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Oxidative-stress-induced production of pyocyanin by *Xanthomonas campestris* and its effect on the indicator target organism, *Escherichia coli* [1]

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Pyocyanin, a potential antimicrobial agent, was secreted by *Xanthomonas campestris*. Treatments with agents causing oxidative stress in the organism caused up to 4.4-fold increase in specific pyocyanin production. Pyocyanin added in the extracellular space did not affect growth rate of *X. campestris*, but decreased maximum cell concentration and specific product formation. However, the growth of *Escherichia coli*, the indicator target organism, was affected by pyocyanin. There was also a significant increase in the intracellular reactive oxygen species (ROS) concentration and antioxidant enzyme [catalase, superoxide dismutase (SOD)] concentrations, in the presence of pyocyanin. The intracellular ROS concentrations in *E. coli* formed upon exposure to pyocyanin, which is an indicator of the toxicity, was dependent on the growth phase of the organism. Studies with mutants of *E. coli* showed that intracellular ROS concentration was not significantly affected by the absence of the regulon *OxyR*, but, was significantly higher in cases when the regulon *rpoS* or the genes *katG* or *katE* were absent. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 266–272.

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Introduction

Pyocyanin is a phenazine compound that occurs in nature in secretions of the pathogen *Pseudomonas* and affects the growth and viability of a wide range of microorganisms [26]. Therefore, pyocyanin can be extracted from the broth in which cells producing pyocyanin are grown, and used as an antimicrobial agent. During our investigations, we found pyocyanin secretion by *Xanthomonas campestris*, a plant pathogen, which has not been reported earlier. *Xanthomonas* uses pyocyanin, possibly to counter the host and to reduce the competition from other organisms, during infection.

When *Xanthomonas* infects plant tissues, the extent of oxidative stress due to reactive oxygen species (ROS) formed in the pathogen, is an important determinant of the pathogenicity [30]. The invading species, *Xanthomonas*, encounter increasing oxidative stress caused by the plant [3] that has to be overcome for successful infection. Thus, if *Xanthomonas* uses pyocyanin as a counteragent, it is possible that the mechanism of pyocyanin synthesis and secretion is enhanced by oxidative stress caused by the ROS. To test this hypothesis, we generated ROS in *X. campestris* through various extracellular treatments and studied pyocyanin secretion as an adaptive response.

Further, the damage caused by pyocyanin in other target organisms is also mediated through ROS. The reduced form of pyocyanin is an unstable free radical that reacts rapidly with molecular oxygen to create the ROS [11]. The electron flow from biological pathways is diverted to increase the production of intracellular reactive oxygen products, leading to cell death [10,12]. However, the cell employs antioxidant enzymes such as catalase and superoxide dismutase (SOD) to counter the ROS [9,16]. The expression of catalase is highly dependent on the growth phase in *Escherichia coli*, because activity of regulons such as *OxyR* and *rpoS* are growth-phase dependent [15,18]. For example, *OxyR* regulates a set of proteins, including catalase expression controlled by katG, expressed in log phase when subjected to oxidative stress, whereas *rpoS* regulates expression of a set of proteins in the stationary phase, including catalase expression by *katE* [29]. Therefore, pyocyanin toxicity could be dependent on the growth phase of the bacteria, which has not been reported earlier. In this study, we have also investigated growth-phase-dependent pyocyanin toxicity in *E. coli*, the indicator target organism, through studies on suitable mutants. This will also provide information for the use of pyocyanin as an antimicrobial agent.

Materials and methods

Bacterial strains and culture conditions

X. campestris (MTCC 2286, IMTECH, Chandigarh, India) and *E. coli* K-12 (MG 1655 [5]) strains along with mutated *E. coli* MG 1655 strains [5] such as Δ*OxyR*, Δ*katG*, Δ*rpoS*, Δ*katE*, Δ*SoxRS* and a double-mutant of *katG*, *katE*, were used in this study. The growth medium and conditions for *X. campestris* were the same as used in our earlier study [27]. The growth medium and conditions for *E. coli* cells were as prescribed by Dukan and Touati [5].

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Extraction of pyocyanin

The procedure employed by Hassett *et al.* [11] for pyocyanin extraction from *Pseudomonas aeruginosa* was followed. The extracted pyocyanin was used in all experiments involving exposure of cells to pyocyanin.

