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Genomic environment of *cue*R and *cop*A genes for prodigiosin biosynthesis by *Serratia marcescens* SB08

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Abstract

Serratia strains produce prodigiosin, which are a red linear tripyrrole antibiotic and a member of prodiginines. Prodigiosin is of potent clinical interest because it is reported to have anti fungal, anti bacterial, anti protozoal, anti malarial, immunosuppressive and anti cancer activities. The prodigiosin biosynthesis gene cluster (pig cluster) from Serratia marcescens SB08 has been sequenced and expressed in heterologous hosts. The gene responsible for prodigiosin production by Serratia marcescens SB08 was identified by PCR and agarose gel electrophoresis. The regions between cueR and pigA and pigN and copA were amplified by PCR using the primers cueR, PE1, ab77 and PE2 which resulted in product size of 488 bp and 183 bp respectively. These findings could suggest that the pigment gene cluster might be under cueR-mediated control and / or that one or more of the pigment enzymes required copper, or a related metal, as a cofactor.

Keywords: *cop*A; *cue*R; Gene cluster; Prodigiosin biosynthesis; *Serratia marcescens* SB08

Introduction

The red pigment prodigiosin is a member of the family prodiginines, produced by some *Serratia* species, actinomycetes and a few other bacteria [1] and is also a linear tripyrrole and a typical secondary metabolite, appearing only in the later stages of bacterial growth [2]. Prodigiosins have no defined role in the physiology of the strains that produce them, but reported to have antifungal, antibacterial and antiprotozoal / antimalarial activities [3, 4]. Montaner *et al.* [5] showed that prodigiosin extracted from *S. marcescens* could induce apoptosis in haematopoietic cancer cell lines including acute T-cell leukemia, myeloma and Burkitt's lymphoma, with little effect on non-malignant cell lines. Prodigiosins and their synthetic derivatives have potent and specific immunosuppressive activity, with novel targets clearly distinct from other drugs [6, 7, 8, 9]. Consequently, prodigiosins are of interest to scientific community to investigate their gene and its role in prodigiosin production which would ultimately expose the efficacy of their potential clinical and other industrial utilities.

Serratia sp. ATCC 39006 has expressed the red pigment prodigiosin gene cluster. This pigment gene cluster is also present in *Erwinia carotovora* subsp. *carotovora*, though it was not expressed in several other members of the *Enterobacteriaceae*, including *E. coli* [10]. In

Serratia 39006 the production of prodigiosin is regulated by multiple factors, including a quorum-sensing system, via -LuxIR homologues, *Sma*I and *Sma*R [10, 11].

According to Cerdeno *et al.* [12], the *Serratia* pigment gene clusters contain 14 candidate genes common to *Serratia* and *Streptomyces coelicolor* and are arranged from *pig*A through to *pig*N. Four candidate genes are unassigned and the remaining candidate genes are supposed to be involved in the post-translational modification of some of the proteins in the cluster. The order of the genes is conserved between the *Serratia* sp 39006 and *Serratia marcescens* ATCC274 and the corresponding 14 predicted proteins are similar in size and share significant amino acid identities between the species. However, the relative genomic contexts of the clusters and their regulation are very different.

Slater *et al.* [11] reported that *Serratia* 39006 pigment gene cluster contains an additional gene, tentatively designated as *pig*O. RT-PCR and primer extension have confirmed that there is transcriptional read through between *pig*N and -*pig*O, consistent with *pig*O being part of the pig operon in that strain. The *Serratia* 39006 pigment gene cluster (*pig*A – O) is transcribed as a polycistronic message from a promoter upstream of *pig*A and so it is presumed that the pigment cluster (*pig*A–*pig*N) of *Serratia marcescens* 274 is also transcribed as a polycistronic message.

*Pig*A has good sequence identity with a wide range of acyl-CoA dehydrogenases. Sequence alignment with human isovaleryl-CoA dehydrogenase, the crystal structure of which has been determined, showed that the majority of amino acids around the flavin and the phosphopantetheinyl moiety of CoA are conserved but the amino acids involved in binding the adenosyl group of CoA are not conserved [13]. This supports the suggestion that the substrate for PigA is a prolyl PCP rather than prolyl-CoA.

