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Pyocyanin Isolated from a Marine Microbial Population: Synergistic Production between Two Distinct Bacterial Species and Mode of Action

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Summary

Marine microbial populations collected from the Hawaiian Islands were screened for antimicrobial activity. A blue metabolite was identified from mixed cell cultures, but production was not evident in pure cultures. Experiments designed to probe the synergistic role of the microorganisms are presented. Full characterization of the blue natural product, pyocyanin, is provided including corrections made to ^{1}H and ^{13}C-NMR assignments of the molecule misreported in the chemical literature and yeast transcriptome analysis. The transcriptional effects were consistent with the compound's purported role as an inducer of oxidative stress and damage and illustrates the overall potential of the method to reveal the primary biological/cellular effects of a natural product. The experiments outlined here might serve as a general paradigm for identification of natural products arising from microbial communities and investigation of their respective interactions.

Introduction

Natural products and their structural analogs account for approximately 60% of new small-molecule drugs to enter the market over the past 20 years [1]. Notable triumphs include Taxol, produced from the Pacific yew tree and one of the most successful anticancer agents of the past decade [2], as well as vancomycin, often referred to as the drug of last resort, the last line of defense for treatment of drug-resistant *Staphylococcus aureus* infections [3]. Both drugs have generated $1 billion in annual sales [3, 4].

Traditional methods to mining natural products entail organism collection, extraction, bioassay-guided purification, and structure determination. Among possible sources, the marine environment has provided an abundant supply of natural products with a diverse array of structures and bioactivities, including antimicrobial, immunosuppressive, and anticancer agents [5, 6]. Indeed, ziconotide, the synthetic form of the cone snail peptide w-cenotoxin M-VII-A, a neuron-specific N-type calcium channel blocker, has recently entered the market for treatment of severe chronic pain [7].

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[2] These authors contributed equally to the experimental portion of this work.
[3] This article is dedicated to Professor Peter G. Schultz in honor of his 50th birthday.

Sponges and "culturable" microorganisms are common resources for the discovery of marine-derived therapeutics. Microorganisms can offer the added advantage of providing a continuous supply of the natural product, albeit it is frequently debated as to whether the sponge itself or microorganisms living within the sponge environment are responsible for natural product production [8–10]. The general strategy for identifying microorganisms from marine habitats typically involves screening microbial isolates for bioactivity. Here, we investigate marine microbial communities for antibiotic/antifungal activity, bearing the hypothesis in mind that natural product production, i.e., the regulation and expression of their respective biosynthetic pathways, could be coupled with microbial interactions. Indeed, bacteria isolated from marine snow particle-microbial communities have been shown to display potent antagonistic effects, offering some support for this mechanism [11, 12].

Results and Discussion

Isolation of Pup14

Samples were collected from the north shore of Oahu, Hawaii. Ocean floor sediments (up to a depth of 40 ft) were obtained as well as culture swabs of submerged rocks. The microorganism specimens were plated on bacterial or fungal plates employing standard literature protocols [13]. Microbial communities were inoculated into liquid media and cultured in shake flasks. Among the microorganism samples examined, Pup14 (Pupukea sample 14, bacterial plate) gave an unusual green phenotype following ~72 hr of culturing; while the cells remained white, the medium turned green. Extraction of the medium resulted in an aqua blue color. Crude extracts of the Pup14 sample were found to be moderately active against *E. coli* and yeast (IC_{50} = 25 μg/ml). Interestingly, the Pup14 sample showed that cells propagated from single colonies and grown in culture could not generate the green phenotype, suggesting microbial crosstalk—that natural product production was in some way coupled with microbial interactions.

Establishing Microbial Synergism of Pup14A and Pup14B

Therefore, to investigate the possibility that microbial interplay was necessary for stimulating natural product production, we initially took a split-pool approach. Sixty single colonies were picked and maintained individually on marine agar plates. Colonies were first pooled ten at a time, assayed for the green phenotype, and upon confirmation, the colonies were further split and pooled until it was determined that two microorganisms, designated here as Pup14A and Pup14B, were necessary for production of the blue metabolite (Figure 1).

At this point, two basic scenarios were envisioned. Either one strain is the inducer and one the producer or one strain generates a metabolic precursor that is subsequently converted by the other to give the final product. To decipher between these possible

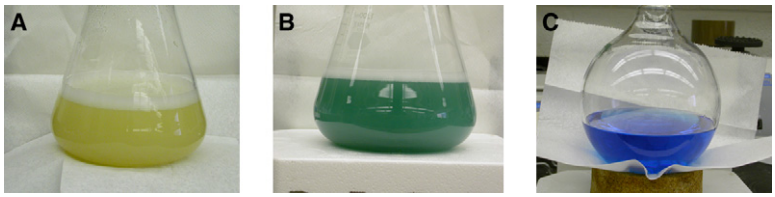


Figure 1. Photographs of Pup14A and Pup14B Cultures and Extracts Are Shown

(A) Pup14A and Pup14B following 24 hr of culturing at 30°C, 250 rpm.

(B) Pup14A and Pup14B following 72 hr of culturing at 30°C, 250 rpm.

(C) Pup14 crude extract generated by extraction of the medium (72 hr) with methylene chloride.

mechanisms, we performed a series of experiments. We first examined the natural product for toxicity against these strains. Overnight cultures of Pup14A and Pup14B were generated, diluted to an OD_{600} 0.1, and aliquoted into a 96-well plate. To each culture was added ethanol vehicle (serving as a control) or the purified blue metabolite to give final concentrations of 10 μg/ml, 100 μg/ml, and 1 mg/ml. The cultures were incubated with shaking for an additional 12 hr, and cell density was measured with a Biotek microplate reader. Cellular cytoxicity was, however, not observed with either Pup14A or Pup14B.

We, therefore, varied the ratio of Pup14A and Pup14B inoculated, which revealed that equal volumes of each microorganism were most effective at inducing natural product production. Skewing the ratio in favor of Pup14A over Pup14B resulted in a loss of induction, whereas inoculating with negligible amounts of Pup14A versus that of Pup14B still gave the green phenotype (and similar levels of production), but with delayed onset. This suggested a mechanism whereby Pup14A is the inducer and Pup14B is the producer. Moreover, as the natural product was produced in similar quantities in either case (whether equal or skewed cell ratios were utilized), the mechanism of activation does not likely involve the generation of an intermediate by one strain followed by metabolite transfer and biosynthesis by the other strain to give the final product.