Treatment procedures

The HOCl treatment procedure for *X. campestris* is the same as that used in our related study [17]. Briefly, cells were exposed to freshly prepared HOCl in three stages. Each stage consisted of HOCl exposure, followed by 40 min of incubation in the dark, at 30°C, with gentle shaking. The concentrations of HOCl used for exposure at the beginning of each stage were [in mmol (g cell)$^{-1}$]: 0.66 (stage I), 4.65 (stage II) and 20 (stage III). After the third incubation, the free chlorine left in the flasks was quenched using sterile sodium thiosulfate.

The menadione and H_2O_2 treatment procedures were similar, but, the concentrations of the treatment agents were different. With menadione, the concentrations were: 8.4 (stage I), 30 (stage II), and 100 (stage III) mmol (g cell)$^{-1}$. With H_2O_2, the concentrations were: 0.18 (stage I), 0.47 (stage II) and 2.18 (stage III) mol (g cell)$^{-1}$.

Analyses

The xanthan concentration was determined through dry weight measurement after isolation through alcohol precipitation followed by centrifugation at 10,000 g for 45 min [8]. The cell concentrations were estimated using OD at 600 nm and were also cross-checked with plating results. Spectrophotometric estimations of catalase enzyme were done using hydrogen peroxide degradation [1], and the pyrogallol autooxidation method was used for SOD estimation [19]. Standard enzymes (Sigma Chemical Co.), i.e., catalase (*Aspergillus niger*) and SOD (*E. coli*) were used for standard graphs. The total protein concentration was estimated by the Bradford method. The pyocyanin concentrations before and after oxidant treatments were estimated [4]. Infrared spectroscopic analysis [20] and NMR spectroscopic analysis of pyocyanin extracted from *X. campestris* cells were carried out using a Nicolet Impact 400 IR spectrophotometer and a Varian FT-NMR spectrophotometer, respectively.

Spin trapping of free radicals

To measure oxygen centered radicals formed after exposure to 100 μM pyocyanin extracted from *X. campestris* a spin trapping technique [25] was employed. Cell densities were adjusted to an optical density at 600 nm of 0.5, which corresponded to a cell density of 0.13 g l^{-1}. The cells were incubated for 5 min at 37°C in a shaking (100 rpm) water bath. 5,5-Dimethyl-pyrroline-1-oxide (DMPO) (Sigma, St. Louis, MO, USA) (100 mM) was then added to the lysed cells. The free radical levels were estimated using electron spin resonance spectroscopy using a Varian ESR spectrometer.

Quantification of free radicals

The free radical concentration is proportional to the area under the absorption curve [2], which was obtained by double integration of the obtained derivative spectrum, using computer programs. The free radical concentrations were obtained by comparison with concentrations of the known standard [13]. The background free radical levels (obtained when no pyocyanin was added) were subtracted to obtain the effect of pyocyanin alone.

Results

Pyocyanin is produced by X. campestris

When wild-type *X. campestris* cells were grown in batch culture, pyocyanin was produced at 2.5 mg l^{-1}, which corresponded to 4.2 mg (g cells)$^{-1}$. Preliminary identification tests for pyocyanin such as formation of colored compounds upon boiling, or reactions with a dilute alkaline solution (0.1 M NaOH), acetic acid (0.1 M) [11,28], were performed on the dried extract and the presence of pyocyanin was inferred. Further, identification studies using IR and NMR spectroscopy were performed on the extract and the results were compared with those obtained using synthetic pyocyanin (phenazonium methosulfate) (Figures 1 and 2). In IR spectroscopy, there was a good correspondence between spectra of the sample and reference (Figure 1), in the expected wave number range of 910–665 cm^{-1} for fused heterocyclic ring compounds [21]. In NMR spectroscopy, the chemical shift spectra (Figure 2) between 7.8 and 9.2 ppm [22], showed good correspondence between the sample and the reference. These results clearly show that pyocyanin is secreted by *X. campestris*.

The glucose concentration in the medium influences the pyocyanin yield from *Pseudomonas* significantly [28] and therefore, we studied the effect of initial glucose concentration on the pyocyanin yield from *X. campestris* grown in batch culture. The results shown in Table 1 indicate that maximum pyocyanin production in wild-type cells resulted when the medium contained the lowest initial glucose concentration of 0.5 g l^{-1}. This information is expected to be useful in formulation of the growth medium for optimal production of pyocyanin.

