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Biochemical conditions for the production of polysialic acid by *Pasteurella haemolytica* A2

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The capsular polysaccharide of *Pasteurella haemolytica* A2 consists of a linear polymer of *N*-acetylneuraminic acid (Neu5Ac) with α(2–8) linkages. When the bacterium was grown at 37°C for 90 h in 250 ml shake flasks at 200 rpm in Brain heart infusion broth (BHIB), it accumulated, attaining a level of 60 μg/ml. Release of this polymer was strictly regulated by the growth temperature, and above 40° no production was detected. The pathway for the biosynthesis of this sialic acid capsular polymer was also examined in *P. haemolytica* A2 and was seen to involve the sequential presence of three enzymatic activities: Neu5Ac lyase activity, which synthesizes Neu5Ac by condensation of *N*-acetyl-*D*-mannosamine and pyruvate with apparent Km values of 91 mM and 73 mM, respectively; a CMP-Neu5Ac synthetase, which catalyzes the production of CMP-Neu5Ac from Neu5Ac and CTP with apparent Km values of 2 mM and 0.5 mM, respectively, and finally a membrane-associated polysialyltransferase, which catalyzes the incorporation of sialic acid from CMP-Neu5Ac into polymeric products with an apparent CMP-Neu5Ac Km of 250 μM.

Keywords: **Polysialic acid biosynthesis, *Pasteurella haemolytica***

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

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 septicemia 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 variations in capsular polysaccharides [3–6]. The presence of the capsule has been demonstrated to be age-dependent [7] and is believed to play a role in the pathogenicity of the organism [8, 9].

P. haemolytica serotype A2, the strain studied here, is able to produce a capsular polysaccharide that consists of a (2–8)-α linked polymer of *N*-acetylneuraminic acid (Neu5Ac); namely, colominic acid or polysialic acid (PA) [10]. This is identical to the capsular polysaccharides of *Neisseria meningitidis* group B and *Escherichia coli* K1. Production of colominic acid by *P. haemolytica* A2 and other bacterial organisms explains the difficulties found by workers attempting to develop extract vaccines from these organisms [11]. This polymer is a particularly poor immunogen [12, 13], which is attributed to tolerance due to cross-reactive tissue polysialylated components of eukaryotic cells such as cell adhesion molecules (N-CAM) [14–16]. The exact mechanism(s) by which sialic acid imparts virulence to *P. haemolytica* A2 is obscure, especially since this polysaccharide does not generally form a readily observable capsule. The overall mechanism for the synthesis and regulation of polysaccharide production in this organism has not yet been defined. In view of the importance of knowledge of the biosynthetic characteristics of polysialic acid (PA) from *P. haemolytica*, and owing to the usefulness of this polymer in the development of new vaccines and diagnostic reagents, an understanding of the different parameters able to regulate the biosynthetic mechanisms, as well as of the enzymatic activities involved in the process, certainly seems justified.

In this study, we examined both the physico-chemical conditions that affect PA production and the biosynthetic pathway of this polysaccharide in *P. haemolytica* A2, identifying the enzymes directly involved in its synthesis.

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Materials and methods

Materials

Neu5Ac, ManNAc, resorcinol, Pyr, Pep, D-xylose, hexadecyltrimethylammonium bromide, vitamins, 2-thiobarbituric acid, L-proline, periodic acid, DTT, sodium arsenite, bovine serum albumin, CMP-Neu5Ac, cytidine 5′triphosphate and L-amino acids were supplied by Sigma Chem. Co. (St. Louis, Missouri). *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, U.K.). Tryptic soy broth (TSB), Casamino acids, and yeast nitrogen base (YNB) were from Difco (Detroit, MI). Brain heart infusion broth (BHIB) was from Pronadisa (Madrid, Spain). Other reagents used were of analytical quality.

Methods

Culture media and growth conditions

P. haemolytica A2 (A.T.C.C. 29694) was obtained from the "Colección Española de Cultivos Tipo" (CECT 924). Strains were kept lyophilized, and to avoid loss of PA production capacity, the lyophilizates 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. The cellular suspension was stored at −70°C in 50% (v/v) glycerol (preinoculum). Experimental cultures were grown by inoculating 50 ml of the required medium (see below) in 250 ml Erlenmeyer flasks with 50 µl of the preinoculum.

Media

The media used in this work were medium BHIB (37 g × 1^{-1}); medium Tryptic Soy Broth (TSB) (30 g × 1^{-1}); and medium Luria-Bertani (LB) (g × 1^{-1}): NaCl, 10; yeast extract, 5; and tryptone, 10.

