Regulation of capsular polysialic acid biosynthesis by temperature in *Pasteurella haemolytica* A2

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Abstract The capsular polysaccharide of *Pasteurella haemolytica* A2 consists of a linear polymer of *N*-acetylneuraminic acid (Neu5Ac) with α(2–8) linkages. The production of this polymer is strictly regulated by the growth temperature and above 40°C no production is detected. Analysis of the enzymatic activities directly involved in its biosynthesis reveals that Neu5Ac lyase, CMP-Neu5Ac synthetase and polysialyltransferase are involved in this regulation. Very low activities were found in *P. haemolytica* grown at 43°C (at least 25 times lower than those observed when the growth temperature was 37°C). The synthesis of these enzymes increased rapidly when bacteria grown at 43°C were transferred to 37°C and decreased dramatically when cells grown at 37°C were transferred to 43°C. These findings indicate that the cellular growth temperature regulates the synthesis of these enzymes and hence the concentration of the intermediates necessary for capsular polysaccharide genesis in *P. haemolytica* A2.

Key words: Polysialic acid biosynthesis; *Pasteurella haemolytica*

1. Introduction

Pasteurella haemolytica is a Gram-negative bacterium responsible for economically important diseases in ruminants, including pneumonic pasteurellosis or shipping fever in cattle, and pneumonic pasteurellosis and septicaemia in sheep [1,2]. Traditionally *P. haemolytica* has been subdivided into two biotypes, A and T, based on biochemical characteristics, and 17 serotypes, based on biochemical variations in capsular polysaccharides [3–6]. The presence of the capsule is believed to play a role in the pathogenicity of this microorganism [7,8].

P. haemolytica A2 is able to produce a capsular polysaccharide (a linear homopolymer of α(2–8)-linked Neu5Ac), known as colominic acid or polysialic acid (PA) [9]. In this bacterium, PA biosynthesis follows a pathway similar to that observed for *Escherichia coli* K1 [10,11] and involves the following enzymatic steps [12]: (i) synthesis of Neu5Ac by the enzyme *N*-acetylneuraminate lyase, (ii) synthesis of CMP-Neu5Ac by the enzyme CMP-Neu5Ac synthetase and (iii) incorporation of sialic acid from CMP-Neu5Ac into polymeric products through sialyltransferase activity.

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Abbreviations: Neu5Ac or sialic acid, *N*-acetyl-D-neuraminic acid; ManNAc, *N*-acetyl-D-mannosamine; Pyr, pyruvate; DTT, dithiothreitol; PA, polysialic acid; CTP, cytidine 5′-triphosphate; CMP-Neu5Ac, cytidine 5′-monophospho-*N*-acetylneuraminic acid

The key importance of growth temperature in the expression of bacterial products has been described previously [13,14]. Thus, in *E. coli* K1 and *E. coli* K92 a decrease in growth temperature diminishes the capacity of PA production and at temperatures below 20°C, although growth continues, PA production is completely halted [15–17]. The absence of PA synthesis in these micro-organisms is due to a regulatory temperature control of the CMP-Neu5Ac synthetase enzyme because no activity is found when both bacteria are grown at this restrictive temperature (20°C) [15,17]. Previous results have shown that in *P. haemolytica* A2 a decrease in the growth temperature also produces a gradual decrease in PA release, although this never ceases completely because below 28.5°C the bacterium is unable to grow [12]. However, when this bacterium is grown above 37°C, growth remains practically constant but PA production decreases dramatically and is negligible at 43°C [12]. In this work, we describe the effect of the cellular growth temperature on PA biosynthesis and the relationship between this event and the synthesis of the enzymes directly involved in the formation of the polymer.

2. Materials and methods

2.1. Chemicals

Neu5Ac, ManNAc, resorcinol, Pyr, 2-thiobarbituric acid, periodic acid, DTT, CTP, sodium arsenite, bovine serum albumin, CPM-Neu5Ac and cytidine 5′-triphosphate were supplied by Sigma Chem. Co. (St. Louis, MO, USA). *N*-Acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (300 Ci/mol) and cytidine 5′-monophospho-*N*-acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (267 Ci/mol) were purchased from Amersham International (Amersham, Bucks, UK). Brain heart infusion broth (BHIB) was from Pronodisa (Madrid, Spain). Other reagents used were of analytical quality.