Pyocyanin production in X. campestris is enhanced by ROS generators

Although the effects of pyocyanin on other target bacteria such as *E. coli* have been extensively studied, the mechanism of pyocyanin production, even in the well-known *Pseudomonas* system, remains unclear. As pyocyanin is produced by two pathogens, i.e., *Xanthomonas* and *Pseudomonas*, which encounter oxidative stress during infection [3,11], it is plausible that ROS, which are responsible for the oxidative stress, enhance pyocyanin synthesis and secretion. Also, ROS can act as modulators of cell function [23]. To investigate the mechanism of pyocyanin production, we studied the pyocyanin production in *X. campestris* cells as an adaptive response to treatments with generators of ROS, such as hypochlorous acid (HOCl), menadione and hydrogen peroxide (H_2O_2). The pyocyanin concentration produced in the log phase (5 h) of wild-type cells was 4.2 mg (g cell)$^{-1}$. HOCl treatment induced a 4.4-fold increase in pyocyanin produced compared to that produced by wild-type cells. Similarly, menadione treatment increased pyocyanin production by 4.0-fold and H_2O_2 treatment increased it by 2.6-fold.

As the highest enhancement was obtained with HOCl treatment, further ROS generations were made with HOCl. Also, the enhancement in pyocyanin production due to HOCl treatment (Table 1) was higher (4- to 5-fold) at higher initial glucose concentrations (20 and 40 g l^{-1}) compared to only a 1.6-fold enhancement at 0.5 g l^{-1} initial glucose concentration.