Analytical methods

Purification of polysialic acid. Polysialic acid was purified by a modification of the method described by Ferrero *et al*. [17]. Cells grown for 100 h at 37°C in BHIB medium were separated by centrifugation (13.000 × g, 10 min), and the clear supernatant was concentrated (55-fold) and consecutively dialyzed by ultrafiltration through a Filtron concentrator using a membrane minissette exclusion size <10,000 Mr (Filtron Technology Corporation, Northboro, MA). The concentrate was precipitated with hexadecyltrimethylammonium bromide. The fraction precipitating between 0.4 and 0.6% (w/v) containing all the polysialic acid was collected by centrifugation (20,000 × g, 20 min) and resuspended in 5 ml of 1 M $CaCl_2$. Then, polysialic acid was precipitated with 2.5 vol. of ethanol at −20°C and resuspended in 25 mM sodium acetate pH 4.5. This operation was repeated twice to ensure a high degree of purification. The final pellet—containing polysialic acid—was resuspended in water, dialyzed, and freeze dried. The quantity of polysialic acid was determined by the Svennerholm method [18]. Purity was higher than 95%.

Cell-free extracts and enzymic assays

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 Tris-HCl 50 mM pH8, and resuspended in Tris-HCl 50 mM pH 8, containing 25 mM $MgCl_2$ and 1 mM dithiothreitol (DTT). The cellular suspension (20 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 × g, 20 min at 4°C. The pellet was discarded, and the supernatant was ultracentrifuged at 200.000 × g for 60 min at 4°C. Supernatants were used for the evaluation of both enzyme activities—*N*-acetyl-D-neuraminic acid lyase (Neu5Ac lyase) and cytidine 5′-monophosphate *N*-acetyl neuraminic acid synthetase (CMP-Neu5Ac synthetase)—according to the methodologies described by Rodríguez-Aparicio *et al.* [19] and González-Clemente *et al.* [20], respectively. In both cases, one unit of enzyme was defined as the amount of enzyme that synthesizes 1 µmol of product (Neu5Ac for Neu5Ac lyase and CMP-Neu5Ac for CMP-Neu5Ac synthetase)/min at 37°C under the respective assay conditions. Specific activities were expressed as units/mg of protein.

The ultracentrifugation precipitate (membrane-rich fraction) was resuspended in Tris-HCl 50 mM, pH 8, and used to evaluate polysialyltransferase activity according to the method described by Rodríguez-Aparicio *et al.* [21]. One unit of this enzyme was defined as the amount of enzyme that synthesizes 1 pmol of product ([^{14}C]-Neu5Ac binding to the acceptor)/min at 33°C under the assay conditions. Specific activity was expressed as units/mg of protein.

Proteins were measured by the method of Bradford [22] using bovine serum albumin as standard. Buffers identical to those containing the protein samples were used as blanks.

Results and discussion

Growth and polysialic acid production in different media

Traditionally, complex media have been employed for the growth of *P. haemolytica* A2 [23, 24], and although defined media have also been used [25] the relationship between growth and polysialic acid production has never been checked. Analysis of different defined media that are ideal for the growth and maximal production of PA by other bacteria that also produce this capsular polysaccharide [21, 26] revealed that none of them permit the growth of *P. haemolytica* A2, even when they were supplied with amino acids, vitamins and yeast extract (data not shown). This result confirms the complex nutritional requirements of this microorganism. Moreover, as shown in Table 1, when

Table 1. Growth and polysialic acid (PA) production from ***P. haemolytica*** A2 after 100 h of growth in complex media

Medium	A_{540nm}	*pH*	*Polysialic acid* ($\mu g\text{-}ml^{-1}$)	*Specific production* μg PA/Unit A_{540nm}
BHIB	2.2	6.1	62.0	28.2
TSB	0.8	6.2	20.0	25.0
LB	3.3	6.0	18.0	5.4

complex media were employed (see Materials and Methods), *P. haemolytica* did grow but the final titers of growth and polysialic acid production varied strongly. In this sense, the highest growth was obtained in LB medium, with the lowest PA production, whereas the highest production was obtained in BHIB medium. By contrast, TSB medium afforded the lowest growth and an intermediate level of PA production. The addition of amino acids, yeast extract, and vitamins to these media did not modify this situation. These observations indicate that there is no relationship between the highest level of growth and the highest level of PA production and show that maximal, total, and specific production is achieved with BHIB medium.

Comparison of the growth profiles and PA production patterns showed that the polysaccharide is first detectable as from the early logarithmic phase of growth (10–15 h), reaching a maximum after 70 h of incubation (Figure 1a) when the medium used is BHIB (the medium in which *P. haemolytica* A2 produces the highest level of PA). By contraat in LB (the medium in which *P. haemolytica* A2 shows the highest level of growth) the bacterium shows a long lag phase and PA is only detectable in the stationary phase of growth (Figure 1b). The delay in growth and PA release accounts for the lower PA production attained in LB medium. In view of these results, BHIB was chosen as the most suitable medium for this work.