A small-molecule exchange assay was performed where the cells of one were introduced into the media of the other and vice versa. The experiment was carried out at two different time points, 24 and 72 hr, respectively. Two sets of Pup14A and Pup14B cultures were thus generated and harvested by centrifugation at the appropriate time point, and the media were sterile-filtered. The cells of Pup14A were introduced into the media of Pup14B and vice versa (Pup14B cells introduced into the media of Pup14A). Induction, however, was not observed in either case. Therefore, small-molecule production by Pup14A alone was not sufficient for activation. Induction of the green phenotype might require cell-cell contact, or more complex mechanisms could be at work, such as metabolite exchange between microorganisms [14–16].

To address this issue and decipher between these two mechanisms, a Boyden chamber (consisting of two wells separated by a membrane) or transwell assay was carried out (Figure 2A). The producing strain, Pup14B, was inoculated into the bottom chamber, and Pup14A, the activating strain, into the top chamber. The cells were incubated at 30°C in an incubator, without shaking, for 2 days, extracted with methylene chloride, and compared visually to a set of controls where: (1) Pup14B was inoculated into the bottom chamber of one well (the top chamber containing nothing), or (2) Pup14A and Pup14B were mixed together (no membrane separation). Activation of the blue phenotype was observed when Pup14A and Pup14B were mixed together or when separated by a membrane (Figures 2C and 2D). In fact, activation was slightly enhanced when there was no cell contact (Figure 2D). As expected, no activation was observed when Pup14B was cultured alone (Figure 2B). Additionally, to ensure that the microorganisms could not penetrate the membrane, both Pup14A and Pup14B were each added to the top chamber of separate wells with the bottom chamber containing medium alone and incubated for 2 days. In neither case was translocation to the bottom chamber observed. The medium in the bottom chamber remained clear. The results from these experiments favor a mechanism in which metabolite exchange between the two microorganisms is necessary to induce the green phenotype.

To determine whether the interaction was specific between Pup14A and Pup14B or whether any microorganism could activate Pup14B, the microorganism was cultured with an assortment of other microorganisms. Pup14B was cultured for a week with microorganisms Hon6, Pup16, Surf1, and KM1. Pup14A was also cultured with these same microorganisms serving as a control set. While none of the Pup14A mixed cultures gave the green phenotype, Pup14B cultured with KM1 resulted in activation of the strain, albeit to a lesser extent than when cultured with Pup14A. Microorganisms were sent to the Centrallbureau voor Schimmelcultures in the Netherlands (CBS) for bacterial classification. The results are depicted in Table 1. Pup14A was identified as *Enterobacter sp.* and Pup14B as *Pseudomonas aeruginosa*, a quorum-sensing bacterium [17–19]. Quorum sensing is a phenomenon used by diverse bacteria

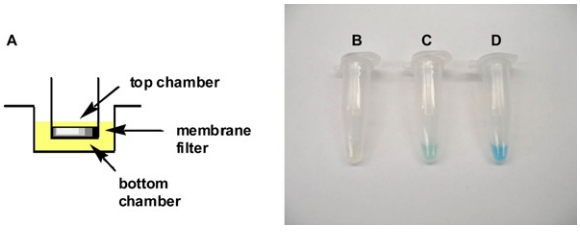


Figure 2. Results of Boyden Chamber Assay

(A) Pictorial diagram of the transwell chamber.

(B–D) Organic extracts obtained from Boyden chamber assay are shown: (B) Pup14B alone (bottom chamber), (C) Pup14A and Pup14B mixed together in the same well, and (D) Pup14A (top chamber) and Pup14B (bottom chamber).

Table 1. Species Identification of Microorganisms

Strain	Species Identification
Pup14A	*Enterobacter*
Pup14B	*Pseudomonas aeruginosa*
Hon6	New species, 98% identical to *Chromohalobacter*
Pup16	*Rhodotorula mucilaginosa*
KM1	*Enterobacter*

to communicate intercellularly, enabling the organism to express specific genes in a coordinated fashion, leading to a rapid and full-blown virulence cascade [15, 16]. A complex network of regulatory factors govern production of secondary metabolites in *Pseudomonas aeruginosa* that involve two quorum-sensing systems, *las* and *rhl* [20–24]. At least three signaling molecules have been identified that act as intercellular communication signals. The acyl homoserine lactone signals, *N*-(2-oxododecanoyl)homoserine lactone and *N*-butyryl homoserine lactone, are each autoinducers, together controlling 6%–11% of the *Pseudomonas* genome [20, 25–27]. The third intercellular signal is a quinolone compound, 2-heptyl-3-hydroxy-4-quinolone, that serves as a regulatory link between the *las* and *rhl* quorum-sensing systems and controls the synthesis of multiple virulence factors [20, 21]. In many strains of *Pseudomonas aeruginosa*, a basal level of autoinducer is produced at low culture densities, and as the population grows, autoinducer concentrations increase. On reaching a threshold concentration, the autoinducers activate the virulence cascade, including that of secondary metabolite production [28, 23]. Pup14B on the other hand, the *Pseudomonas aeruginosa* strain we have isolated, did not induce the blue phenotype in culture without activation by another strain (Pup14A or KM1), reflecting some likely evolutionary differences with this strain. Intriguingly, KM1 like Pup14A is of the genus *Enterobacter*, accounting for its ability to activate production of the compound. Both *Pseudomonas sp.* and *Enterobacter sp.* have been identified in microbial biofilms among a variety of virulent pathogens, some of which have been shown to be regulated by acylhomoserine lactone (AHL) quorum-sensing signals [29]. Moreover, cell-free supernatants of *Pseudomonas aeruginosa* and *Enterobacter agglomerans*, were shown to contain diketopiperizines (DKPs) capable of activating an *N*-acylhomoserine lactone (AHL) biosensor [30].

Characterization of the Natural Product

Concurrent to these investigations, we obtained an X-ray crystal structure of the blue natural product (Figure 3). Literature evaluation of *Pseudomonas aerginosa* taken together with the X-ray crystal structure data revealed that the identity of the compound was likely pyocyanin; however, full characterization on the compound was lacking. Therefore, in addition to providing X-ray analysis, 1D ^{1}H-NMR, and ^{13}C-NMR data, we performed 2D-NMR analyses of the compound including H,H-COSY, HMBC, HSQC, and DEPT (Table 2). The results revealed that some of the ^{1}H-NMR and ^{13}C-NMR signals in the spectra of the compound had been misassigned in the 1989 report [31]. The proton at carbon 3 was assigned to carbon 6, and vice versa.