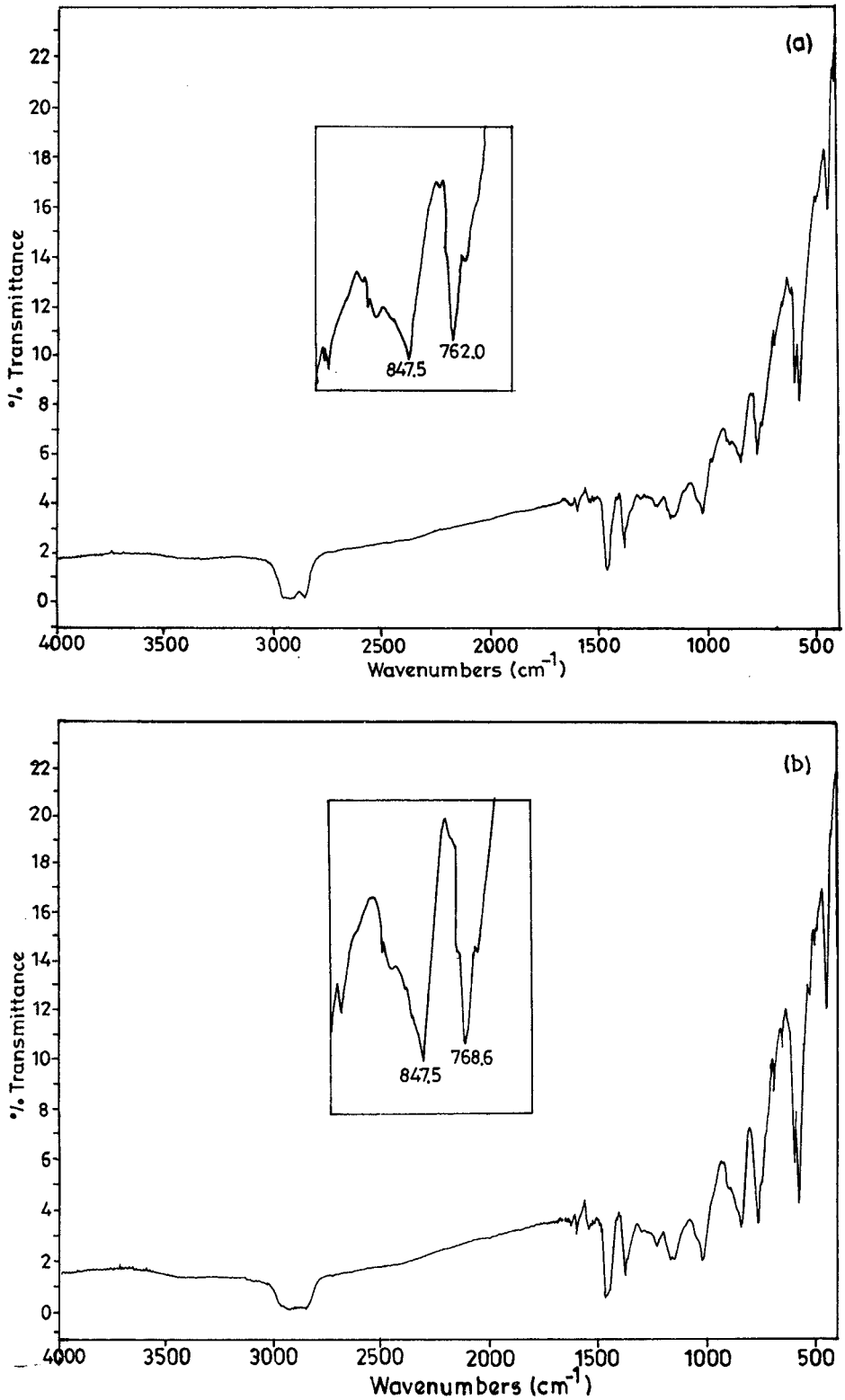

Figure 1 Infrared spectra of (a) pyocyanin sample extracted from the medium in which *X. campestris* cells were grown and (b) synthetic pyocyanin (phenazonium methosulfate).

Identification of the free radical types

The important ROS in the cell are the superoxide, hydroxyl and peroxyl free radicals [7]. The adducts of these species with DMPO give characteristic ESR derivative spectra that differ in the number of hyperfine splits [2,24]. This difference arises from the differences in interaction of the magnetic moment of the trapped