In this paper we report the organization of the prodigiosin biosynthetic gene (pig) clusters in *Serratia marcescens* SB08 and we define the genomic context of the pigment gene cluster with the objective to find whether the *cue*R / *cop*A genes flank the pigment cluster.

Materials and Methods

DNA extraction and quantification

The cells of *Serratia marcescens* SB08 were extracted by centrifugation at 5,000 rpm for 10 minutes at 7^{o}C and resuspended in 600 µL of saline solution (0.15 M NaCl) containing 0.1 M ethylenediaminetetraacetic acid (EDTA), pH 8.0. After a second centrifugation the pellet was resuspended in 300 µL of saline-EDTA. Then 200 µL of 25% (w/v) sodium dodecyl sulfate (SDS) was added, and the mixture was homogenized and incubated at 60^{0}C for 10 minutes. Sodium chloride solution (5.0 M) was added to achieve a concentration of 1.4 M NaCl and the lysate was immediately cooled and then centrifuged at 8,000 rpm for 15 minutes. The supernatant was transferred to a new microfuge tube for deproteinization with phenolchloroform. Total DNA was precipitated with isopropanol and the precipitate was resuspended in Tris- EDTA (TE) buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0). The pure working DNA was stored at -70^{o}C for longer periods and preferably at – 20^{o}C for short period for repeated uses in precisely coded and parafilm sealed microfuge tube [14].

Primer selection

The nucleotide sequence was analyzed by NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). BLAST (http://www.ncbi.nlm.nih. gov/BLAST) and PFAM (http://www.sanger.ac.uk/Software/Pfam) searches were performed to identify putative gene function. Primer sequences were analyzed using the software for cross

homologies against each other. Synthesized primers were purchased from Bangalore Genei Pvt., Bangalore, India.

Target gene	Primer	Sequence (5' – 3')	PCR product size (bp)	Reference
*cue*R	cueR PE1	TCGTAAAAACGAATCGTC GCAAAACTCTGAGCGGATTCGC	488	Gene ID: 2955378
*cop*A	ab77 PE2	GAAACACTTAACCTGACG CGCAGTTCATGCAGGACAGC	183	Gene ID: 2653903

Primer sequence

***cue*R** (copper efflux regulator)
GeneID: 2955378
Sense strand 5'- TCGTAAAAACGAATCGTC - 3'
Antisense strand 5'- GCAAAACTCTGAGCGGATTCGC - 3'

***cop*A (copper-transporting P-type ATPase)**
GeneID: 2653903
Sense strand 5' – GAAACACTTAACCTGACG – 3'
Antisense strand 5'-CGCAGTTCATGCAGGACAGC- 3'

PCR amplification

Each set of PCR reaction was carried out in duplex. DNA samples were subjected to PCR amplifications. All the genes studied were genotyped by amplifying 100 – 200 ng of DNA in a 22 – cycle three step PCR (Eppendorf Thermal Cycler, Germany) under the following conditions.

The PCR reaction systems consisted of 10 Mm Tris–HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM deoxynucleotide triphosphates (dNTPs), 25 µM of each primer, 1 U *Taq* DNA polymerase (Bangalore Genei, India), and 100 ng of DNA in a final volume of 25µl. Degenerate primers based on the *cop*A gene (5'– GAAACACTTAACCTGACG –3' and 5'– CGCAGTTCATGCAGGACAGC-3') and *cue*R gene (5' – TCGTAAAAACGAATCGTC - 3' and 5'-GCAAAACTCTGAGCGGATTCGC -3') were used.

The amplifications were carried out in a thermal cycler. Each cycle consisted of a 1 min denaturation step (94^{o}C), a 30 s annealing step (55^{o}C), and a 30 s extension step (72^{o}C). Finally, products were extended for 10 min at the completion of 22 amplification cycles. The PCR products (amplicons) were subjected to electrophoresis in 2.0% agarose (Sigma, USA) gel containing ethidium bromide 1% (Merck – Germany) in TBE buffer 140 mA. A DNA ladder was also run to confirm the amplification of the desired gene.