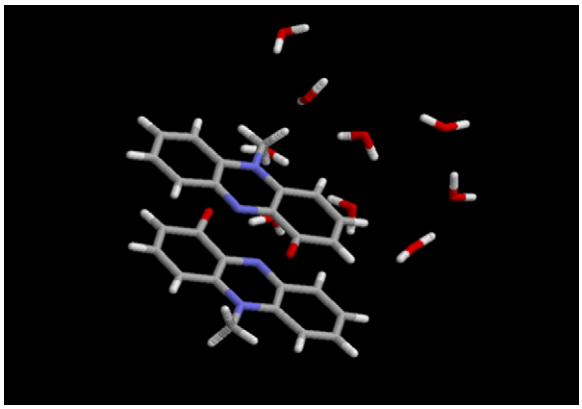


Figure 3. X-Ray Crystal Structure of Pyocyanin
Gray, carbon; blue, nitrogen; white, hydrogen; red, oxygen.

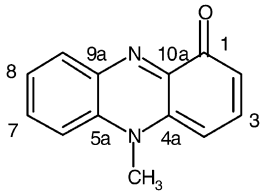
H,H-COSY showed coupling between protons 2, 3, and 4. Since protons at positions 3 and 6 have similar chemical shifts, 8.12 and 7.99 ppm, respectively, the data were misinterpreted. Moreover, ^{13}C-NMR peaks corresponding to carbons 5a and 9 and carbons 3 and 10a were also reversed in their assignments. Carbon positions 3 and 9, located at 147.3 and 134.0 ppm, were readily assigned on the basis of HSQC and DEPT experiments, which revealed the presence of an attached proton at these sites. This revealed an inversion of peak assignments between these carbons and carbons 10a and 5a that have very similar chemical shifts, 146.7 and 134.4 ppm, respectively. Spectral data on the compound are provided in Table 2 with corrections in place and *J*-values listed.

Quorum Control of Pyocyanin

Intriguingly, two seven-gene phenazine biosynthetic loci have been identified from *Pseudomonas aeruginosa* PAO1 [32]. The two operons, designated as *phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*, exhibit high homology to phenazine biosynthetic operons from *P. fluorescens*, *P. aureofaciens*, and *P. chlororaphis* PCL1391 [33–35]. Additionally, Southern hybridization analysis for the presence of the core phenazine biosynthetic genes (*phzA-G*) in 30 bacterial strains (including other *P. aeruginosa* strains) showed that the cluster could be detected in 21 of these strains [32]. Two additional genes (*phzM* and *phzS*) located in the vicinity of the *phzA1B1C1D1E1F1G1* operon in PAO1 have been shown to convert phenazine-1-carboxylic acid (PCA) to pyocyanin [32]. Upstream of the *phzA1B1C1D1E1F1G1* operon is a well-conserved putative promoter element required for quorum control, the *las* box, but does not precede the *phzA2B2C2D2E2F2G* locus [27]. Instead, a gene, *qscR*, encodes a protein that negatively regulates expression of a number of quorum-sensing controlled genes including the *phzA1B1C1D1E1F1G1* operon [36, 74].

We examined Pup14B for the presence of the gene cluster and its quorum-sensing region by PCR analysis, employing oligonucleotide probes flanking both the *las* box and *qscR* regions of the two biosynthetic loci and their respective upstream regions. PCR products were

Table 2. Pyocyanin Spectral Data



Position	^{1}H δ, mult; *J* (Hz)	^{13}C δ	COSY	HMBC	HSQC	DEPT
1		177.5				C
2	6.68, d; 8.7	115.5	H-3-H-2	C-3, C-4	H-2	CH
3	7.99, t; 8.4	147.3	H-2-H-3, H-4-H-3		H-3	CH
4	6.66, d; 8.4	95.3	H-3-H-4	C-2, C-3	H-4	CH
4a		136.4		5-Me		C
5a		134.4		5-Me		C
6	8.12, m	116.8	H-7-H-6	C-7,C-8	H-6	CH
7	8.12, m	138.1	H-6-H-7, H-8-H-7	C-8	H-7	CH
8	7.79, t; 7.0	128.0	H-7-H-8, H-9-H-8	C-6, C-7	H-8	CH
9	8.38, d; 8.4	134.0	H-8-H-9		H-9	CH
9a		138.5		C-7, C-8		C
10a		146.7		C-2, C-4		C
5-Me	4.29, s (3H)	36.7			H-5	CH_3

All spectra were recorded in methanol-d_4. ^{1}H-NMR (300 MHz) spectra were recorded on a Varian Inova-300 spectrometer. ^{13}C-NMR (125 MHz) and 2D-NMR spectra were recorded on a Bruker ARX500 spectrometer.

obtained in both cases and sequencing confirmed that both of these regions are intact in the Pup14B strain (BLAST alignments are provided as Supplemental Data available with this article online). As with *P. aeruginosa* PAO1, these results might suggest that the presence of two differentially regulated phenazine biosynthetic operons might give the bacterium the ability to more easily modulate phenazine production and adapt to environmental signals or growth phase [32].

Transcriptional Response to Pyocyanin Treatment

To further characterize pyocyanin, we investigated the mode of action of the compound by profiling the effects of the compound on the yeast transcriptome with Affymetrix oligonucleotide microarrays (Yeast S98 Array). Data were obtained in duplicate at two concentrations of agent, 25 and 250 μg/ml, at 6 hr of drug exposure (Table 3). Differential mRNA gene expression (≥2.5-fold) was observed in 45 transcripts of the approximately 6200 genes that comprise the yeast genome. The results are consistent with the proposed role of the drug as an inducer of oxidative damage [37–40]. The primary transcriptional response consisted of genes involved in oxidative damage and stress, cell-wall maintenance and synthesis, and DNA damage. Secondary effects consisted of genes involved in protein synthesis, drug resistance, transport, stress response, as well as metabolic genes, cell-cycle-related effects, and several unclassified transcripts.