2.2. Culture media and growth conditions

P. haemolytica A2 (ATCC 29694) was obtained from the Colección Española de Cultivos Tipo (CECT 924). Following the methodology described [12], the lyophilisates were incubated for 24 h in a rotary shaker (250 rev/min) at 37°C in 250 ml Erlenmeyer flasks containing 50 ml of BHIB (37 g/l). The cellular suspension was stored at −70°C in 50% (v/v) glycerol (preinoculum). Experimental cultures were grown by inoculating 400 ml of BHIB medium in 2500 ml Erlenmeyer flasks with 400 μl of the above preinoculum at the required temperature and time. Cell growth was monitored at absorbance 540 nm (A_{540}).

2.3. PA determinations

The sialyl polymer produced by *P. haemolytica* A2 was analysed by the Svennerholm [18] methodology, described elsewhere [16].

2.4. Cell-free extracts and enzymatic assays

Cell-free extracts of *P. haemolytica* A2 were obtained as previously described [12]. Briefly, *P. haemolytica* A2 was grown in BHIB medium for the time required. Bacteria were collected by centrifugation (10 000×*g*, 10 min at 4°C), washed twice with 50 mM Tris-HCl, pH 8, and resuspended in 50 mM Tris-HCl, pH 8, containing 25 mM

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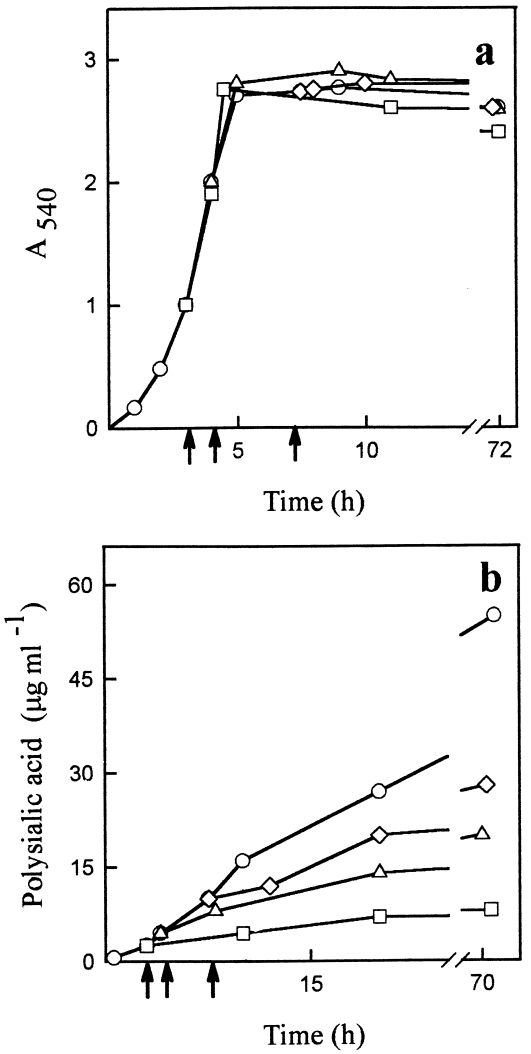

Fig. 1. Effect of incubation temperature on cellular growth (a) and PA production (b) when cultures of *P. haemolytica* A2 grown at 37°C (○) were transferred to 43°C after 3 h (□), 4 h (△) or 7.5 h (◇) of incubation. Arrows indicate the time at which the temperature transfer was made. Each value is the mean of triplicate experiments.

$MgCl_2$ and 1 mM dithiothreitol (DTT). The cellular suspension (25 ml) was disrupted with 20 g of glass beads (Ballotini, 0.17–0.18 mm diameter) using a Braun MSK mechanical disintegrator. Cell debris was eliminated by centrifugation at $10\,000 \times g$ for 20 min at 4°C. The pellet was discarded and the supernatant was ultracentifuged at $200\,000 \times g$ for 60 min at 4°C. Supernatants were used for the evaluation of both *N*-acetyl-D-neuraminic acid lyase (Neu5Ac lyase) and cytidine 5′-monophosphate-*N*-acetylneuraminic acid synthetase (CMP-Neu5Ac synthetase) activities according to the methodologies described by Rodríguez-Aparicio et al. [10] and González-Clemente et al. [15], respectively. In both cases, one unit of enzyme activity was defined as the amount of enzyme that synthesises 1 μmol of product (Neu5Ac for Neu5Ac lyase and CMP-Neu5Ac for CMP-Neu5Ac synthetase) per minute at 37°C under the respective assay conditions. Specific activities were expressed as U/mg of protein.