Agarose gel electrophoresis

Fifty ml of the 1X TAE buffer was taken in a conical flask and 0.5 g of agarose was added. It was melted completely into a clear solution, using a microwave oven. Molten agarose was cooled to 50°C and ethidium bromide was added to final concentration of 0.5 µg ml^{-1} (5 µl of 10 mg ml^{-1} solution of ethidium bromide to 100 ml gel mixture). Molten agarose was poured gently avoiding bubbles into a pre-set template with well – forming comb (an agarose gel template tank) cleaned with 70% ethanol and its ends were sealed with adhesive tapes. It was horizontally kept undisturbed till the agarose solidifies. After 30 – 45 minutes, the comb and the sealing tapes were removed without damaging the wells. The template was

mounted in an appropriate electrophoresis tank and the tank was filled with 1X TBE buffer just to immerse the gel up to 1mm. The samples were loaded into the wells in the gel. The electrophoresis was performed at 50 volts, using 1X TBE as the running buffer. When the dye, Bromophenol blue had migrated a distance sufficient for separation of the plasmid DNA fragments (when it had crossed more than 2/3 of the length of the gel, Bromophenol blue co - migrates with 500 bp fragments) it was switched off.

Silver staining

The gel was subjected to stain in silver nitrate to visualize the bands. The gel was carefully removed from the plate and washed in the washing solution containing 1 mL of 37% formaldehyde and 40% ethanol and 60% distilled water with slow shaking for 10 minutes. After decanting the washing solution, the gel was immersed in 0.2% of sodium thio sulphate solution for 1 – 2 minutes with gentle shaking and the gel was washed twice with distilled water. After this the gel was soaked in 0.1% silver nitrate solution for 15 minutes in dark with gentle shaking. The silver nitrate solution was collected into a dark bottle and the gel was washed again with distilled water. Later the developing solution containing 0.75 g sodium hydroxide dissolved in 50 mL of water along with 200 µL of formaldehyde (37%) was added in dark condition to develop the gel. When sufficient intensity of band developed, the reaction was stopped by addition of solution containing 5% of acetic acid. The gel was preserved in preserving solution. Later the gel was documented in bio-rad documentation unit (USA).

Southern blot hybridization

DNA fragment containing an internal part or the nearly complete *cop*A and *cue*R gene were labelled with digoxigenin as described by the manufacturer (Boerhinger Mannbeim). The genomic DNA was isolated and after electrophoresis, the DNA was denatured and transferred to a nylon membrane (Hybond N). After baking, the membrane was prehybridized and hybridized in 5×SSC, 0.5% blocking reagent – (Boerhinger Mannheim), 0.1% sarkosyl and 0.02% SDS. Washings, antibody incubation and signal detection with AMPFD (Boehringer Mannheim) were as described by the manufacturer.

Results

Genomic context of the pigment clusters

Serratia marcescens SB08 was examined for *cue*R (copper efflux regulator) and *cop*A (copper-transporting P-type ATPase) genes which flank the pigment cluster. The regions between *cue*R and *pig*A and *pig*N and *cop*A amplified by PCR using the primers cueR, PE1, ab77 and PE2 resulted in product size of 488 bp and 183 bp respectively (figure 1 and 2).

The *Serratia* pigment gene clusters contained 14 genes and were arranged from *pig*A to *pig*N. The order of the genes was conserved and the corresponding 14 predicted proteins were similar in size and shared significant amino acid identities. But the relative genomic context of *Serratia marcescens* (pigment cluster *pig* A – N) was transcribed as a polycistronic message from a promoter upstream of *pig*A. The product of the 5' gene showed sequence similarity to NADH oxidases and contained the pyridine nucleotide-disulphide oxidoreductase dimerization domain. The 3' gene encoded a hypothetical protein with no known significant homologues.