The oxidative damage, cell-wall biosynthesis, and DNA damage effects observed with pyocyanin treatment are each fully consistent with the agent's documented ability to redox cycle and oxidize glutathione (L-γ-glutamylcysteinylglycine) [41, 37]. A map correlating pyocyanin's biochemical actions with its transcriptional effects is depicted in Figure 4. Under aerobic conditions, pyocyanin is reduced by NADH or NADPH by electron transfer, passing those electrons to O_2 to give superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). These oxidative species result in the oxidation of glutathione (GSH, the primary oxygen detoxification species and chemical modulator of redox potential within the eukaryotic cytosol) [42–44] to its oxidized state (GSSG). In this regard, glutathionine peroxidase (GPX2) was upregulated by 4.7-fold. Glutathione peroxidase neutralizes destructive peroxides by catalyzing their reduction using glutathione as an electron source [45]. Pyocyanin can also directly oxidize glutathione to form pyocyanin radical and superoxide radical ($O_2^{\bullet -}$), placing further oxidative stress on the cell [41]. Reactive oxygen species generated through pyocyanin exposure can also oxidize residues of proteins and elicit oxidative-stress-defense systems. Methionine sulfoxide reductase, for example, an oxidative damage-repair protein that acts by catalyzing the reduction of methionine sulfoxide and other methyl sulfoxides to methyl sulfides, was upregulated in its expression by 2.6-fold, consistent with this notion [46]. Other oxidative stress proteins upregulated with pyocyanin treatment included two alcohol dehydrogenases, YPL088W and YNL134C, differentially expressed by 3.1-fold and 3.7-fold, respectively, at the high dose [48]. Although their exact substrates are unknown, both have previously been associated with oxygen stress response. Likewise, we observed upregulation of glutathione *S*-transferase by 2.4-fold, a class of enzymes recruited for xenobiotic detoxification [42, 47]. Somewhat paradoxically, three genes normally upregulated by increases in oxygen concentration were found to be downregulated. These were *ctt1*, a cytoplasmic catalase (−2.6-fold) [49], and two cytochrome *c* encoding transcripts, *cyc7* (−3.3-fold and −4.3-fold at low and high doses, respectively) and *cyc1* (−2.6-fold at high dose), which function in respiration and electron transport [50]. Downregulation of

Table 3. Transcriptional Effects of Pyocyanin on Yeast

Probe Set	ORF/Gene Description	25 μg/ml	250 μg/ml
Protein-Synthesis Related			
10210_at	YLR136C/TIS11, tRNA-specific adenosine deaminase; transcription factor	—	4.9
10226_at	YLR107W/REX3, RNA exonuclease involved in RNA processing/degradation	—	2.8
9413_f_at	YMR230W/RPS10B, ribosomal protein	—	3.2
5957_at	YMR322c/SNO4, possible chaperon and cysteine protease	—	2.8
9667_at	RRN6P,RRN7P/rRNA transcription factors (RNAP I specific initiation factor)	—	3.0
10976_at	YJR047C/ANB1 translation initiation factor IF5A.2	—	−4.7
Oxygen-Stress Related			
5697_at	YER042w/MXR1, responsible for the reduction of methionine sulfoxide	—	2.6
7109_at	YBR244W/GPX2, glutathione peroxidase	—	4.7
7847_at	YPL088W/similar to aryl alcohol dehydrogenase	2.4	3.1
8988_at	YNL134C/putative alcohol dehydrogenase implicated in oxygen stress response	—	3.7
4924_at	YGR088W/*ctt1* cytoplasmic catalase T	−2.6	—
5751_at	YEL039C/*cyc7* iso-2-cytochrome *c*	−3.3	−4.3
10977_at	YJR048W/*cyc1* cytochrome *c* isoform 1	−2.6	—
General Drug Resistance			
10358_at	YLL028W/TPO1, vacuolar polyamine/H+ antiporter	—	4.1
4573_at	YHL035C/VMR1, multidrug resistance protein, ABC protein	—	2.5
4778_at	YGR213C/RTA1, integral membrane protein involved in 7-aminocholesterol resistance	—	5.9
8923_at	YNLO65W/AQR1, membrane transporter	—	4.7
9117_at	YNL231C/PDR16, protein involved in lipid synthesis and multidrug resistance	—	2.5
9328_at	YMR279C/member of a family of multidrug resistance proteins	10.6	—
Transport Proteins			
4936_at	YGR055W/MUP1, high affinity methionine permease	—	3.0
10852_at	YJR150C/DAN1 protein only expressed during anaerobic growth	—	−2.9
10795_at	YKL217W/JEN1 lactate and pyruvate permease	−2.5	—
9065_at	YNL194C/integral membrane protein localized to cell periphery	−2.3	−4.4
8528_at	YOR11w/AUS1 ATP-binding cassette transporter	—	−2.8
4388_at	YHR139C/SPS100, sporulation-specific wall maturation and cell-differentiation protein	—	3.2
6113_at	YDR371w/CTS2, sporulation-specific chitinase involved in cellular differentiation	—	4.7
7409_at	YBL043W/ECM13 involved in cell-wall synthesis	—	2.5
10853_at	YJR151C/DAN4 cell-wall mannoprotein, similar to YKL224c, Sta1p	—	−2.6
8526_at	YOR009W/TIR1 cell-wall mannoprotein, involved in cell-wall maintanance	—	−4.0
8527_at	YOR010C/TIR2 cold shock induced protein	—	−3.3
9628_at	YMR006C/PLB2 lysophospholipase/phospholipase B	—	−3.6
DNA Related			
5970_at	YDR501w/PLM2, plasmid maintenance protein; mutant shows 2mu-m plasmid instability	8.6	7.3
4045_s_at	YIL066C/RNR3, ribonucleotide reductase, large subunit expressed only after DNA damage	8.4	—
Amino Acid Metabolism			
5196_at	YGL184C/STR3, cystathionine beta-lyase	2.0	3.6
6915_at	YCL064C/CHA1, L-serine/L-threonine deaminase	6.1	12.6
Isoprenoid Metabolism			
8642_at	YOL101C/IZH4 implicated in zinc homeostasis, membrane protein	—	−8.1
9854_at	YLR450W/HMG2 3-hydroxy-3-methylglutaryl-coenzyme A reductase A	—	−2.6
Cell Cycle			
8380_at	YOR178c/GAC1 ser/thr phosphoprotein phosphatase, cell-cycle checkpoint	—	−2.5
Other			
4194_at	YIL117C/PRM5, pheromone-regulated protein, induced during cell-integrity signaling	—	2.6
5372_at	YFL014W/HSP12, heat-shock protein	—	2.6
8338_at	YOR226C/mitochondrial matrix protein required for synthesis of iron sulfur proteins	—	3.0
8399_at	YOR152C/protein of unknown function localized to ER	—	2.9
8399_at	YOR107W/RGS2, negative regulator of glucose induced cAMP signaling pathway	—	2.7
9305_at	YMR299C/DYN3, light intermediate chain of dynein	—	3.8
9567_at	YMR081C/ISF1 involved in suppression of mitochondrial splicing defect	—	−2.7

cytochrome *c* peroxidase genes has been observed with treatment of *Rhodobacter sphaeroides* with hydrogen peroxide [51]. The mechanism by which organisms cope with damage inflicted by reactive oxygen species varies from organism to organism, whereby nonessential genes are downregulated [51]. The involvement of catalase and superoxide dismutase (SOD) have both been detected in yeast exposed to pyocyanin at 0.5–1.5 hr posttreatment [38]. The absence of these genes from the transcriptional profile (measured at