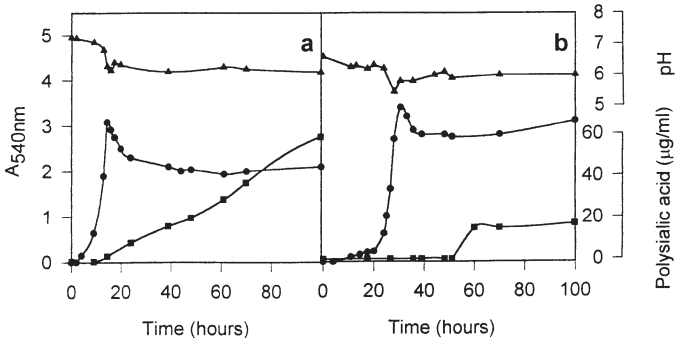


Figure 1. Production of polysialic acid (■), growth (●), and pH determination (▲) when *P. haemolytica* A2 was grown in BHIB (a) or LB (b) medium.

Effects of different physical parameters on growth and PA synthesis

Study of the effect of temperature on the growth of *P. haemolytica* A2 (Figure 2) revealed that this bacterium does not grow below 28.5°C and the level of growth observed as from this temperature onward remains practically constant, at least up to 43°C. Regarding PA production (total and specific), the highest values correspond to 37°C. Above this temperature, production decreases dramatically and is negligible at 40°C (Figure 2). These results contrast with previously reported findings for other bacteria that produce PA, such as *E. coli* K1 and *E. coli* K92; these microorganisms are able to grow below 28.5°C and at 20°C, although as growth continues, the quantity of polymer released is negligible [20–21, 26].

When the effect of shaking was studied (Table 2), we observed that regarding growth no substantial modifications occur between 250 and 100 rpm, and only the total elimination of shaking elicited a significantly lower rate of growth. However, with respect to PA production (total and specific), the highest titers were achieved at 200 rpm (Table 2), and a substantial reduction (50% of specific production) was observed when shaking was performed at 100 rpm. This level of specific production remained constant even when shaking had ceased. The difference between the titer of PA at 200 rpm and those obtained at other rpms with less shaking or none at all points to the importance of oxygen levels in the biosynthesis of this polysaccharide.

Analysis of the enzymatic activities involved in PA biosynthesis by P. haemolytica A2

P. haemolytica A2 is able to synthesize a capsular polysaccharide consisting of an (2–8)-α linked polymer of *N*-acetylneuraminic acid identical to the capsular polysialic acid of *E. coli* K1 and *N. meningitidis* group B [10]. However, no

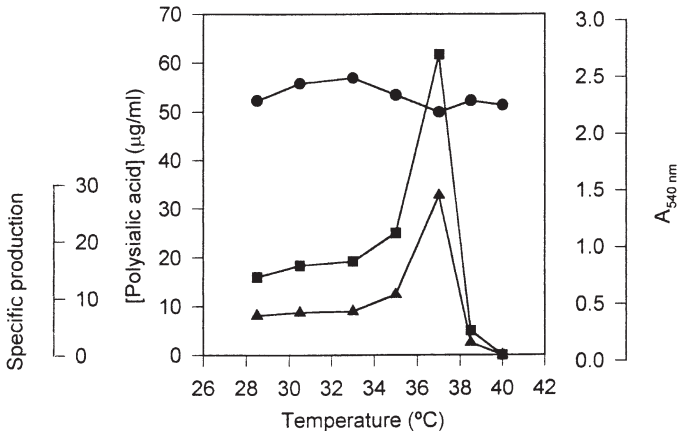


Figure 2. Effect of temperature on growth (●), total PA production (■), and specific PA production (μg PA/A_{540nm}) (▲) by *P. haemolytica* A2 grown in BHIB medium at 200 rpm at 100 h of growth.