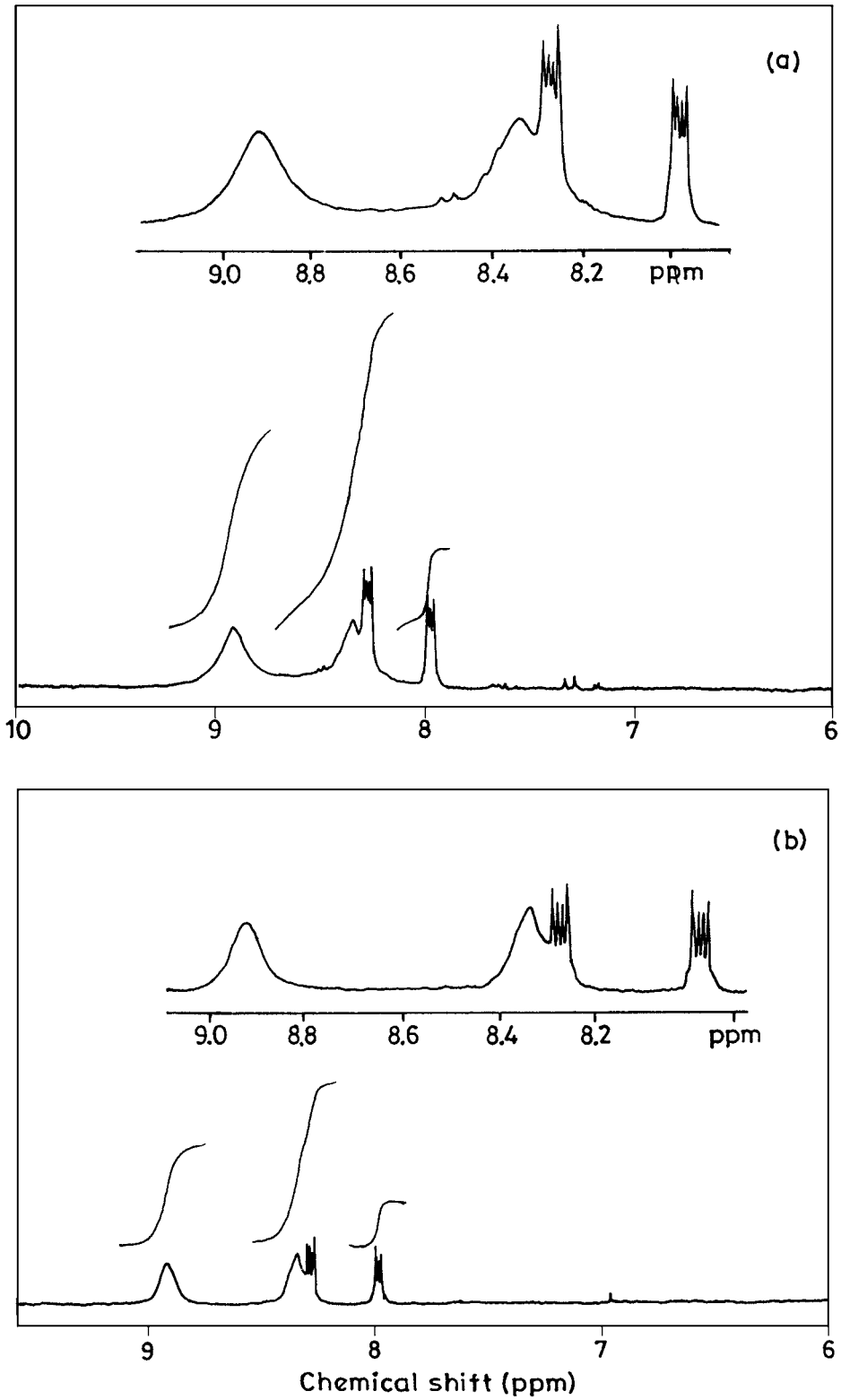

Figure 2 Nuclear magnetic resonance spectra of (a) pyocyanin sample extracted from the medium in which *X. campestris* cells were grown and (b) synthetic pyocyanin (phenazonium methosulfate).

radical with that of the α-hydrogen and the nitroxide nitrogen on DMPO [14]. The types of ROS were identified using the above and only superoxide radicals (with three hyperfine splits) were found in this study.

Table 1 Effect of initial glucose concentration on pyocyanin levels obtained from wild-type and HOCl-treated *X. campestris* cells

Initial glucose concentration [g l^{-1}]	Pyocyanin level (wild-type cells) [mg (g cell)$^{-1}$]	Pyocyanin level (HOCl-treated cells) [mg (g cell)$^{-1}$]
0.5	170.0±11.3	272.2±10.4
1.0	58.2±6.4	97.9±6.2
5.0	23.8±3.2	78.9±2.3
10.0	9.6±1.9	34.3±3.5
40.0	4.2±1.8	18.5±2.6

The average values from three experiments along with the standard deviations are presented.

Effect of extracellular pyocyanin on X. campestris

Although pyocyanin exhibits bactericidal action on other target bacteria, the producer, *Pseudomonas*, is unaffected by it [11]. Therefore, we studied the effect of 100 μM pyocyanin added externally on *X. campestris*. Growth rates were not significantly affected (0.24 vs. 0.248 h^{-1}) by pyocyanin; however, the maximum cell concentration was 45% higher in the presence of pyocyanin. Pyocyanin enhances growth of *P. aeruginosa* [28]. However, xanthan gum production decreased from 2.03 to 1.35 g (g cell)$^{-1}$, in the presence of pyocyanin.

Free radicals generated in presence of pyocyanin are an indirect measure of pyocyanin-induced toxicity in bacteria [10,12]. In the presence of pyocyanin, no free radicals were detected (Figure 3). Furthermore, no significant difference in concentrations of the antioxidant enzymes SOD and catalase, which protect the bacterium from free radicals, was found in presence or absence of pyocyanin.

Effect of extracellular pyocyanin on E. coli

The maximum cell concentration was reduced by 1.7-fold and the growth rate was reduced by 12% when *E. coli* was grown in the presence of 100 μM pyocyanin. Furthermore, 0.38 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were found intracellularly in log-phase cells in the presence of pyocyanin, whereas no free radicals were detected in its absence. A typical ESR spectrum obtained in this study, which indicates the presence of superoxide radicals in the presence of pyocyanin, is presented in Figure 3. Also, no free radicals were detected in the stationary phase of cells grown in the presence or the absence of pyocyanin. Furthermore, the intracellular SOD levels were 4.5-fold higher and the intracellular catalase levels were 2-fold higher in log-phase cells grown in the presence of pyocyanin compared to cells grown in the absence of pyocyanin.