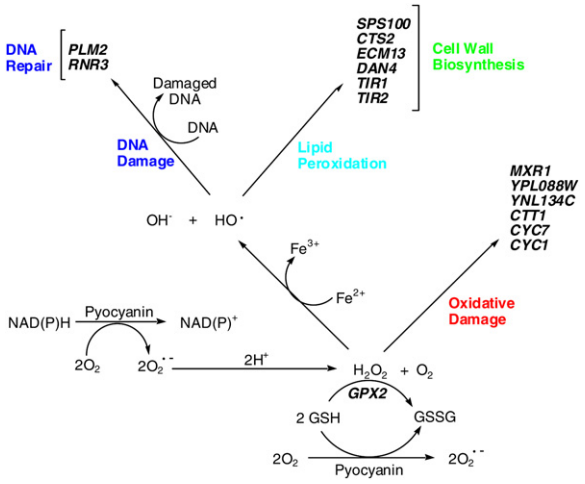


Figure 4. Correlation of Gene Changes Generated from Yeast Transcriptional Profile Data

6 hr) could thus reflect the timing of the response or half-life of the mRNA of these genes.

The production of hydrogen peroxide by pyocyanin treatment was further exemplified by the upregulation of genes associated with DNA damage. Although hydrogen peroxide (H_2O_2) itself does not inflict DNA damage, it is readily converted to hydroxyl radicals by transition metals such as iron (via the Fenton reaction) (Figure 4), resulting in the production of DNA radicals [52, 53]. Pyocyanin itself has also been shown to bind double-stranded DNA (with no base specificity) and inhibit DNA template controlled RNA synthesis [76]. Two DNA damage-response proteins, RNR3 and PLM2, were upregulated with pyocyanin treatment. RNR3, the large subunit of ribonucleotide reductase, was upregulated by 8.4-fold. Ribonucleotide reductases catalyze the reduction of nucleotide triphosphates to their corresponding deoxynucleotide triphosphates, playing a central role in DNA synthesis [54] and in response to DNA damage [55, 56]. PLM2 is a plasmid-maintenance protein and was upregulated in its expression up to 8.6-fold. Expression of this gene product is coupled with DNA damage [57] and has been shown to activate genes involved in DNA damage checkpoint and repair [58].

The damaging effects of hydroxyl radicals on DNA notwithstanding [52, 53], lipoperoxidation can also result, causing enhanced cell-wall permeability [51, 59–61]. Interestingly, a variety of cell-wall proteins were differentially expressed with pyocyanin treatment. While these effects have not been previously documented with pyocyanin exposure, they are consistent with the ability of the compound to redox cycle and generate reactive oxygen species. For example, SPS100, a protein that plays a protective role during the early stages of spore-wall formation, was enhanced in its expression by 3.2-fold [62]. CTS2, a sporulation-specific chitinase expressed during spore-wall synthesis was upregulated by 4.7-fold. Deletion of CTS2 has been shown to decrease surface density of the spore [63]. ECM13, another protein involved in cell wall biosynthesis, was increased in its expression by 2.5-fold, although the exact function of this gene has yet to be established [64]. On the contrary, cell-wall mannoproteins, DAN4, TIR1, and TIR2, were shown to be downregulated with pyocyanin treatment (−2.6-fold, −4.0-fold, and −3.3-fold, respectively). Each of these genes is responsible for the synthesis of cell-wall mannoproteins that are necessary for anaerobic growth [65]. Similarly, PLB2 a phospholipase involved in lipid metabolism was also downregulated. Presumably, these are nonessential genes for the survival of the organism under these oxidative conditions [66].

Collectively, the gene changes resulting from pyocyanin support the purported role of this compound as an inducer of oxidative damage. Although the transcriptional effects of the natural product have not been previously reported, we were intrigued to find that differentially expressed transcripts roughly paralleled those observed with hydrogen peroxide treatment [51, 67–71]. Pyocyanin has also been screened against a yeast deletion library as a means to identify potential protein targets [38]. Targets identified in this study also supported the ability of this compound to redox cycle and promote oxidative stress encompassing proteins involved in the cell cycle, electron transport and respiration, epidermal cell growth, protein sorting, vesicle transport, and the vacuolar ATPase [38].

Confirmation of Selected Gene Changes by RT-PCR

Validation of the transcriptional effects induced by pyocyanin treatment was demonstrated by semiquantitative reverse transcriptase (RT)-PCR of an arbitrary subset of the gene changes, including glutathione peroxidase (YBR244W), catabolic serine (threonine) dehydratase, iso-2-cytochrome *c* (YEL039C), and cystathionine β-lyase (YGL184C). The procedure, while less sensitive and quantitative than real-time PCR, is capable of confirming a transcriptional response, the presence or absence of a transcriptional effect. Normalization of the RNA/cDNA was based upon glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was unaffected by drug exposure. The results are provided as Supplemental Data.

Significance

In summary, we probed the hypothesis that microbial interactions in the marine environment can impact natural product production. Marine microbial populations were cultured and examined for antibiotic activity. Among the mixed cultures examined was a bacterium that was found to act synergistically with select microbial species to produce a blue compound. The bacterium was identified as *Pseudomonas aeruginosa*, and the blue metabolite as pyocyanin. Full structural analysis of pyocyanin, including X-ray analysis and 2D-NMR, was carried out. Corrections were made to ^{1}H-NMR and ^{13}C-NMR assignments of the compound that were misreported in the chemical literature. Taken together, the data gleaned from these investigations suggest the importance of investigating not only organic extracts generated from single colonies, but also those from mixed microbial populations for bioactivity. The strategy could lead to the discovery of otherwise unexpressed compounds. While the procedures detailed here were for the microbial

production of a blue metabolite, which certainly facilitated our analyses, the approach is broadly applicable toward the investigation of metabolites of other organisms. The primary procedural modification would involve examination and comparison of extracts generated from microbial communities and pure cultures by TLC and LC/MS.

