Constant values which characterize the inactivation of racemic sarin and of racemic soman by cyclodextrins under the conditions indicated[a]

		pH	α-CD	β-CD	γ-CD
Sarin	Concentration range of CD used (mM)		2.5–50	1.5–10	10–50
	k_2 min^{-1}	8.0	$1.6 \pm 0.2 \times 10^{-1}$		
	k_2 min^{-1}	9.0		$9.7 \pm 0.7 \times 10^{-2}$	
	k_2^{max} min^{-1}		$2.0 \pm 0.2 \times 10^{3}$	$1.2 \pm 0.1 \times 10^{2}$	
	K_d mM	8.0	40 ± 10		
	K_d mM	9.0		4.9 ± 0.7	
	k_2/K_d M^{-1} min^{-1}	8.0			1.1 ± 0.1[b]
	k_2^{max}/K_d M^{-1} min^{-1}		$5.0 \pm 1.4 \times 10^{4}$	$2.5 \pm 0.4 \times 10^{4}$	$1.4 \pm 0.1 \times 10^{4}$
Soman[c]	Concentration range of CD used (mM)		7.5–50	0.2–4.0	2.5–50
	k_2 min^{-1}	7.4	$3.9 \pm 0.4 \times 10^{-2}$	$5.9 \pm 0.6 \times 10^{-2}$	
	k_2 min^{-1}	8.0			$2.4 \pm 0.2 \times 10^{-2}$
	k_2^{max} min^{-1}		$2.0 \pm 0.2 \times 10^{3}$	$3.0 \pm 0.3 \times 10^{3}$	$3.0 \pm 0.2 \times 10^{2}$
	K_d mM	7.4	18 ± 5	0.53 ± 0.05	
	K_d mM	8.0			5.5 ± 1.1
	k_2^{max}/K_d M^{-1} min^{-1}		$1.1 \pm 0.3 \times 10^{5}$	$5.6 \pm 0.8 \times 10^{6}$	$5.4 \pm 1.2 \times 10^{4}$

[a] All measurements were made in 10 mM Tris buffer, ionic strength 0.155 and at 25 °C. [b] Slope of the plot of k_{CD}–k_0 against $|\gamma\text{-CD}|_0$ which was a straight line. [c] Values corresponding to inactivation of soman by β-cyclodextrin are taken from[8].

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Relationship of prodigiosin condensing enzyme activity to the biosynthesis of prodigiosin and its precursors in *Serratia marcescens*

L.K.N. Cho, J.A. Lowe+, R.B. Maguire and J.C. Tsang*

Department of Chemistry, Illinois State University, Normal (Illinois 61761, USA), 30 June 1986

Summary. Prodigiosin condensing enzyme (PCE) activities were present in *Serratia marcescens* wild type 08, mutants OF, WF and 9-3-3. Their specific activities exhibited different maxima and at different times during the late log phase or the early stationary phase of cell growth. The levels of prodigiosin and its precursors also showed a significant increase at this period. The results support that prodigiosin and/or its precursors are secondary metabolites. The ubiquity of the PCE activity in mutants deficient in prodigiosin biosynthesis suggest that this particular enzyme may also be present in non-pigmented clinical isolates.
Key words. Serratia marcescens; growth; prodigiosin; prodigiosin condensing enzyme; secondary metabolites.

Prodigiosin is a characteristic red pigment synthesized by *Serratia marcescens*. This pigment has been shown to have some antibiotic properties. Biosynthesis of prodigiosin has been suggested to be mediated by plasmids[1,2] or transferred by a transducing phage[3]. In general, genes containing the genetic information for biosynthesis of secondary metabolites such as antibiotics, are both chromosomal and extrachromosomal[4]. In at least one case, structural genes for antibiotic synthesis have been located on plasmids[5]. Normally the structural genes are chromosomal, whereas regulatory genes controlling the expression of the genetic information appear to be extrachromosomal[4,6,7]. Recently, cloning and expression in *Escherichia coli* of *Serratia marcescens* genes encoding prodigiosin biosynthesis supported the idea that genes responsible for the production of prodigiosin lie mainly on the chromosome and that prodigiosin production is probably not mediated by a plasmid[8].

