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EFFECT OF 2,4-DINITROPHENOL, CHLORAMPHENICOL AND KCN ON PRODIGIOSIN PRODUCTION BY POLYMYXIN B TREATED NON-PROLIFERATING CULTURES OF *SERRATIA MARCESCENS*

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RESUMEN

Efecto del 2,4-dinitrofenol, cloranfenicol y KCN sobre la biosíntesis de prodigiosina por cultivos no proliferantes de *Serratia marcescens* en presencia de Polimixina B

La biosíntesis de prodigiosina por cultivos no proliferantes de *Serratia marcescens* (NPC) inducidos con L-prolina se amplifica por el efecto de la adición de algunos antibióticos, especialmente la polimixina B. El presente trabajo describe la cinética de producción de pigmento por NPC tratados con polimixina B e inducidos con L-prolina en presencia de 2,4-dinitrofenol, KCN y cloranfenicol. Los resultados demuestran que el transporte electrónico y la síntesis proteica son imprescindibles para la cromogénesis en estas condiciones, mientras que el bloqueo de la fosforilación oxidativa no evita la producción de pigmento.

Palabras clave: *Serratia marcescens*. Producción prodigiosina.

SUMMARY

Prodigiosin biosynthesis by proline-induced non-proliferating cultures of *Serratia marcescens* is enhanced by some antibiotics specially polymyxin B. This paper reports kinetics of prodigiosin production by proline induced polymyxin-treated non-proliferating cultures of *S. marcescens* in the presence of 2,4-dinitrophenol, KCN or chloramphenicol. Results show that protein synthesis and electron transport are needfull for prodigiosin production in the presence of polymyxin B whereas oxidative phosphorylation is not.

Keywords: *Serratia marcescens*. Prodigiosin production.

INTRODUCTION

Some strains of the enterobacteria *Serratia marcescens* are characterized by their ability to produce a red pigment named prodigiosin. Chemically prodigiosin has a linear tripyrrole structure whose biosynthetic pathway is as yet unclear. Various studies on the environmental factors affecting prodigiosin production have been published (WILLIAMS *et al.*, 1971b).

Polymyxin B is a polypeptide antibiotic that can interact with the outer and the inner membrane of gramnegative bacteria. This compound also acts as an inhibitor of prodigiosin production in *S. marcescens* cultures (TSANG FENG, 1983).

Under certain conditions, prodigiosin biosynthesis can be separated from bacterial growth in *Serratia marcescens* (WILLIAMS *et al.*, 1971a; QADRI WILLIAMS, 1972). According to Williams and Qadri these systems are named by the acronym NPC (non-proliferating cells).

Cultures of *Serratia marcescens* are unable to synthesize prodigiosin when the incubation temperature is 38 °C or higher (WILLIAMS *et al.*, 1971b). When the cultures in which pigmentation was inhibited by temperature, are

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shifted to 28 °C the ability to produce pigment is restored.

Washed NPC are unable to produce pigment but pigmentation can be induced in such cultures by the addition of some amino acids to the bacterial suspensions (SCOTT *et al.*, 1976; QADRI WILLIAMS, 1973, 1974; WILLIAMS *et al.*, 1976).

TSANG KALLVY (1971) suggested that prodigiosin could be found associated to the outer membrane components of the bacterial cell wall. VIÑAS (1981) and VIÑAS *et al.* (1983) showed that prodigiosin is mostly localized on the external face of the inner membrane.

Although the cellular localization of prodigiosin is not yet fully established, it seems clear that the biosynthesis and localization of prodigiosin is strongly related to the bacterial envelope physiology.

In 1983, Lauferska *et al.* demonstrated the enhancement of prodigiosin production in NPC by polymyxin B. This antibiotic shows an opposite effect in NPC and in growing cultures. On the other hand, Robert P. Williams (personal communication) has observed similar phenomena with other related antibiotics.

MATERIALS AND METHODS

Bacterial strains: *Serratia marcescens* ATCC 274 was used in all experiences of the present work. Bacterial cultures were maintained on Trypticase Soy aga (TSA, BBL) slants. For all experiments the bacterial strain was cultured in Trypticase Soy broth (TSB, BBL) at 30 °C and subcultured in the appropriate conditions.