Experimental Procedures

Instrumentation and General Methods

Unless otherwise specified, biochemical reagents were obtained from Sigma Biochemicals (St. Louis, MO). Instant Ocean utilized in marine medium preparations was obtained from Petco (College Station, TX). All other medium reagents were obtained from VWR International (West Chester, PA). Dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), and methanol (MeOH) were purchased from EMD, while ammonium hydroxide (NH_4OH) was purchased from EM Science (VWR International, West Chester, PA). All were used without further purification. Flash column chromatography was performed with grade 62 silica gel (EMD, 60–200 mesh). ^{1}H-NMR spectra were obtained with a Varian Inova-300 spectrometer (Palo Alto, CA), in methanol-d_4 (Aldrich; Milwaukee, WI). ^{1}H-NMR (300 MHz) spectra are reported as follows: chemical shifts in ppm downfield from TMS, the internal standard (δ-scale); multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet), integration, and coupling constant (Hz). ^{13}C- and other 2D NMR spectra were obtained on a Bruker ARX500 spectrometer. ^{13}C-NMR (125 MHz) spectra were recorded in proton decoupled mode and reported in ppm. Preparative high-performance liquid chromatography (HPLC) separations were conducted on a Varian ProStar 230 solvent delivery module equipped with a Varian ProStar 330 Photodiode Array Detector (Palo Alto, CA) by using a Phenomenex Luna 5μ Silica (250 × 10.0 mm) column (Torrance, CA). Infrared (IR) spectra were obtained on a Perkin-Elmer FT-IR spectrometer (Boston, MA) with absorption peaks reported in wave number (cm^{-1}). Mass spectra were recorded on a PE Sciex with an API Qstar Pulsar (Concord, Ontario, Canada). The Affymetrix platform (Santa Clara, CA) was used in all GeneChip experiments. Cell-density measurements were made with a Biotek μQuant plate reader.

Organisms

All marine microorganisms utilized in these experiments were obtained from field collections. Hon6 (isolate 6) was acquired from Honokohau, Maui, Pup16 (isolate 14) from Pupukea, Oahu, and KM1 (isolate 1) from the Florida Keys. Pup14A and Pup14B were also obtained from Pupukea (sample 14). The identity of each of the microorganisms was assigned by the Centralbureau voor Schimmelcultures (CBS) in the Netherlands. The yeast *S. cerevisiae* wild-type strain (#404; BY4741; MATa his3[δ]1 leu2[δ]0 met15[δ]0 ura3[δ]0) was obtained from Dr. Michael Kladde at the Department of Biochemistry, Texas A&M University. *E. coli* DH10B was obtained from Invitrogen (Carlsbad, CA).

Field Collection of Marine Microbial Samples

Marine agar plates were prepared with slight modification to standard literature protocols [13]. Yeast extract (2 g), bactopeptone (2 g), glucose (4 g), and bacto-agar (Difco, 20 g) were combined in 1 liter of seawater, prepared by mixing Instant Ocean (38 g, Petco) with deionized distilled water. The marine agar plates were further supplemented with either 10 mg/l of miconazole or a combination of chloramphenicol (10 mg/l) and kanamycin (10 mg/l) for culturing of bacteria or fungi, respectively. Culture swabs (Becton Dickinson, supplier VWR International; West Chester, PA) were utilized to swab submerged rocks, and autoclaved vials were used to collect ocean floor sediments. Samples were applied to both types of marine agar plates and incubated at room temperature for one week. Single colonies were generated by repeated streaking of microorganisms onto fresh plates. To generate marine microbial populations, cell scrapers (Costar, supplier VWR International; West Chester, PA) were applied to the original plates, and the cells resuspended and cultured in marine broth.

IC_{50} Determination

Overnight cultures (5 ml) of *S. cerevisiae* or *E. coli* were generated. *S. cerevisiae* was cultured in YPD broth (30°C, 250 rpm) [72] and *E. coli* in LB medium [73]. Cultures were diluted to an OD_{600} 0.1 and aliquoted (100 μl per well) into a 96-well plate. To each culture was added 1 μl of serial dilutions of the crude extract (solubilized in ethanol) or ethanol alone. All assays were executed in triplicate. The 96-well plate was incubated at 30°C, 250 rpm for 12 hr. Empty wells were filled with 100 μl of autoclaved water to prevent evaporation during incubation. Cell density was measured on a Biotek μQuant plate reader (Winooski, Vermont).

Examination of Synergism

Split/Pool Analysis

Single colonies (60) were streaked and individually numbered on marine agar plates. The cells were each individually resuspended in marine broth and brought to an OD_{600} 1.0. Initially, the cells were each cultured ten at a time. To 5 ml aliquots of marine broth was added 10 μl of each cell sample (in pools of ten, ten cell samples/5 ml marine broth). The samples were cultured for 3–4 days. Several pools gave the green phenotype. We focused on one of those pools and repeated the assay again but pooling two samples at a time (two cell samples/5 ml marine broth). As a control, we also cultured each sample individually, but no induction was observed in this case.

Varying the Ratio of Pup14A to Pup14B

Pup14A and Pup14B were streaked onto marine agar plates and grown to confluency. The cells were resuspended in marine broth and brought to an OD_{600} 1.0. The concentration of Pup14A (200 μl) was held constant and mixed with different amounts of Pup14B (at 1, 10, 50, 100, and 200 μl) and vice versa in marine broth (5 ml). The concentration of Pup14B (200 μl) was held constant and the amount of Pup14A varied (at 1, 10, 50, 100, and 200 μl).

Inhibition Assays

Pup14A and Pup14B were cultured overnight at 30°C, 250 rpm. Cultures were diluted to an OD_{600} 0.1 and aliquoted (100 μl per well) into a 96-well plate. To each culture was added 1 μl of pyocyanin (solubilized in ethanol; stock solutions of 1, 10, and 100 mg/ml to give final concentrations of 10 μg/ml, 100 μg/ml, and 1 mg/ml) or ethanol alone. All assays were executed in triplicate. The 96-well plate was incubated at 30°C, 250 rpm for 12 hr. Empty wells were filled with 100 μl of autoclaved water to prevent evaporation during incubation. Cell density was measured on a Biotek μQuant plate reader (Winooski, Vermont).