Many clinical isolates of *S. marcescens* are non-pigmented and multiply resistant to antibiotics. When resistance plasmids of non-pigmented strains were transferred to pigmented strains of *S. marcescens*, spontaneous loss of production occurred. This has been suggested as a possible explanation for the predominance of multiply resistant non-pigmented *Serratia* clinical isolates[9,10]. However, it was suggested that the decrease in pigmentation associated with RP4 carriage resulted from plasmid suppression of growth rate[11]. Therefore, the relationship between the presence of resistance plasmids and pigment production remains unclear. Perhaps prodigiosin serves a survival function in nature where nutrients are limited for microbial growth. On the other hand, in clinical isolates, when adequate nutrients are available for all species present, prodigiosin production may not be necessary.

A bifurcated pathway was proposed for the biosynthesis of prodigiosin[12–14]. The final step in prodigiosin biosynthesis involves the Prodigiosin Condensing Enzyme (PCE) which condenses two substrate precursors: a monopyrrole, 2-methyl-3-amylpyrrole (MAP) and a bipyrrole, 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC). Mutants have been isolated which are blocked in various steps in either the MAP or MBC path-

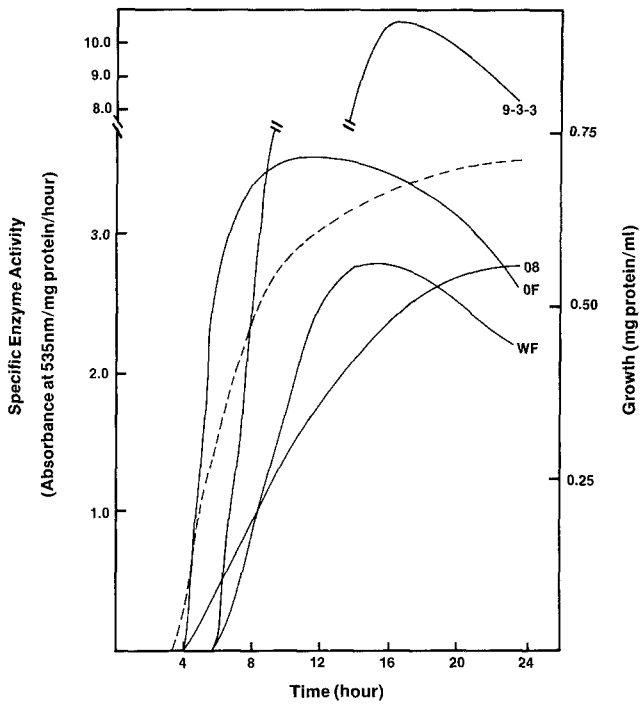

Figure 1. Relationship of growth kinetics to prodigiosin condensing enzyme activities of 4 strains of *Serratia marcescens* (----) Growth; (——) Prodigiosin condensing enzyme activity.

way[13,14]. Mutant OF is blocked late in the synthesis of MBC and accumulates 4-hydroxy-2,2′-bipyrrole-5-carboxaldehyde (HBC). This leads to the production of norprodigiosin, a hydroxy analogue of prodigiosin[15]. WF and 9-3-3 are non-pigmented mutants which are blocked in the synthesis of one of the two corresponding precursors, and produce MAP and MBC, respectively[13]. The presence of PCE has been reported in mutant 9-3-3[16]. Recently, Feng and Tsang[17] reported that the PCE in mutant WF could be released from isolated cell envelopes using neutral detergents. One of our objectives was to quantify and relate the PCE activities to cell growth in *S. marcescens* using pigmented strains: wild type 08 and mutant OF; and non-pigmented strains: mutants WF and 9-3-3. In addition, an attempt was made to correlate the levels of prodigiosin and its precursors with PCE activities and cell growth.

Materials and methods. Bactopeptone (0.5%)-Glycerol (1%) (PG) broth was used for bacterial cultivation. Wild type strain 08, and mutants OF, WF and 9-3-3 were grown in 100 ml of PG broth and 1 ml aliquots of each culture were taken at 2-h intervals for 24 h. Cell growth was monitored by the total cellular protein[18] at each interval. PCE activities were also assayed at each interval. A substrate mixture containing MBC and the monopyrrole 2,4-dimethyl-3-ethylpyrrole (DEP) was used for the PCE assay. The condensation of MBC with DEP yields a prodigiosin analogue with spectral properties nearly identical with prodigiosin[16]. MBC, the bipyrrole, was extracted with dichloromethane from the supernatant of mutant 9-3-3 cultures grown in batch[19]. DEP, an analogue of MAP, was used in place of MAP because MAP is neither commercially available nor easily extracted from mutant WF cells.