Growth-phase-dependent pyocyanin effect on E. coli

ROS were found in the log phase, but not in the stationary phase, of *E. coli* grown in the presence of pyocyanin. The expression of the anti-oxidant enzyme, catalase, which protects the cell from ROS, is growth-phase dependent in *E. coli* [6]. Under stress conditions, catalase expression is under the control of the *OxyR* regulon in the log phase and the *rpoS* regulon in the stationary phase [5,29]. These regulons control expression of the catalase genes *katG* and *katE*, in the log and stationary phases, respectively. Therefore, to investigate whether pyocyanin toxicity results from its effect on the

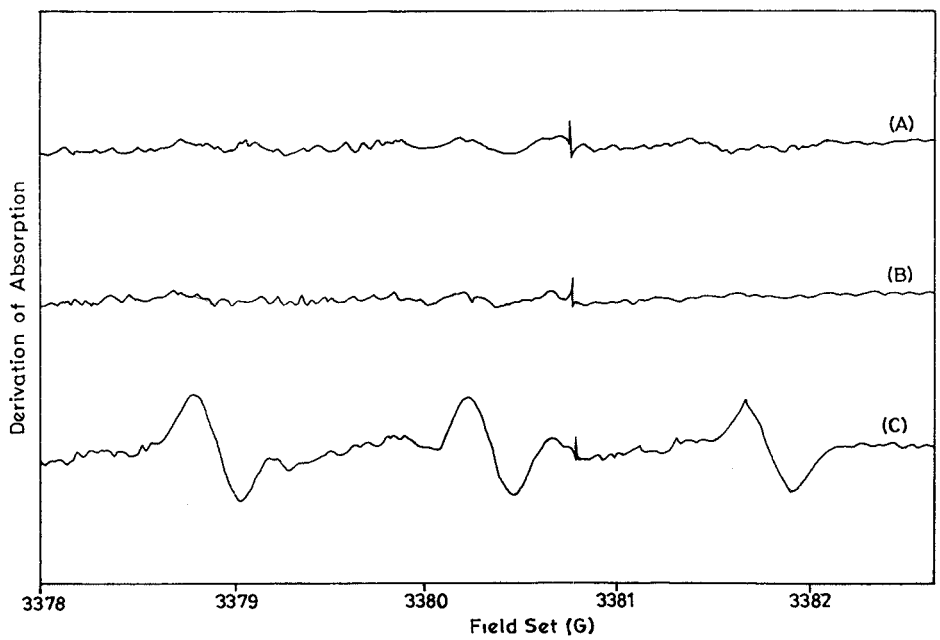

Figure 3 Electron spin resonance derivative spectra: (A) *X. campestris* cells grown in the absence of pyocyanin, (B) *X. campestris* cells grown in the presence of 100 μM pyocyanin and (C) a typical spectrum of superoxide radicals obtained from *E. coli* cells. Field set 3380 G; microwave frequency, 9.5 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; *g*, 2.00304.

Table 2 Free radical levels obtained with various cell types and mutants in the presence of 100 μM pyocyanin

Cell type	Growth phase	Free radical type	Free radical concentration [mmol (g cell)$^{-1}$]
X. campestris			
Wild-type	log	none	none
E. coli			
Wild-type	log	superoxide	0.38±0.03
Wild-type	stationary	none	none
Δ*OxyR*	log	superoxide	0.45±0.02
Δ*katG*	log	superoxide	0.74±0.03
Δ*rpoS*	stationary	superoxide	1.39±0.06
Δ*katE*	stationary	superoxide	0.68±0.04
katG katE double mutant	log	superoxide	1.01±0.02
katG katE double mutant	stationary	superoxide	0.61±0.02
Δ*SoxRS*	log	superoxide	0.81±0.07

The average values of free radical concentration obtained from ESR measurements of three different samples of the same cell type or mutant, on different days, are presented along with the standard deviations. The free radical levels obtained in the absence of pyocyanin (background levels) have been subtracted.

relevant regulons and genes, we studied the free radical levels generated by pyocyanin in various deletion mutants. The deletion mutants employed in this study were Δ*OxyR*, Δ*katG*, Δ*rpoS*, Δ*katE*, and a double mutant of *katG* and *katE*. We compared the levels of free radicals generated by 100 μM pyocyanin in these mutants to that generated in wild-type *E. coli* cells in the log and stationary phases.

