The bacterial pigment xanthomonadin offers protection against photodamage

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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 the 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.**

Key words: Bacterial blight of rice; Xanthomonadin pigment; Photoprotection; Inhibition of lipid peroxidation

1. Introduction

Xanthomonas oryzae pv. *oryzae* is the causal agent of a serious disease of the rice plant called bacterial leaf blight [1]. Most members of the genus xanthomonas produce yellow membrane bound, brominated aryl polyene pigments called xanthomonadins [1–3]. The chemical structure of such a pigment is given in Fig. 1. The presence of these pigments exclusively in the genus xanthomonas has led them to be used as diagnostic and taxonomic markers [1]. The functional role of the pigment in xanthomonas 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 [4–6]. The polyene moiety in carotenoids has been implied to be necessary for photoprotective action [7].

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 [8], hinting at such a role for xanthomonadin. We have investigated this issue deeper in this communication, using wild-type and pigment-deficient mutants from two different pathotypes of the rice pathogen *X. 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. 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.

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2. Materials and methods

2.1. Bacterial strains

The bacterial strains, relevant characteristics and their references are listed in Table 1. The *X. oryzae* pv. *oryzae* strains used in this work were grown in Peptone Sucrose (PS; [9]) medium at 28°C. Rifampicin was added at a final concentration of 50 μg/ml (to prevent contamination). Rif^r derivatives of wild-type *X. oryzae* pv. *oryzae* strains were obtained and pigment-deficient mutants from these rif^r derivatives were isolated by visual inspection after ethyl methane sulfonate (EMS) mutagenesis. Toluidine blue O, EMS and egg PC (phosphatidyl choline) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Isolation of pigment-deficient mutants

Pigment-deficient mutants of *X. oryzae* pv. *oryzae* were obtained by EMS mutagenesis at a frequency of 1% following a mutagenesis protocol [10] that resulted in 99% lethality. BXO47 is a pigment-deficient mutant derived from the wild-type *X. oryzae* pv. *oryzae* strain BXO43. Similarly, BXO712 is a pigment-deficient mutant derived after EMS mutagenesis of the *X. oryzae* pv. *oryzae* strain BXO711 (see Table 1). The two wild-type strains BXO43 and BXO711 belong to different pathotypes of *X. oryzae* pv. *oryzae* [11]. A 17 kb region containing genes required for xanthomonadin biosynthesis was isolated (L. Rajagopal, unpublished data) by screening a genomic library of *X. oryzae* pv. *oryzae* [12] using the xanthomonadin clone from *X. campestris* [13] as a probe. This clone was mobilized into BXO712 using methods as described [14]. The resulting strain BXO713 was restored for pigment production. The vector, pUFR034 [15] used in the construction of this library was also mobilized separately into BXO712. The resulting strain BXO714 remains pigment deficient and serves as a control.

2.3. Xanthomonadin extraction

Approximately equal cell numbers (1×10^8 CFU (colony forming units) ml^{-1}) of each *X. oryzae* pv. *oryzae* strain were extracted with methanol as previously described [16] 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.

2.4. 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 1×Minimal A [10] and resuspended to the original volume with 1×Minimal A. A 1.5 ml cell suspension containing approximately 1×10^8 CFU/ml was added in a presterilized quartz cuvette and toluidine blue was added at a final concentration of 5 μM, as described previously [8]. A small magnetic bead was introduced into the cuvette for gentle stirring.

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The cuvette was placed in a fluorimeter (Hitachi: F4000 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/cm^2) [17]. Ten μl aliquots were removed at different time points, i.e. 0, 30, 60, 90, 120 min, diluted in 1×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. Percentage survival = (total no. of CFU at one time interval/total no. of CFU at '0' min)×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.

2.5. Irradiation of egg PC liposomes

A 1 mg/ml solution of egg PC liposomes (small unilamellar vesicles or SUV) was prepared as described previously [18]. 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.

2.6. Lipid peroxidation assay

The thiobarbituric acid assay (TBA; [19,20]) 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:

$$\text{irradiated blank} - \text{unirradiated blank} = T \text{ (total peroxidation)},$$

$$\text{irradiated } X_{\text{wt}} - \text{unirradiated } X_{\text{wt}} = x(\text{wt}),$$

$$\text{irradiated } X_{\text{mut}} - \text{unirradiated } X_{\text{mut}} = x(\text{mut}),$$

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%;