The mixture was incubated at 27 °C for 30 min. Any prodigiosin formed was then extracted with 1% acetic acid in methanol. After centrifugation, the supernatant was read at 535 nm. This serves as a measure of PCE activity. Aliquots were also taken to determine the levels of prodigiosin and norprodigiosin or its precursors synthesized by the corresponding mutants. For wild type 08, cells were first centrifuged and washed. Prodigiosin was extracted with 1% acetic acid in methanol and read at 535 nm. The procedures for mutant OF were the same except that the norprodigiosin extracted was read at 498 nm. MBC was monitored in supernatant of mutant 9-3-3 cultures by taking the difference in absorbance at 365 nm and 400 nm[20]. MAP was monitored by a syntrophic pigment synthesis method[21,22]. This method involves the cross-feeding of MBC to growing mutant

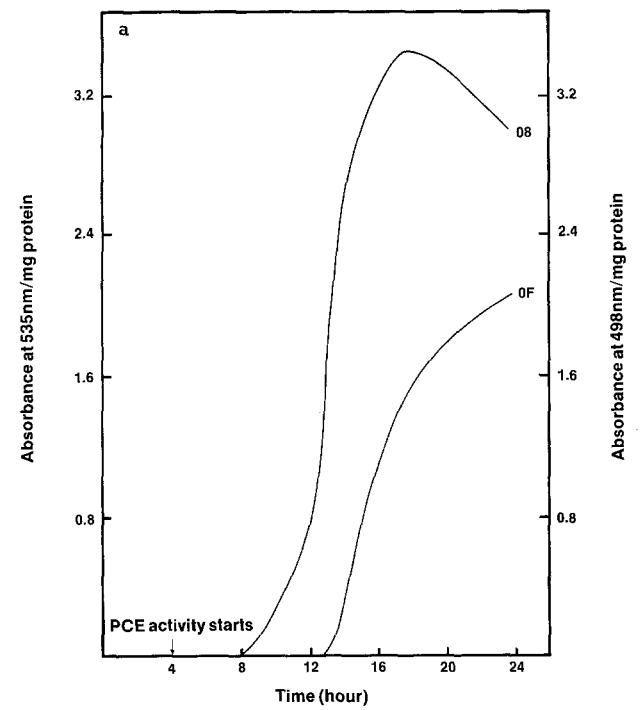

Figure 2. Progress of biosynthesis of prodigiosin and its precursors in *Serratia marcescens*. Biosynthesis of prodigiosin in strain 08 and norprodigiosin in mutant OF.

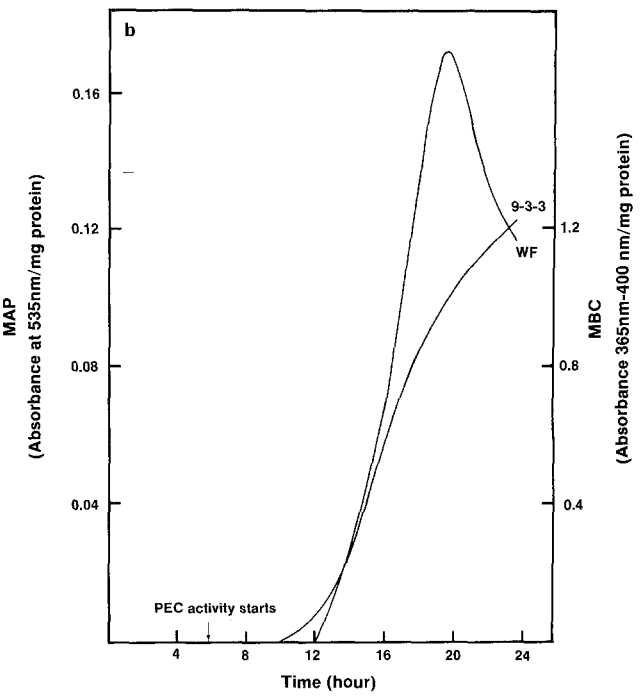

Figure 3. Progress of biosynthesis of prodigiosin and its precursors in *Serratia marcescens*. Biosynthesis of MAP in mutant WF and MBC in mutant 9-3-3.

WF cells. The pigment thus formed is proportional to the levels of MAP present.