Preparation of non-proliferating systems: NPC were prepared by culturing *Serratia marcescens* ATCC 274 in 100 ml TSB contained in 500 ml Erlenmeyer flasks and incubated at 38 °C in a reciprocal shaker water bath during 24 hours. Under these conditions no pigment was produced (WILLIAMS *et al.*, 1971b). After the incubation period, bacteria were harvested by centrifugation in an A 8.24 rotor of a Centrikon H-401 high speed centrifuge at 8000 rpm during 15 minutes. The bacteria were washed twice in NaCl (0.85% w/v) and then distributed in 500 ml Erlenmeyer flasks with 50 ml each. The NPC were further incubated at 27 °C in a reciprocal shaker (Salvis) in presence of inducer amino acid (L-proline, 10 mg/ml), polymyxin B and other compounds as specified for every experiment in the corresponding legends for figures. Polymyxin B was added to the NPC at a concentration of 300 µg/ml. This concentration is optimal for the enhancement of prodigiosin production (LAUFERSKA *et al.*, 1983). Chloramphenicol, KCN and 2,4-dinitrophenol were added to a concentration of 60 µg/ml, 75 µg/ml and 10^{-3} M respectively. The addition of these compounds was carried out at different times after amino acid induction and polymyxin B treatment.

Analytical procedures: The levels of prodigiosin were measured spectrophotometrically at a wave lenght of 535 nm with a single beam spectrophotometer Perkin-Elmer Mod 558. Samples of 4 ml were centrifuged at 5000 rpm for 20 min and the resulting pellet was then suspended in ethanol-HCl as indicated by WILLIAMS *et al.* (1961). After keeping the suspension at 4 °C overnight, the samples were again centrifuged and the pigment in the supernatant monitored. The amount of prodigiosin was calculated by the specific absorbance of prodigiosin (51.5×10^3 L/g·cm) (WILLIAMS *et al.*, 1961).

Protein was determined by the method of LOWRY *et al.* (1951) with bovine serum albumin as standard. The reading was performed at 600 nm since at this wave lenght pigment absorbance is negligible, KCN was detected by the Prussian blue test.

RESULTS

2,4-DNP acts as an uncoupler agent in the oxidative phosphorylation. Addition of 2,4-DNP to a level of 10^{-3} M at different time modifies pigment production. The pigment levels reached in the presence of this compound were lower than those detected in the absence of the uncoupler. If the time of addition was greater

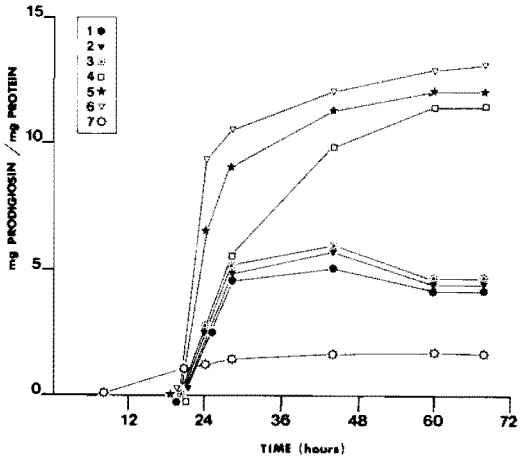

FIGURE 1. Kinetics of Prodigiosin production in NPC treated with 2,4-dinitrophenol at different times; 1) 0h; 2) 2h; 3) 4h; 4) 12 h; 5) 24h; 6) control without 2,4-dinitrophenol; 7) control with L-proline (10 mg/ml) and without Polymyxin B.

Cinéticas de producción de progidiosina en sistemas no proliferantes en presencia de L-prolina y Polimixina B tratados con 2,4-dinitrophenol a distintos tiempos; 1) 0h; 2) 2h; 3) 4h; 5) 24h; 6) control en ausencia de 2,4-dinitrophenol; 7) control con 10 mg/ml de Prolina en ausencia de Polimixina B.