In *Serratia marcescens* SB08, the genes flanking the pigment gene cluster encoded protein similar to *cue*R and *cop*A. *Cue*R was the transcriptional regulator of *cop*A and *cop*A was a copper transporting P-type. The *cop*A and *cue*R genes and their homologues were typically contiguous.

As shown in PCR product (figure 1 and 2), *cue*R – *cop*A generated product revealing that *cue*R and *cop*A flanked the pigment cluster. The *cue*R and *cop*A homologues flanked by the pigment cluster in *Serratia marcescens* SB08 strain at 488 and 183 bp showed the predicted functions of pigment gene products and the red homologues of each pigment proteins.

The above findings could suggest that the pigment cluster may be under *cue*R mediated control and that one or more of the pigment enzymes require copper as a cofactor. The *cue*R / *cop*A system is involved in copper efflux. It is therefore intriguing that prodigiosin has been shown to affect copper dependent DNA cleavage and is conceivable that intracellular copper availability must be regulated in concert with prodigiosin synthesis to avoid DNA damage.

Southern blotting using *Serratia marcescens* SB08

The *Serratia marcescens* SB08 pigment gene cluster cosmid (pPIG4) was digested, DIG labelled using random prime (Roche) and used to probe genomic DNA from the same strain. The strain had bands, many corresponding to the restriction pattern from which the probe was generated, suggesting that restriction sites were conserved between many of the pigment gene clusters.

The results showed that *cop*A had a role in copper homeostasis in *Serratia marcescens* SB08 and was also clearly a factor impinging on regulation of the secondary metabolite, prodigiosin.

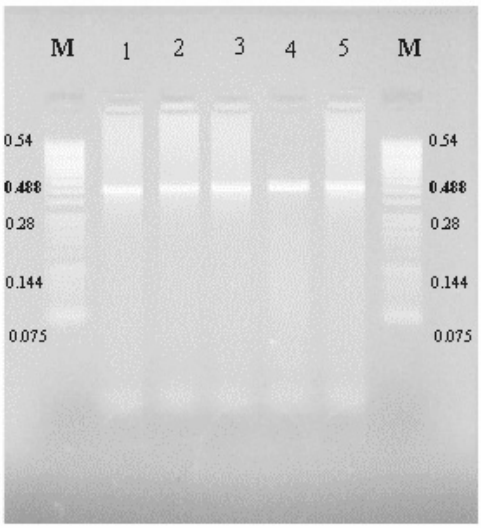

Figure 1 – Agarose gel electrophoresis of *cue*R gene. Migration was from top (cathode) to bottom (anode). Electrophoresis was done at 50 V in a 1.5 % agarose slab gel with (SIGMA- USA) ethidium bromide 1% (MERCK- GERMANY). Finally examined under UV Transilluminator (BIO-RAD, USA). Lane 1 - 5 represents the *cue*R gene and lane M represents MSPI/DNA digest 15 bp marker

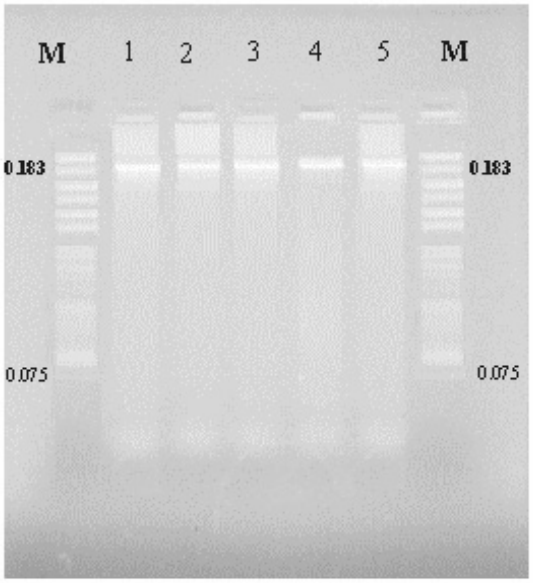

Figure 2 - Agarose gel electrophoresis of *cop*A gene. Migration was from top (cathode) to bottom (anode). Electrophoresis was done at 50 V in a 1.5 % agarose slab gel with (SIGMA- USA) ethidium bromide 1% (MERCK- GERMANY). Finally examined under UV Transilluminator (BIO-RAD, USA). Lane 1 - 5 represents the *cop*A gene and lane M represents MSPI/DNA digest 10 bp marker