The ultracentrifugation precipitate (membrane-rich fraction) was resuspended in 50 mM Tris-HCl, pH 8, and used to evaluate polysialyltransferase activity according to the method described by Rodríguez-Aparicio et al. [16]. One unit of this enzyme activity was defined as the amount of enzyme that synthesises 1 pmol of product ([^{14}C]Neu5Ac binding to the acceptor) per minute at 33°C under the assay conditions. Specific activity was expressed as U/mg of protein.

Proteins were measured by the method of Bradford [19], using bovine serum albumin as standard. Buffers identical to those containing the protein samples were used as blanks.

3. Results and discussion

Previous studies have addressed the role of growth temperature in the expression of different products [14,15,17]. Here we observed that this control by temperature also affects the capsular polysaccharide of *P. haemolytica* A2. Thus, when this

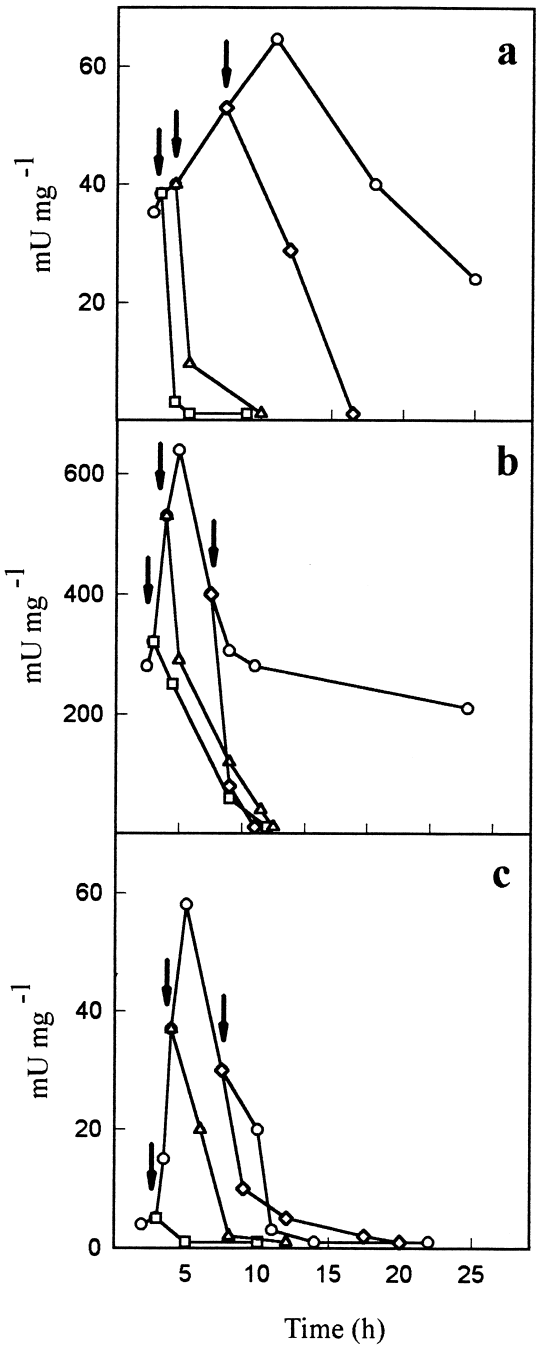

Fig. 2. Effect of incubation temperature on enzymatic activities: Neu5Ac lyase (a), CMP-Neu5Ac synthetase (b) and sialyltransferase (c), when cultures of *P. haemolytica* A2 grown at 37°C (○) were transferred to 43°C after 3 h (□), 4 h (△) or 7.5 (◇) of incubation. Arrows indicate the time of transfer. Each value is the mean of triplicate experiments.