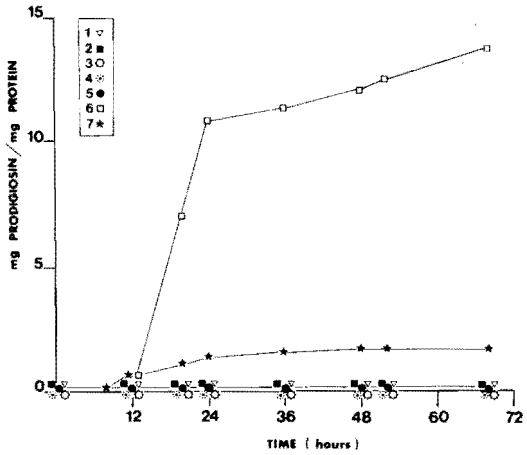

FIGURE 2a. Kinetics of prodigiosin production in L-proline induced and polymyxin B treated NPC in chloramphenicol presence. Chloramphenicol was added at 1) 0h; 2) 2h; 3) 4h; 4) 6h; 5) 8h; 6) control without chloramphenicol and 7) control without chloramphenicol nor Polymyxin B.

Cinéticas de producción de prodigiosina en NPC inducidas con L-prolina y tratadas con polimixina B en presencia de cloranfenicol a distintos tiempos 1) 0h; 2) 2h; 3) 4h; 4) 6h; 5) 8h; 6) control sin cloranfenicol y 7) control sin cloranfenicol ni polimixina B.

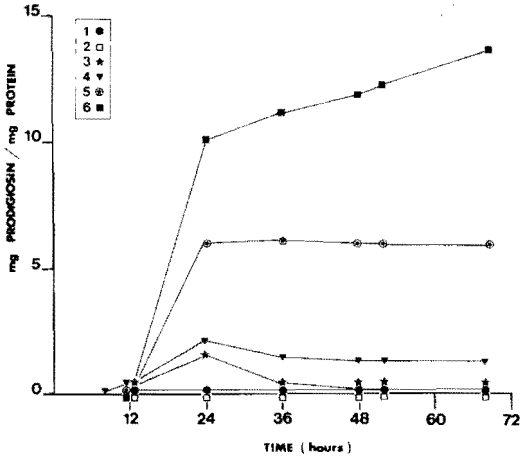

FIGURE 2b. Kinetics of prodigiosin production in L-proline induced and polymyxin B treated NPC in chloramphenicol presence. Chloramphenicol was added at 1) 10h; 2) 12h; 3) 14h; 4) 16h; 5) 18h; 6) 24h.

Cinéticas de producción de prodigiosina en NPC inducidas con L-prolina y tratadas con polimixina B en presencia de cloranfenicol a distintos tiempos 1) 10h; 2) 12h; 3) 14h; 4) 16h; 5) 18h; 6) 24h.

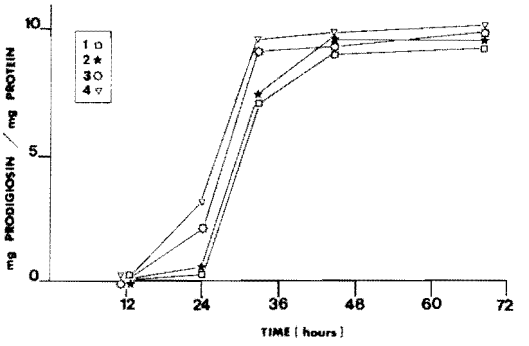

FIGURE 3a. Kinetics of prodigiosin production in L-proline and polymyxin B treated NPC with KCN added at different time. 1) 0h; 2) 5h; 3) 6h; 4) 7h.

Cinéticas de producción de prodigiosina en NPC tratadas con L-prolina y polimixina B a los que se añadió KCN a distintos tiempos. 1) 0h; 2) 5h; 3) 6h; 4) 7h.

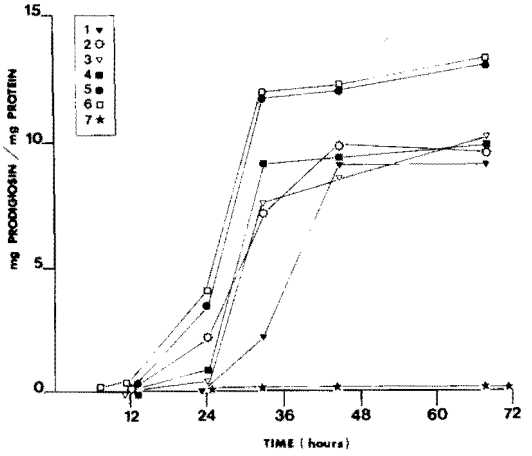

FIGURE 3b. Kinetics of prodigiosin production in L-proline and polymyxin B treated NPC with KCN added at different time 1) 8h; 2) 9h; 3) 10h; 4) 12h; 5) 24h; 6) control without KCN and 7) KCN added every 6 h.

