanolic extract was added to liposomes containing toluidine blue, in a dose dependent manner, and then irradiated at 640 nm. Following irradiation, the amounts of peroxidized lipids were estimated by the well-known TBA assay (Gutterridge, 1982). Figure 4.6 shows the results obtained with methanolic extracts of BX043 and pigment deficient mutant BX047. As is evident from the graph, the percentage of peroxidized lipids in the presence of increasing amounts of xanthomonadin from wild type BX043 is reduced to less than 20%, while with extracts from the pigment deficient mutant BX047 the percentage of peroxidized lipids is about 80% even with increasing amounts of the extract. The slight drop in peroxidation seen with the mutant extract may be due to intermediates in pigment biosynthesis that are present in BX047.

Likewise, the methanolic extract from BX0711 provides over 50% protection to lipids from peroxidation when compared to the mutant BX0712, in which case again the level of peroxidation remains static at 80% even with increasing amounts of the extract (Figure 4.7). Unirradiated and xanthomonadin-free controls were included for each wild type-mutant set. The experiment was repeated twice for each set. The slight differences in the amount of protection observed
Figure 4.6 & 4.7: Xanthomonadin protects lipids from peroxidation in liposomes.

Liposomes were prepared and xanthomonadin was added to liposomes containing toluidine blue and irradiated for a period of 90 min., following which the amount of peroxidized lipids was calculated as described in Materials and Methods. Methanolic extracts of xanthomonadin were prepared from these strains as described in Materials and Methods. The amount of xanthomonadin is expressed as \( \mu l \times 10^8 \) CFU because the exact structure (and the molar extinction coefficient) of the xanthomonadin pigment(s) of *X. oryzae* pv. *oryzae* is not known.

In Figure 4.6: BX043 is a pigment proficient strain and BX047 is a pigment deficient mutant derived from BX043.

In Figure 4.7: BX0711 is a pigment proficient strain and BX0712 is a pigment deficient mutant derived from BX0711.
between the two wild type strains may be due to differences in the extraction procedure and the concentration of the pigment intermediates in them. (The strain BX043 seems to have a greater amount of the protectant in it in comparison to BX0711; this seems apparent in Figures 4.3/4.4 as well).

We also extracted the pigment from the strain BX0713 (the pigment proficient strain into which the xanthomonadin biosynthetic genes were introduced on a plasmid) using methanol and tested the product for its ability to inhibit lipid peroxidation in egg PC SUV. The inhibition displayed by this extract was around 52-54% which compares well with the 55-60% inhibition displayed by the pigment isolated from wild type strains at equal concentrations.

Discussion

Xanthomonadin is a pigment found exclusively in the plant pathogenic genus *Xanthomonas*. Though the exact role of xanthomonadins in the life cycle of the pathogen has not been elucidated, previous reports indicate that it may provide protection to bacteria against photobiological damage (Jenkins and Starr, 1982). The kinetics of survival indicate that two independent pigment deficient mutants of *X. oryzae* pv. *oryzae* are hypersensitive to photodamage compared to the corresponding wild type (pigmented) strains. These results therefore confirm the earlier results with *X. juglandis*. The results from the lipid peroxidation study provide further clues on the mechanism of protection by xanthomonadin. Photoinduced lipid peroxidation of liposomes were conducted based on the assumption that xanthomonadin, a
membrane-bound pigment, might serve to protect membranes from photodamage. The finding that extracts of xanthomonadin from wild type strains provide >50% protection against lipid peroxidation suggests that xanthomonadin may function to prevent peroxidation of bacterial cell membranes. As an internal control, we have included extracts from pigment deficient mutants which show < 20% decrease in peroxidation. This drop may be due to intermediates in the xanthomonadin biosynthetic pathway present in the pigment deficient mutants that may offer minimal protection.

The observation that xanthomonadin, a membrane bound pigment, can protect lipids from peroxidation suggests that it may serve to protect the bacterial membrane from oxidative damage. *X. oryzae* pv. *oryzae* may be exposed to photobiological damage caused by reactive oxygen species during the phase of its life cycle in which it is present on the leaf surface and is exposed to light and air (Alvarez et al. 1989). The observation that pigment deficient mutants are reduced for virulence upon epiphytic inoculation (discussed in Chapter 3) is consistent with this possibility. The novel observation that xanthomonadin can function as an antioxidant *in vitro* provides the opportunity to explore possibilities that it could function like other synthetic antioxidants used for the protection of polymers and foodstuff against oxidative damage.
References