Small-Molecule Induction Assay

Pup14A and Pup14B were cultured on marine agar plates for two days as described above. The agar plates were then sliced into pieces. Agar from one half of each plate was added to separate 2 liter Erlenmeyer flasks containing 1 liter of marine broth. Two sets of each culture (corresponding to a total of four 2 liter flasks) were allowed to grow at 30°C at 250 rpm for 24 and 72 hr, respectively, and subsequently centrifuged. The media from each culture were filtered through a 0.2 μm filter (Nalgene) and transferred to autoclaved flasks (2 liter). Pup14A cells (24 and 72 hr) were added to the medium of Pup14B (24 and 72 hr) and vice versa. Cultures were again placed in a rotary shaker at 30°C for 3 days. As a control, the other half of each plate (Pup14A and Pup14B) was cocultured in 1 liter of marine medium for 2–3 days until the coloration of the medium turned from yellow to green.

Boyden Chamber Assay

The Boyden chamber assay was performed with Corning transwell plates (0.4 μm pore diameter, 24 mm diameter, 6-well, Corning, NY). Marine broth (1 ml) was added to the top and bottom chambers of the wells of the plate and subsequently inoculated with the appropriate microbial strains. The plates were incubated at 30°C for 2 days without shaking. Following incubation, the cultures (from the bottom chamber of the wells) were extracted with methylene chloride and centrifuged to give two distinct layers, and the organics were transferred to fresh tubes and concentrated to ~100 μl volume for comparison.

Induction of Blue Phenotype with Different Strains

Microorganisms Pup14A and Pup14B, Hon6, Pup16, Surf 1, and KM1 were cultured on marine agar plates to confluency and resuspended in marine medium to give an OD_{600} 1.0. Pup14A or

Pup14B (200 μl) was added to 5 ml of marine broth and mixed with 200 μl of Hon6, Pup16, or KM1. As a control, Pup14A and Pup14B (200 μl each) were mixed and cultured. All cultures were grown at 30°C, 250 rpm for a period of a week.

Samples were evaporated to dryness, redissolved in dichloromethane, and examined by HPLC. An isocratic solvent system of 87:13 CH_2Cl_2/5% NH_4OH in MeOH was employed. Under these conditions, pyocyanin elutes with a retention time (R_t) of 7.23 min. Pyocyanin gives a characteristic blue phenotype and is easily detected upon collection. Purified pyocyanin was collected, concentrated in vacuo and its identity confirmed by mass spectrometry.

Isolation and Characterization of Pyocyanin

Purification Conditions

Liquid cultures (2 l) were extracted twice with 1 l of dichloromethane. Fractions were combined, and the solvent evaporated under vacuum. The organic residue was partially purified by flash-column chromatography with silica gel as the stationary phase and 10:1:0.001 CH_2Cl_2/MeOH/NH_4OH as the mobile phase. Fractions were analyzed by TLC utilizing the same conditions. Fractions containing the blue compound were combined and the solvent evaporated to dryness. The remaining semipure residue was dissolved in 1 ml dichloromethane and precipitated with 2 ml hexanes. After standing on ice for 5 min, the sample was centrifuged at 14,000 rpm at 4°C for 1 min to pellet the blue material and the solvent aspirated off. The precipitation procedure was repeated five times.

HPLC Conditions

Further purification of pyocyanin was performed via high performance liquid chromatography (HPLC). The blue organic residue was dissolved in dichloromethane and purified by HPLC utilizing an isocratic solvent system (87:13 CH_2Cl_2/5% NH_4OH in MeOH). The purified pyocyanin was collected with a retention time (R_t) of 8.63 min, concentrated in vacuo, and stored at −80°C. Approximately 2 mg of pyocyanin can be generated per liter of cells.

Crystallization Conditions

Pyocyanin (1 mg) was dissolved in 0.5 ml 1:1 CH_2Cl_2:$CHCl_3$ and placed into a test tube equipped with a female ground glass joint. The joint was connected to a Dean-Stark trap which contained 6 ml hexanes in the collection tube. The test tube was submerged in an ice bath. The remainder of the apparatus was maintained at room temperature. Green-blue crystals formed within 48 hr.

Characterization Data

^{1}H-NMR (300 MHz, methanol-d_4) 8.38 (d, J = 8.4 Hz, 1H), 8.12 (m, 2H), 7.99 (t, J = 8.4Hz, 1H), 7.79 (t, J = 7.0 Hz, 1H), 6.67 (dd, J = 8.4, 4.1 Hz, 2H), 3.95 (s, 3H); ^{13}C NMR (75 MHz, Methanol-d_4) 177.5, 147.3, 146.7, 138.5, 138.1, 136.4, 134.4, 134.0, 128.0, 116.8, 115.5, 95.3, 36.7; IR (neat solid) 2982, 1599, 1454 cm^{-1}; HRMS (ESI) for $C_{13}H_{11}N_2O$ $(M+H)^+$: calcd 211.0871, found 211.0885.

Spectral data are published as Supplemental Data. X-ray data are available through the Cambridge Crystallographic Data Centre (deposition number: CCDC 626277).

Detection of Phenazine Biosynthetic Loci- and Quorum-Sensing Regions

P. aeruginosa strain Pup14B was cultured as detailed above and genomic DNA isolated from 125 mg of the cell pellet. The pellet was washed twice with 1.5 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.5]) and resuspended in 2 ml of TE buffer. To this was added 2 ml of lysis buffer (0.5 M EDTA, 1% w/v SDS [pH 9.0]) and 1 mg of proteinase K (Sigma-Aldrich). The sample was incubated at 50°C for 16 hr. The solution volume was doubled with water, extracted with chloroform, and the DNA isolated by spooling [73]. The DNA was resuspended in 500 μl of TE and treated with 10 μl of 1 mg/ml RNase A for 2 hr at 37°C. This solution was extracted with an equal volume of chloroform, the DNA precipitated with isopropanol, and the pellet washed with 70% ethanol. The DNA pellet was dried and respended in 250 ul of TE buffer.