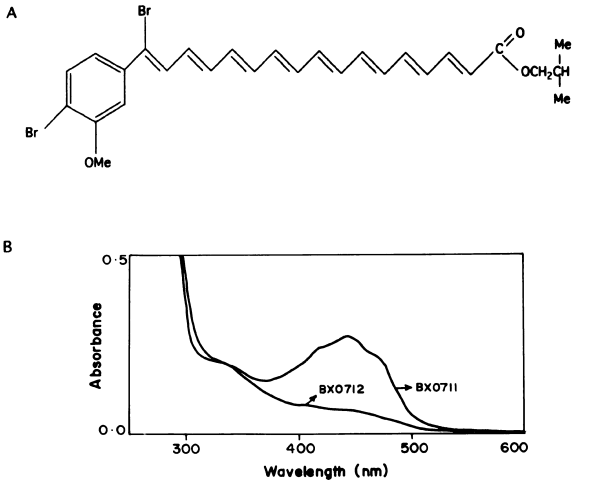

Fig. 1. A: Structure of xanthomonadin I [2]. B: Absorption spectra of methanolic extracts from *Xanthomonas oryzae* pv. *oryzae* strains. Methanolic extracts of xanthomonadin were prepared and absorption spectra recorded as described in Section 2. BXO711 is a pigment-proficient strain and BXO712 is a pigment-deficient mutant derived from BXO711.

$$\text{Percentage (\%) lipid peroxidation for } T_{x(\text{wt})} = x(\text{wt})/T \times 100,$$

$$\text{similarly \% lipid peroxidation for } T_{x(\text{mut})} = x(\text{mut})/T \times 100.$$

3. Results and discussion

3.1. Isolation of pigment-deficient mutants

Pigment-deficient mutants of *X. oryzae* pv. *oryzae* were obtained by EMS mutagenesis (see Section 2). BXO47 and BXO712 are pigment-deficient mutants derived from wild-type strains BXO43 and BXO711 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 Fig. 1B. 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 to be due to intermediates in pigment biosynthesis that may be present.

Table 1
Bacterial strains and plasmids

Strain	Relevant characteristics	Reference
Plasmids		
pUFR034	IncW, kan^r, Mob$^+$, mob(P), lacZα$^+$, Par$^+$, *cos*	[15]
pLR9	pUFR034+17 kb insert containing genes required for xanthomonadin biosynthesis	unpublished data
Xanthomonas oryzae pv. *oryzae* strains[a]		
BXO1	laboratory wild-type; an Indian isolate	lab collection
BXO43	rif^r derivative of BXO1	lab collection
BXO47	pig$^-$; rif^r (derived from BXO43)	lab collection
BXO8	a natural isolate from India	lab collection
BXO711	rif^r derivative of BXO8	lab collection
BXO712	pig$^-$; rif^r (derived from BXO711)	this study
BXO713	pig$^+$; rif^r; kan^r+pLR9	this study
BXO714	pig$^-$; rif^r; kan^r+pUFR034	this study

[a]Rif^r denotes resistance to rifampicin; kan^r denotes resistance to kanamycin; pig$^-$ denotes pigment deficiency.

3.2. 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 Section 2. Fig. 2A shows that the pigment-deficient mutant BXO47 shows 10–100-fold greater sensitivity to photokilling than the pigmented strain BXO43. 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. Thus in the presence of both the photosensitizer and light, the wild-type mutant is able to survive better than the pigment-deficient mutant. As further confirmation, we included another wild-type *X. oryzae* pv. *oryzae* strain BXO711 and a pigment-deficient mutant derived from it, i.e. BXO712. The results plotted in Fig. 2B indicate that the mutant strain BXO712 shows 10–100-fold greater sensitivity to photokilling when compared to the pigmented strain BXO711. Percentage survivals in dye-free and light-free controls in these cases too gave similar values as with the BXO43-BXO47 pair. This experiment was also repeated thrice. These results therefore indicate that pigment-deficient mutants of *X. oryzae* pv. *oryzae* show hypersensitivity to photodamage thereby confirming the earlier studies with *X. juglandis* [8].