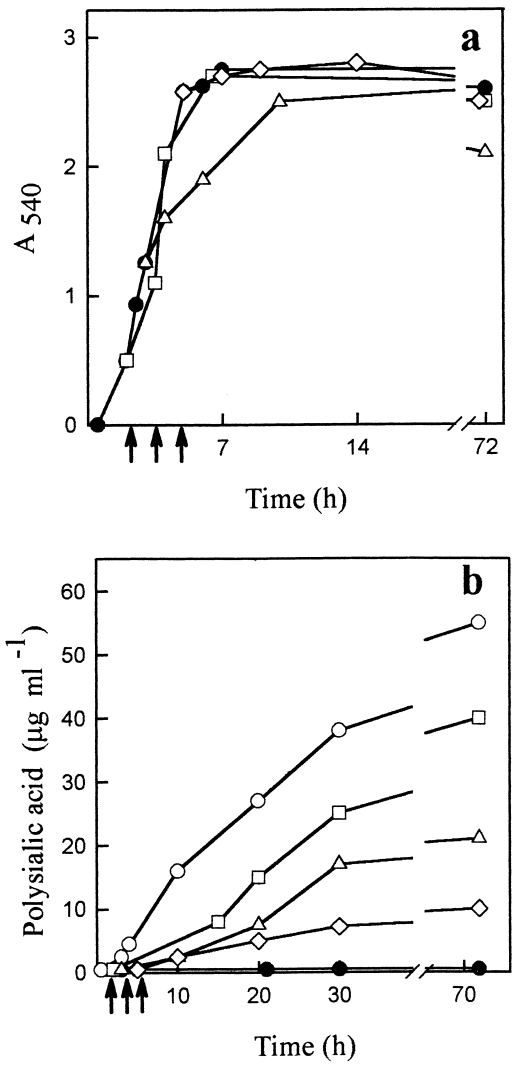

Fig. 3. Effect of incubation temperature on cellular growth (a) and PA production (b) when cultures of *P. haemolytica* A2 grown at 43°C (●) were transferred to 37°C after 2 h (□), 3 h (△) or 5 h (◇) of incubation. (○) PA production when cells were grown at 37°C from time 0. Arrows indicate the time of temperature transfer. Each value is the mean of triplicate experiments.

bacterium is grown at temperatures above 40°C the quantity of PA synthesised is negligible [12]. Fig. 1a shows that when *P. haemolytica* A2 is grown at 37°C and is then transferred to 43°C at different times after incubation (3, 4 and 7.5 h) no substantial modifications in the growth level occur. However, the PA production profiles (Fig. 1b) were strictly dependent on the time of transfer. In this sense, the quantity of PA obtained decreased with progressively shorter times of transfer to 43°C.

In these experiments, analysis of the presence of the enzymes directly involved in PA biosynthesis (Neu5Ac lyase, CMP-Neu5Ac synthetase and sialyltransferase) [12] revealed that after transfer all enzymatic activities decreased dramatically, no activity at all being detected at 10 h after the temperature change (Fig. 2).

These results suggest that the production of PA in *P. haemolytica* A2 and the enzymatic activities involved in PA biosynthesis are intimately related to and regulated by the temperature of cellular growth.

When the temperature change was made from 43 to 37°C at different incubation times (2, 3 and 5 h) no significant modifications in the cellular growth pattern were observed (Fig. 3a) and, as before, PA production was dependent on the time of transfer. In this case, the highest values of PA production were obtained with the shortest transfer times (Fig. 3b). Analysis of the presence of the enzymes involved in PA biosynthesis along the period when *P. haemolytica* was grown at

