EFFECT OF POLYMYXIN B ON THE SYNTHESIS OF PRODIGIOSIN AND ITS PRECURSORS IN *SERRATIA MARCESCENS*

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Prodigiosin is a characteristic red pigment antibiotic synthesized in *Serratia marcescens*[1]. In a previous study, we attempted to use the membrane active cyclicpeptide antibiotic, polymyxin B (PB), as a probe on the site of biosynthesis of the pigment in *S. marcescens* 08[2]. It was shown by the agar gel diffusion technique that PB inhibited pigment synthesis and the minimal concentration of pigment inhibition was estimated to be 12.5 μg/ml. At this concentration, PB caused a reduction of over 80% of the pigment formation. However, it was not known if the reduction was the result of a decrease of synthesis of the precursors or an increase of degradation of the pigment. The objective of this investigation was to analyze the pigment and its precursor components in *S. marcescens* when the cells are grown in the presence of PB.

Three strains of *S. marcescens*, strain 08 (pigmented, polymyxin B resistant), mutant WF (nonpigmented, polymyxin B resistant), and mutant 9–3–3 (nonpigmented, polymyxin B sensitive) were used in this study. The minimal inhibitory concentrations of PB on the three strains were $>1{,}000$ μg/ml (08), $>1{,}000$ μg/ml (WF) and 1.95 μg/ml (9–3–3). Mutant WF can only synthesize one of the two precursors required for prodigiosin synthesis (2-methyl-3-amylpyrrole, MAP), while mutant 9–3–3 synthesizes the other precursor, 4-methoxy-2,2-bipyrrole-5-carboxaldehyde (MBC). Bacto peptone (0.5%) - glycerol (1%) broth (PGB) or Tryptic soy broth (TSB) was used for bacterial cultivation. Bacteria were grown in a 500-ml Erlenmeyer flask containing 150 ml of broth in the presence or in the absence of PB in a shaker bath at 25°C. The concentration of PB (Aerosporin, Burroughs Wellcome) used was 12.5 μg/ml for strain 08 and mutant WF and 2 μg/ml for mutant 9–3–3. After 24 hours growth (mutant WF) or 48 hours growth (strain 08 and mutant 9–3–3), the optimal times for pigment or precursors productions, the bacterial culture was centrifuged at $10{,}000\times g$ in a Beckman Model J 2–21 centrifuge to separate the cells from the growth medium.

The pigment of strain 08 was extracted with acetone as previously described[2]. The amount of pigment extracted was determined spectrophotometrically at 537 nm. The precursors, MAP or MBC, in the growth medium of mutant WF or mutant 9–3–3 were extracted with dichloromethane according to the procedure reported previously[4]. The extracted MAP was monitored at 223 nm. The difference in absorbance between 365 nm and 400 nm was used to express the amount of MBC present[3]. Synthropic pigment synthesis technique was used to confirm the identity of the precursors[4]. In addition to spectrophotometric analysis, the extracted pigment and precursors were subjected to high performance liquid chromatographic (HPLC) analysis. An isocratic chromatograph (Beckman model 330) with a 10 μl sample loop injector and a variable wavelength UV-visible detector were used. The column was a 25 cm$\times$4.6 mm (i.d.) reversed phase column packed with Lichrosorb RP-18 of 10 μm particle size. For the analysis of the pigment, 25% dichloroethylene in methanol with 20 ppm of concentrated HCl was used as a mobile phase and monitored at 537 nm[5]. For the analysis of the precursors, 70% methanol in water was used as a mobile phase and monitored at 223 nm for MAP and 250 nm or 365 nm for MBC[4]. The peak area of the chromatogram was used to express the amount of the various components separated. The identity of the precursor components (MAP or MBC) was confirmed by syntrophic pigment synthesis on the peaks collected. After the cellular protein content of the cultures were determined by the method of LOWRY *et al.*[6] (after the cells were hydrolyzed overnight with NaOH), the amount of the pigment or precursors was expressed as peak area/mg protein.