Cinéticas de producción de prodigiosina en NPC tratados con L-prolina y polimixina B y a los que se añadió KCN a distintos tiempos 1) 8h; 2) 9h; 3) 10h; 4) 12h; 5) 24h; 6) control sin KCN y 7) KCN añadido cada 6 horas.

than 12 hours after induction and polymyxin B treatment, no effect could be observed. Fig 1 shows the kinetics of prodigiosin production in NPC in which 2,4-DNP was added at 0, 2, 4, 12 and 24 hours as compared to a control of L-proline and in a system treated with both L-proline and polymyxin B.

Chloramphenicol acts inhibiting peptidyltransferase function in 70S ribosomes. Addition of chloramphenicol to NPC induced with L-proline and treated with polymyxin B, causes a total inhibition of prodigiosin biosynthesis within the first 12 hours. This effect decreases at longer time and is neglegible at a time higher than 24 hours. Figures 2a and 2b show the kinetics of prodigiosin production in NPC treated with polymyxin B and induced with L-proline in which chloramphenicol was added at various time as compared to a control without chloramphenicol.

Potassium cyanide is an inhibitor of oxygen consumption by the bacteria. Addition of KCN to L-proline induced and polymyxin B treated NPC retards the prodigiosin production. Figures 3A and 3B show the kinetics of pigment production in NPC treated with KCN at different time. One of the NPC suspensions was treated with KCN every six hours and under these conditions no pigment was formed. In those NPC treated only with KCN once, the Prussian blue test was applied to evaluate the presence of KCN. In all cases KCN was undetectable twelve hours after its addition.

DISCUSSION

Polymyxin B enhances the production of the pigment in L-proline induced NPC but is unable to induce pigmentation. LAUFERSKA *et al.* (1983) suggested that the effect of polymyxin B is related to the action of the antibiotic on the bacterial membranes allowing a more effective passage of the inducer amino acid towards the sites of pigment biosynthesis. PIZZIMENTI *et al.* (1983) demonstrated that the availability of the amino acids in the intrabacterial pool is closely related to the pigment biosynthesis.

In order to study the difference of prodigiosin production in NPC treated with polymyxin B from those untreated, chloramphenicol, KCN and 2,4-DNP were used in the experiences described in this paper.

Addition of 2,4-DNP modifies pigment production (see fig 1). Even when the concentration of 2,4-DNP was increased only a partial inhibition was observed. Although oxidative phosphorylation does not appear to be necessary for the pigment biosynthesis, it is possible that 2,4-DNP carries out some independent secondary effects on chromogenesis.

QADRI WILLIAMS (1972), demonstrated that chloramphenicol inhibits prodigiosin biosynthesis in proliferating cultures as well as in NPC. This effect is indistinguishable from that produced by streptomycin and other related antibiotics, so that protein biosynthesis appears to be a necessary condition to allow the iniciation of prodigiosin biosynthesis. However when chloramphenicol was added at times longer than 12 hours after induction and polymyxin B treatment, prodigiosin production was not modified. This fact could be explained tacking into account the results obtained by WILLIAMS *et al.* (1976) that demonstrated that RNA and protein synthesis takes place after amino acids induction.

In KCN treated NPC Prussian blue test demonstrated that KCN consumption was concomitant to pigment inhibition and that KCN exhaustion restored prodigiosin biosynthesis.

On the other hand NPC treated with the compounds used in this work produce pigment of the normal blood-red colour within the first hours of incubation. However if the time of incubation is prolonged longer, a dark colour can be observed. Our point of view is that normal degradation of the pigment is possibly accelerated in the presence of chemical structures that produce secondary effects on prodigiosin.

The chemical structure of prodigiosin as well as the relationship between this pigment and the cytoplasmic membrane could suggest that the biosynthesis of prodigiosin and the energetic metabolism in *Serratia* are related. From this point of view, the experiences reported in this paper point out that the biosynthesis of prodigiosin is strongly related to the energetic state of the inner membrane. On the other hand, the pyrrolic ring forms part of prodigiosin and other energetic pigments as chlorphyls, phycobilins, etc. Further work on the effect of ionophores, cationic transports and amino acid uptake will be necessary to assess the idea presented in this discussion.

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