Results and discussion. The kinetics of cell growth in wild type 08, mutant OF, WF and 9-3-3 were identical as monitored by the cellular protein content (fig. 1). Based on these results, there were no differences in cellular multiplication between pigmented and non-pigmented strains. PCE activities started at the 4th hour for pigmented strains 08 and mutant OF, but later at the 6th hour for the non-pigmented mutants WF and 9-3-3. All four strains showed a gradual increase in specific PCE activities with time. Maximal values were not reached until after the 8th hour (08: 2.7 A_{535}/mg protein and h, 24 h; OF: 3.5 A_{535}/mg protein and h, 10 h; WF: 2.7 A_{535}/mg protein and h, 14 h; 9-3-3: 10.5 A_{535}/mg protein and h, 16 h). It appeared that maximal PCE activities were reached during the late log phase or the early stationary phase of cell growth.

The penultimate step of the prodigiosin biosynthetic pathway is the synthesis of MBC from HBC, a reaction which requires an O-methytransferase enzyme. Recombinant DNA techniques have been employed to isolate and clone *Streptomyces* chromosomal DNA segments coding for this enzyme in order to 'complement' mutants from both *Streptomyces* and *Serratia*[23]. The last step of the biosynthesis of either prodigiosin or norprodigiosin involves a condensation reaction between MAP and MBC in the wild type 08 or between MAP and HBC, the modified bipyrrole in mutant OF. Our studies showed that mutant OF was able to condense DEP and MBC with a PCE activity comparable to that in wild type 08. It is likely that the two enzymes are similar, if not identical, and cannot distinguish HBC from MBC. Mutant 9-3-3 exhibited a three-fold higher maximal specific PCE activity when compared to those of the other three strains. This could possibly be due to the higher permeability of the substrates through the cell envelopes of 9-3-3 and/or a higher intrinsic specific activity of the enzyme in the mutant itself. However, no satisfactory explanation or physiological significance can be assigned to the differences in enzyme activities among the four strains at this time.

The appearance of prodigiosin in wild type 08 and norprodigiosin in mutant OF occurred at the 8th hour and shortly after the 12th hour, respectively (fig. 2). The levels of both pigments increased significantly during the late log phase or the early stationary phase of cell growth. The late decrease in cell associated prodigiosin levels might be attributed to its release into the culture media as a result of cell autolysis. The biosynthesis of the two precursors of prodigiosin are shown in figure 3. Formation of MBC in mutant 9-3-3 and MAP in mutant WF started at the 10th and 12th hour, respectively. Both levels also increased gradually with time. The decrease in the MAP level may be due to its relative instability after release into the growth media. From figures 1 and 3, it can be seen that the PCE activities precede the biosynthesis of both precursors. Evidence has been reported that MAP and MBC pathways share a common intermediate at an early step in their biosynthesis and hence are subject to common control[12].

Various possible functions of prodigiosin have been proposed, such as being an antibiotic[24], a component of a specific receptor for kappa phages[25,26], and a secondary metabolite[27], which serves to remove unwanted intermediates from primary metabolism[28]. Expression of the genes encoding for antibiotic biosynthesis usually does not occur at high growth rates[29]. This phenomenon suggests that during rapid growth either antibiotic synthetases (such as the PCE) are not formed, or if formed, their activity is inhibited, or that precursors are not available in saturating concentrations. A characteristic of secondary metabolites is their delayed maximal biosynthesis until cellular multiplication has ceased. Based on both the PCE activities and pigment and/or precursor levels, our results support the proposition that prodigiosin is a secondary metabolite, and suggest that norprodigiosin in mutant OF, MAP in mutant WF, and MBC in mutant 9-3-3 may behave similarly. It has also been suggested that some clinical isolates may have a defect in the condensing enzyme[30], since pigment(s) other than prodigiosin could be formed when supplied with MBC plus MAP, and MBC alone. If this is true, then why do these three particular mutants: OF, WF and 9-3-3, possess PCE activities while some clinical isolates do not. Further studies are warranted to clarify the presence or absence of PCE activity in various strains of *S. marcescens*.

Prodigiosin and its precursors produced by *S. marcescens* can be assayed easily. Moreover, PCE represents one of the few anabolic membrane-associated enzymes for the biosynthesis of secondary metabolites. This can serve as a model[15] whereby further studies should reveal more about the underlying enzymology and genetics of secondary metabolism.

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* To whom all correspondence should be addressed.
+ Present address: Blackhawk College, East Moline, Illinois 61244, USA.

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