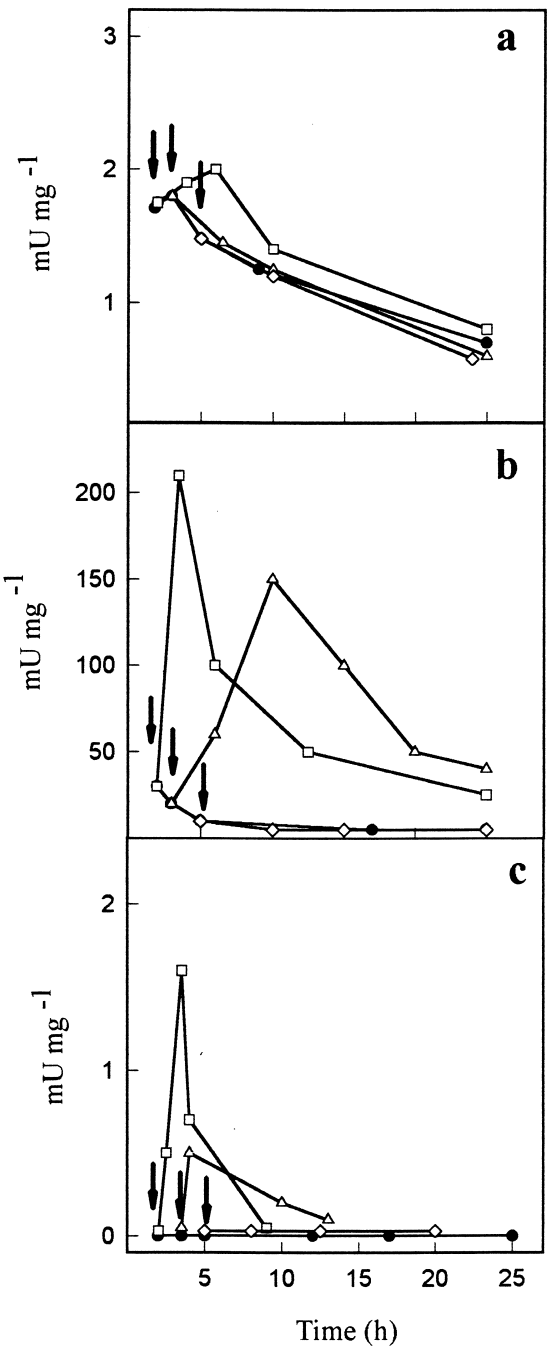

Fig. 4. Effect of incubation temperature on enzymatic activities: Neu5Ac lyase (a), CMP-Neu5Ac synthetase (b) and sialyltransferase (c), when cultures of *P. haemolytica* A2 grown at 43°C (●) were transferred to 37°C after 2 h (□), 3 h (△) or 5 h (◇) of incubation. Arrows indicate the time at which transfer was made. Each value is the mean of triplicate experiments.

43°C revealed that in the case of sialyltransferase no activity was detected (Fig. 4c) while in the case of Neu5Ac lyase and CMP-Neu5Ac synthetase (Fig. 4a,b) the activity levels reached at this growth temperature were 25-fold lower that those observed when the growth temperature was 37°C (Fig. 2a,b). Thus, when the cellular cultures were transferred from 43 to 37°C (Fig. 4) an increase in all the enzymatic activities analysed was observed, as long as the temperature changes were made before 5 h of cellular growth. When the transfer was made after this time no significant differences were obtained with respect to the control growth at 43°C (Fig. 4). Moreover, in no case did the increase observed reach the levels obtained when *P. haemolytica* was grown at 37°C (Fig. 2).

These results explain why even though cells grown at 43°C are transferred during the first stages of incubation (before 2 h; $A_{540} = 0.5$) PA production never reaches maximum values (Fig. 3b).

The fact that PA production and the enzymes involved in its biosynthesis (Neu5Ac lyase, CMP-Neu5Ac synthetase and sialyltransferase) are strictly regulated by the cellular growth temperature indicates that the biosynthetic pathway of PA can be rapidly modulated at the enzymatic level. Surprisingly, this regulatory control acts simultaneously on the whole group of the enzymes and not only on CMP-Neu5Ac synthetase, which in *E. coli* K1 and *E. coli* K92 is the enzyme responsible for this effect [15,17]. These results indicate that in *P. haemolytica* A2 these proteins are subject to the same regulatory system. A similar variation in the half-life of these proteins (or the corresponding mRNAs) or the existence of a 'regulon' involving the genes encoding these enzymes can support these experimental data.

A better understanding of this thermally regulated system should clarify these possibilities and should permit the development of different regulation mechanisms of PA synthesis in *P. haemolytica* A2 that may eventually lead to therapeutic approaches for controlling the pathogenicity of this bacterium with the consequent economic effects. Further research on this topic is currently in progress.

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