The 700 bp sequence upstream of the phzA1 gene was amplified by PCR from the Pup14B genomic DNA with the pyo las F and pyo las R primer set. The reaction contained (in 50 μl) 1× ThermoPol Reaction Buffer, 2.5% DMSO, 250 mM of dNTP's, 2 U Taq DNA polymerase (New England Biolabs, Ipswich, MA), 50 pmol of each primer, and 20 ng of genomic DNA template. Thermal cycling was performed with a PTC-200 thermal cycler (MJ Research, Waltham, MA) by using an initial 2 min denaturing step at 94°C, followed by 30 cycles of 94°C for 30 s, 30 s at the annealing temperature of 55°C, and 1 min at 68°C. After cycling, the reaction was held at 68°C for 7 min. Similarly, the 2041 bp region upstream of the phzA2 gene was amplified in overlapping sections from genomic DNA by using three primer sets, phz2_las forward and reverse (480 bp), phz2_up2 forward and reverse (1102 bp), and phz2_up3 forward and reverse (937 bp). The same reaction conditions and temperature cycles were used, except that the annealing temperature was 59°C for the phz2_las primer pair and 50°C for the phz2_up2 and phz2_up3 primer pairs. The PCR products were gel purified and TOPO-TA cloned (Invitrogen, Carlsbad, CA).

PCR Primers

pyo las, 55°C: forward, 5′-GTAACCCGAGAAGTACCCAAGCG-3′; reverse, 5′-TTTCCCTGTACCGCTGACCGTTC-3′. phz2 las, 59°C: forward, 5′-ACGCCATCGGCCTGCTCAACTG-3′; reverse, 5′-GGTAAACCCTTTCAACCGTTGG-3′. phz2_up2, 50°C: forward, 5′-CTCTTCAGCCTCGTTTCGTC-3′; reverse, 5′-CCCGGAAAGTTGCACTAGC-3′. phz2_up3, 50°C: forward, 5′-GGAACCAACTGTTCCAGCAT-3′; reverse, 5′-CCTGGGTAATTGGACAGGAA-3′.

Cloned fragments were end sequenced with primers PCR2.1seq forward and reverse. Gaps were filled by chromosome walking with the primers 3-4 left, 3-4 right, 2-3 left, and 2-3 right. Sequencing reactions were performed with the Perkin Elmer ABI BigDye Terminator kit according to the manufacturer's instructions and analyzed on an ABI 3130×/Genetic Analyzer by the Gene Technology Lab Core Facility of Texas A&M University. Primer design for these procedures was aided by Primer 3 software (source code available at http://fokker.wi.mit.edu/primer3/) [75].

Sequencing Primers

PCR2.1seq: forward, 5′-CAGGAAACAGCTATGACCATG-3′; reverse, 5′-AGATGCATGCTCGAGCGGCCGCC-3′.

Chromosome Walking Primers

3-4 Left: 5′-CTTTTTAATTGCCTCAGAAT-3′. 2-3 Left: 5′-AGATATGCGGTAATTATGGA-3′. 3-4 Right: 5′-AACCGTAGAACGGCTCTC-3′. 2-3 Right: 5′-GTGATCCAGAGCAGGAAG-3′. Sequence data are published as Supplemental Data.

Yeast Sample Preparation and GeneChip Evaluation

Yeast was cultured overnight in YPD broth [72] to an OD_{600} of 1.0. Total RNA was extracted with an SDS/hot phenol extraction method [72] and treated with DNase as follows. Total RNA (30 μl) was incubated at room temperature with 5 μl of first strand buffer (GIBCO-BRL cDNA Superscript Choice Kit; Carlsbad, CA) and 1 μl of DNase I (RNase-free, Ambion). Samples were heat inactivated by heating the samples at 75°C for 15 min and subsequently purified with an RNeasy kit (QIAGEN, Valencia, CA). The samples (15 μg of total RNA per sample) were provided to the GeneChip facility where they were amplified, biotinylated, and hybridized to GeneChips according to the protocol detailed by Affymetrix (Santa Clara, CA). Microarray data are available through the NCBI Geo Database (accession number: GSE6185).

RT-PCR, Validation of GeneChip Results

RT-PCR was used to quantify the mRNA levels of the following: glutathione peroxidase, catabolic serine (threonine) dehydratase, iso-2-cytochrome *c*, and cystathionine β-lyase. Total RNA was extracted from control cells and cells treated with pyocyanin (25 μg/ml and 250 μg/ml). Following extraction, total RNA (10 μg) was transcribed into single-stranded cDNA with the Superscript Choice system (GIBCO-BRL). PCR reactions were conducted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous amplification standard. PCR conditions were optimized so that amplification of both GAPDH and the cDNA of interest were in the exponential phase (estimated by varying the number of PCR cycles for each template or gene of interest). PCR cycles consisted of: step 1, 5 min denaturation at 95°C; step 2, 1 min of denaturation at 95°C; step 3, 1 min of primer annealing at the appropriate temperature (specified below); step 4, 1 min of extension at 72°C. Steps 2 through 4 were repeated for the requisite number of cycles. PCR products were stained with Sybr Green and fluorescence was measured with a microplate reader.

Primers

Glutathione peroxidase, YBR244W, 44°C: forward, 5′-ATGACCACA TCTTTTTATGATTTAG-3′; reverse, 5′-TCATTTACTTAACAGGCTTTG GATT-3′. Catabolic serine (threonine) dehydratase, YCL064C 45°C: forward, 5′-CATCTTTGTCCTCTATTACAACGGA-3′; reverse, 5′-GGT AGATAAAATCAGGAACACCGGT-3′. Iso-2-cytochrome *c*, YEL039C, 45°C: forward, 5′-CTATTTGGCAGCCTTTGTCATATAA-3′; reverse, 5′-ATGGCTAAAGAAAGTACGGGATTCA-3′. Cystathionine beta-lyase, YGL184C, 45°C: forward, 5′-ATTGTGTTCTTCTAGAGTCT-3′; reverse, 5′-TATTGAACGATTTATGCAGC-3′. GAPDH, YJR009C, 55°C: forward, 5′-ACATTGACATCGCCATTGACTCCAC-3′; reverse, 5′-TTTCATCGTAGGTGGTTTCCTTGTT-3′.

Supplemental Data

Supplemental Data include characterization data on pyocyanin, sequence data demonstrating the presence of the phenazine biosynthetic gene cluster and quorum-sensing region in Pup14B, and RT-PCR analysis on select green changes observed by transcriptional-array profiling. They are available at http://www.structure.org/cgi/content/full/13/12/1349/DC1/.

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Accession Numbers

Microarray data has been entered into the NCBI Geo Database under the accession number GSE6185.