Discussion

In *Serratia* 39006, the genes flanking the pigment encode proteins similar to *cue*R (68% identity and 76% similarity to YbbI, accession number AAK06655) and *cop*A (69% identity and 80% similarity to *Cop*A, accession number Q59385) of *E. coli* [15, 16]. *Cue*R is the transcriptional regulator of *cop*A. *Cue*R is a member of the MerR family of transcriptional regulators, which occur in a range of bacterial genera and respond to a wide variety of external stimuli [16, 17, 18, 19, 20]. *Cop*A is a copper-transporting P-type ATPase efflux pump and a central component of copper homeostasis in *E. coli* [15].

This study, investigated to find whether the *cue*R / *cop*A genes of *Serratia marcescens* SB08 flank the pigment cluster revealed that *cue*R–*cop*A homologues are arranged at either end of the pigment cluster (Figure 3). These findings could suggest that the pigment gene cluster might be under *cue*R-mediated control and / or that one or more of the pigment enzymes required copper, or a related metal, as a cofactor. The *cue*R/*cop*A system is involved in copper efflux [16, 21, 22]. It is therefore intriguing that prodigiosin has been shown to effect Cu-dependent DNA cleavage [23, 24] and so it is conceivable that intracellular copper availability must be regulated in concert with prodigiosin biosynthesis to avoid DNA damage.

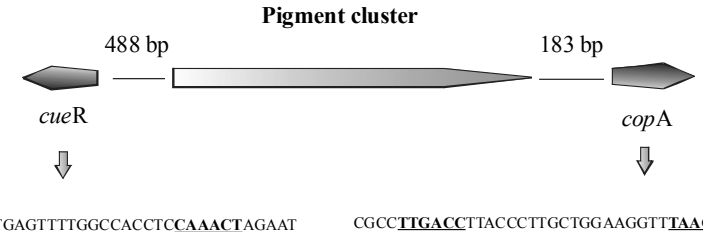

Figure 3 - Promoter regions of the prodigiosin biosynthetic clusters (pigment clusters) from *Serratia marcescens* SB08 (predicted *cue*R and *cop*A promoters)

In *E. coli* K-12 the *cue*R–*cop*A intergenic region contained the *cop*A promoter where *cue*R bound and promoted transcription of *cop*A [16]. This entire intergenic region between *cue*R and *cop*A was very similar to the sequence found in the intergenic region between *pig*N and *cop*A in *Serratia marcescens* SB08. The promoter was highly conserved between *E. coli* K-12 and *Serratia marcescens* SB08, with 32/40 bases identical over the whole region. Sequence analysis of the PCR products from strains Db11, Sma 12 and 17, in which the *cue*R–*cop*A genes were adjacent to each other rather than either side of the pigment gene cluster, had an identical predicted *cop*A promoter region to *Serratia marcescens* SB08. Database searches have shown this promoter to be highly conserved in many bacteria that contain *cue*R and *cop*A homologues, such as *E. coli* O157: H7, *Yersinia pestis, Salmonella typhi* and *Salmonella typhimurium* [25]. Despite the location of the pigment cluster, the presence of the *cop*A promoter, the putative transcriptional terminator and the 183 bp gap between *pig*N and *cop*A might suggest that the *cop*A gene is regulated independently of the pigment genes in *Serratia marcescens* SB08.

Conclusions

The results of our investigation showed that for prodigiosin biosynthesis by *Serratia marcescens* SB08 the pigment gene cluster were flanked by *cue*R and *cop*A which are involved in metal (copper)-dependent regulation and efflux. This study confirms the physiological connection between copper (and other metal) homeostasis and prodigiosin biosynthesized by *Serratia marcescens* SB08.

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