Results of the present study confirmed the previous finding that when cells of *S. marcescens* strain 08 were grown in TSB, there was no concomitant inhibition of cellular growth when the pigment production was significantly reduced by PB (over 90%)[2]. When strain 08 and mutant WF were grown in PGB in the presence of PB at

Table 1. Effect of polymyxin B on cellular protein contents of various strains of *Serratia marcescens*.

Strains	MIC[a] (μg/ml)	Growth media	Concentration of PB in growth media (μg/ml)	Cellular protein content (mg/ml of culture)		Percent change
				Control	PB added	
08	1,000	TSB[b]	12.5	2.12	2.13	0
08	62.5	PGB[c]	12.5	0.78	0.61	−22.0
WF	62.5	PGB	12.5	0.81	0.67	−17.0
9-3-3	1.95	PGB	2.0	1.02	0.51	−50.0

[a] MIC was determined in the corresponding growth media at 37°C by the two-fold dilution technique.
[b] TSB=Tryptic soy broth.
[c] PGB=Bacto peptone - glycerol broth.

Table 2. Effect of polymyxin B on production of pigment and/or its precursors in various strains of *Serratia marcescens*.

Strains	Growth media	Concentration of PB in growth media (μg/ml)	Level of pigment or precursors determined by					
			Spectrophotometer (absorbance units/mg cellular protein)			HPLC (peak area (cm^2)/mg cellular protein)		
			Control	PB added	% Change	Control	PB added	% Change
08	TSB	12.5	0.23	0.02	−91.8	12.36	1.13	−90.9
08	PGB	12.5	7.40	2.22	−70.0	710.90	161.70	−77.2
WF	PGB	12.5	N.A.[a]	N.A.	N.A.	5.68	1.78	−68.7
9-3-3	PGB	2.0	3.14	4.31	+37.3	2.68	3.85	+43.6

[a] N.A.=not available. The MAP precursor was masked by several other contaminating components at 223 nm.

a concentration of 12.5 μg/ml, there was a reduction of growth by about 17% and 22%, respectively (Table 1). In the case of mutant 9-3-3 the cellular growth was reduced as much as 50% by a concentration of PB as low as 2 μg/ml (the MIC of PB on 9-3-3 is 1.95 μg/ml).

The effect of PB on the production of prodigiosin and its precursors was studied by extracting these components from cells or growth media followed by spectrophotometric or HPLC analysis (Table 2). The results of these two approaches are quite comparable. The yield of prodigiosin in strain 08 was reduced by as much as 70 to 77% in comparison with those of the control. The yield of MAP in the PB containing culture of mutant WF was only 31.3% of the control sample. The study of the effect of PB on the synthesis of MBC was complicated by the fact that mutant 9-3-3 was susceptible to PB in both TSB (MIC 1.95 μg/ml) and PGB (MIC 1.95 μg/ml). Cellular protein content of mutant 9-3-3 was reduced by almost 50% when grown in the presence of 2 μg/ml of PB. When the ratio of MBC synthesized to mg of cellular protein was used as an indicator, there was an increase as much as 40% in its synthesis This finding requires further substantiation when a PB-resistant 9-3-3 mutant is available for investigation.

As previously suggested[2], the inhibitory effect of PB on the synthesis of prodigiosin and its precursor(s) could possibly be explained by the fact that PB, as a cationic molecule, could compete for the same sites for the pigment synthesis. However, PB has also been shown to have the ability to activate several degradative enzymes in the outer membrane in Gram-negative bacteria[7,8,9]. We suggest that the outer membrane of *S. marcescens* strain 08 and mutant WF (and may be mutant 9-3-3) damaged by these degradative enzymes in the presence of PB may not provide the favorable environment required for the synthesis of the pigment and the precursor. In our view there is a strong likelihood that the main steps of pigment formation and/or storage might be localized in or adjacent to the outer membrane of *S. marcescens*.

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