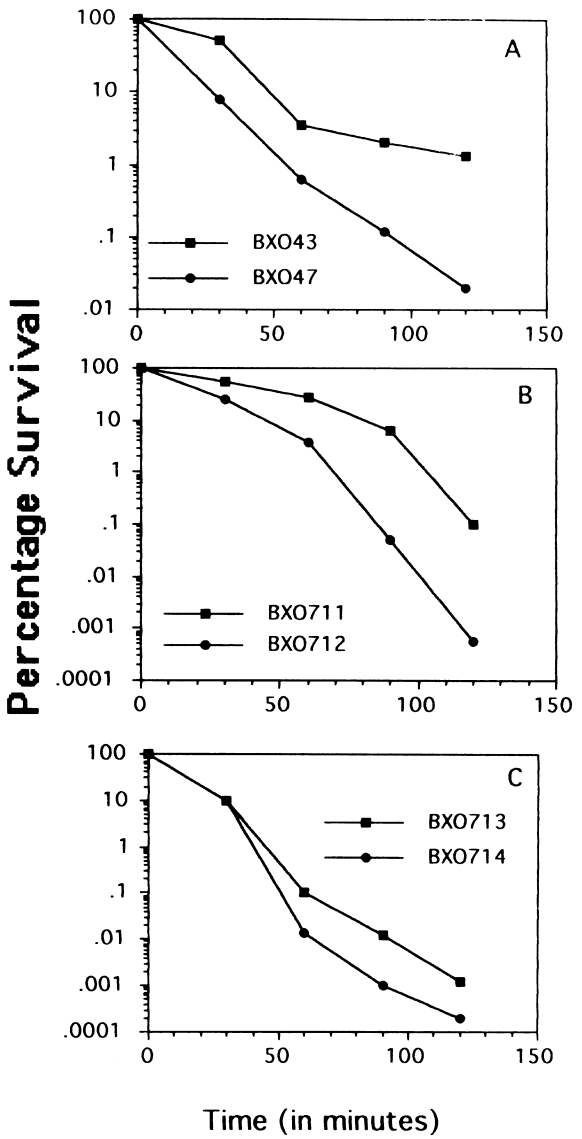

Fig. 2. Kinetics of survival of *X. oryzae* pv. *oryzae* strains after exposure to light and air in the presence of toluidine blue. Percentage survival was calculated for each strain as described in Section 2. The datum shown at each time point represents an average of two independent experiments. Note the logarithmic scale of the Y axis. A: BXO43 is a pigment-proficient strain and BXO47 is a pigment-deficient mutant derived from BXO43. B: BXO711 is a pigment-proficient strain and BXO712 is a pigment-deficient mutant derived from BXO711. C: BXO713 is the pigment-deficient mutant that is complemented for pigment production and is therefore pigment proficient; BXO714 is the pigment-deficient mutant into which only the plasmid pUFR034 has been introduced and is thus pigment deficient.

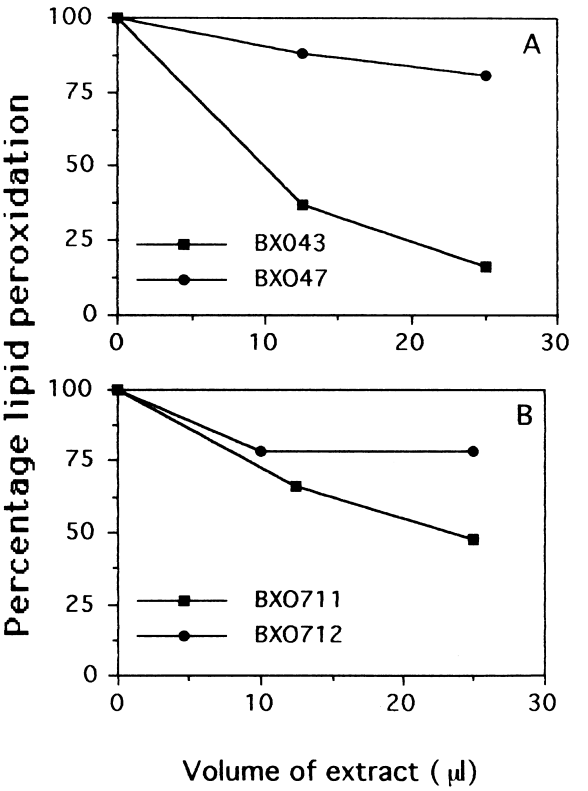

Fig. 3. Xanthomonadin protects lipids from peroxidation in liposomes. Liposomes were prepared and xanthomonadin was added to liposomes containing toluidine blue and irradiated for a period of 90 min, following which the amount of peroxidized lipids was calculated as described in Section 2.

3.3. Clone of xanthomonadin biosynthetic genes restores photoprotection to pigment-deficient strains

In the next set of experiments we worked with strains BXO713 and BXO714 that are both derived from the pigment-deficient strain BXO712. The strain BXO713 was constructed by introducing into BXO712 a plasmid containing the genes for xanthomonadin biosynthesis, while BXO714 was also constructed from BXO712 with a plasmid that did not include the xanthomonadin genes (see Section 2 and Table

1). BXO713 is thus a complemented strain which is pigment proficient, while the pigment-deficient BXO714 serves as a control.

Fig. 2C shows that BXO713 has 10-fold greater number of cells that survive the photodynamic assault of toluidine blue, while the response of BXO714 after 120 min is comparable to that of BXO712. The protection offered in this instance is modest in comparison to what is seen in the pairs in Fig. 2A and B, 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.

3.4. *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 BXO43 and BXO711 as well as from the pigment-deficient mutants BXO47 and BXO712. 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 [20]. Fig. 3A shows the results obtained with methanolic extracts of BXO43 and pigment-deficient mutant BXO47. As is evident from the graph, the percentage of peroxidized lipids in the presence of increasing amounts of xanthomonadin from wild-type BXO43 is reduced to less than 20%, while with extracts from the pigment-deficient mutant BXO47 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 BXO47.

Likewise, the methanolic extract from BXO711 provides over 50% protection to lipids from peroxidation when compared to the mutant BXO712, in which case again the level of peroxidation remains static at 80% even with increasing amounts of the extract (Fig. 3B). 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 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 BXO43 seems to have a greater amount of the protectant in it in comparison to BXO711; this seems apparent in Fig. 2 as well.)

We also extracted the pigment from the strain BXO713 (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.

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 [21]. Alternatively, *X. oryzae* pv. *oryzae* may also be exposed to reactive oxygen species *in planta*, as these are known to be produced as part of the host defense response. 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.

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