When the deletion mutant of *OxyR* (Δ*OxyR*) was grown in the presence of pyocyanin, 0.452 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were obtained in the log phase, which was about 19% higher than that obtained with wild-type cells grown in the presence of pyocyanin. With the Δ*katG* mutants, 0.735 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were obtained when cells were grown in the presence of pyocyanin, which was 1.9-fold of that obtained with wild-type cells grown in the presence of pyocyanin. Thus, in the log phase, in the presence of pyocyanin, absence of the gene, *katG*, resulted in higher superoxide radical levels compared to absence of the regulon, *OxyR*.

When Δ*rpoS* cells were grown in the presence of pyocyanin, 1.394 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were detected in the stationary phase whereas, no radicals (Table 2) were found in stationary-phase wild-type cells. Also, with Δ*katE* cells, 0.682 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were detected in the stationary phase, which was 49% of that found in Δ*rpoS*. Thus, in contrast to the log phase, absence of the regulon *rpoS* resulted in higher superoxide radical levels compared to the absence of the gene, *katE*, in the stationary phase in the presence of pyocyanin.

When the double mutant of *katG katE* was grown with pyocyanin 1.011 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) were obtained in the log phase, which was 2.7-fold of that in the corresponding wild-type cells, and 0.605 mmol (g cell)$^{-1}$ superoxide radicals (Table 2) in the stationary phase compared to no superoxide radicals (Table 2) in the corresponding wild-type cells. The level of superoxide radicals generated in the presence of pyocyanin, in the double mutant of *katG* and *katE*, was comparable to that obtained with the single mutant of *katE*, in the stationary phase, whereas in the log phase it was 38% higher compared to the corresponding single mutant of *katG*.

Studies on the growth-phase-independent protection system against superoxide radicals, *SoxRS*, which controls SOD expression, showed that absence of *SoxRS* resulted in 0.812 mmol (g cell)$^{-1}$ superoxide radicals (Table 2), which was 2.1-fold of that in wild-type cells. This suggests that the *SoxRS* regulon is also involved in protection against pyocyanin-induced superoxide radicals.

Discussion

X. campestris produced pyocyanin and improved pyocyanin production was observed as an adaptive response to treatments that induce ROS. The best improvement in pyocyanin production was observed after HOCl treatment and HOCl is a hydroxyl free radical generator. The superoxide radical generator, menadione, also caused a comparable improvement in pyocyanin production. Both HOCl and menadione are strong inducers of defense mechanisms against hydroxyl and superoxide radicals, respectively [3,5]. Comparatively, the hydroxyl radical producer, H_2O_2, caused a lower improvement in pyocyanin production and H_2O_2 is a weaker inducer of defense mechanisms against hydroxyl radicals in *X. campestris* [3]. Thus, it appears that pyocyanin production is linked to induction of defense mechanisms against free radicals.

Pyocyanin acts as a carrier in increasing the oxygen uptake of bacterial suspensions by several fold [28]. This enhanced oxygen availability explains the increased maximum cell concentration of *X. campestris* obtained in the presence of pyocyanin. However, the limited diffusion of pyocyanin through the membrane, as observed in the case of *P. aeruginosa* [11], may play a role in the observed insensitivity of extracellularly added pyocyanin on *X. campestris*. Also, the antioxidant levels in the presence and absence of pyocyanin are not significantly different, probably because much less pyocyanin enters the cell, which is not sufficient to induce antioxidant enzymes. On the other hand, the sensitivity of *E. coli* to pyocyanin could be due to higher uptake of pyocyanin; it is known that the amount of pyocyanin taken up by *E. coli* is higher than that taken up by *P. aeruginosa* under comparable conditions [11].

In the presence of pyocyanin, the superoxide radical levels obtained with the Δ*OxyR* mutant in the log phase was only 18% higher compared to the wild-type cells. This suggests that *OxyR*, which is normally induced by agents causing oxidative stress [6],

is not substantially induced by pyocyanin. However, absence of *katG* led to a 92% increase in the superoxide radical level, which indicates that *katG* is involved in protection against pyocyanin-induced free radicals in the log phase.

However, in the stationary phase, the absence of the regulon *rpoS* or the gene *katE* caused significant levels of superoxide radical generation compared to no superoxide radicals (Table 2) in the wild-type cells when they were grown in the presence of pyocyanin. This result indicates that the *rpoS–katE* system is able to provide the necessary protection against pyocyanin generated superoxide radicals in the stationary phase. In contrast, in the log phase, the *OxyR–katG* system was not able to provide the necessary protection which may be the reason for pyocyanin toxicity in the log phase.

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