Table 2. Effect of shaking at 37°C on growth of *P. haemolytica* A2 grown in BHIB medium at 100 h of growth

	Shaking (rpm)			
	0	*100*	*200*	*250*
A_{540nm}	1.2	2.0	2.2	2.2
PA (μg/ml)	13.0	26.0	62.0	59.0
PA (specific production)	10.8	13.1	28.2	26.8

Table 3. Effect of the addition of Pyruvate decarboxylase on Neu5Ac synthesis when cell extracts of *P. haemolytica* were used[a]

	μmol Neu5Ac /mg prot	
	− *Pyr decarboxylase*[b]	+ *Pyr decarboxylase*[b]
Pyr + ManNAc	9.8	ND
Pep + Man NAc	8.7	ND

[a]Reaction mixtures were incubated at 37°C for 1 h, using 0.2 M citrate, pH 7.0, as buffer.
[b]Blank reation mixtures containing Pyr decarboxylase (2U) and without crude extracts were used as Pep and Pyr degradation controls, respectively. After incubation at 37°C for 1 h, pyruvate decarboxylase was removed using a Centricon-10 ultrafiltration system and Pep and Pyr were measured spectrophotometrically [19]. Under these conditions, the Pyr disappeared, and the Pep concentration was not modified.
ND, Not detected.

information is available about the enzymatic biosynthetic mechanisms of this polymer in *P. haemolytica* A2. It is possible that this bacterium might use a similar biosynthetic pathway to that of other known bacteria, with the sequential participation of Neu5Ac lyase (which condenses ManNAc and Pyr) or Neu5Ac synthase (which condenses ManNAc and Pep), CMP-Neu5Ac synthase, and a sialyltransferase complex enzyme.

The presence of each enzyme activity was checked at different cell growth times in protein extracts of *P. haemolytica* A2 grown in BHIB medium at 37°C under 200 rpm shaking conditions (see Materials and Methods).

The first enzyme analyzed was the one able to synthesize Neu5Ac. Since in bacteria two proteins have been implicated in this process, we analyzed both Neu5Ac lyase and Neu5Ac synthase using cellular extracts of *P. haemolytica* (see Materials and Methods). The results obtained revealed the presence of both activities (data not shown). However, it is possible that like *E. coli* K1 [19], in *P. haemolytica* A2 a phosphatase activity, present in crude extracts, converts Pep into Pyr and that only a Neu5Ac lyase is responsible for Neu5Ac synthesis in this bacterium. To prevent lyase activity (as previously reported by Rodríguez-Aparicio *et al.* [19], we added 2 units of Pyr decarboxylase (from Yeast, Sigma Chemical Co., St. Louis, MO) to the Neu5Ac synthesis reaction mixtures. Pyr decarboxylase converts Pyr into acetaldehyde, and in the presence of this enzyme, the Neu5Ac must be synthesized only through Neu5Ac synthase action. Thus, when commercial Pyr decarboxylase was present, Neu5Ac synthesis was not detected (Table 3). These results demonstrate that in *P. haemolytica* a Neu5Ac lyase, and not a Neu5Ac synthase, is the enzyme responsible for Neu5Ac synthesis.

As shown in Figure 3A, the Neu5Ac lyase enzyme began to be synthesized as from the early logarithmic phase of growth (8–10 h), and its levels increased slowly, reaching maximum values at 25 h (stationary phase of growth) and thereafter decreasing continuously. This kind of kinetic behavior differs from that observed for *E. coli* K1 lyase, where the maximum quantity of enzyme appears during the logarithmic phase of growth [27]. Indeed, in this case the maximum level detected was three orders of magnitude higher than that observed for the enzyme of *P. haemolytica* A2. Moreover, the presence of the Neu5Ac lyase activity in these bacterial extracts confirms that the mechanism of Neu5Ac synthesis by *P. haemolytica* A2 is similar to that of *E. coli* K1 [19] and that it differs from the mechanism used by *N. meningitidis* group B, where Neu5Ac is synthesized by condensation of ManNAc and phosphoenolpyruvate [28]. These findings also confirm previous results in which the use of a polyclonal antibody revealed the presence of a Neu5Ac lyase protein similar to that of *E. coli* K1 in crude extracts from *P. haemolytica* A2 [27]. Study of the kinetic characteristics of the Neu5Ac lyase enzyme revealed a hyperbolic type of behavior for Pyr and ManNAc (Figure 4) and the Km value calculated for each were 73 and 91 mM, respectively. The substrate affinity of the enzyme from *P. haemolytica* was higher (one order of) than that of enzymes from other sources [27, 29–32]. This, together with the lower amount of Neu5Ac lyase detected in the different extracts (Figure 3A), suggests that *P. haemolytica* does not require as much Neu5Ac as other bacteria that produce a capsular polymer composed of sialic acid.

With respect to CMP-Neu5Ac synthetase, the enzyme that activates Neu5Ac, this was also detected in cellular extracts of *P. haemolytica* A2. Even at early growth times (8–10 h) the protein displayed high activity (Figure 3B). Thereafter, activity decreased, reaching minimum values at 17 of incubation. This behavior is similar to that of the CMP-Neu5Ac synthetases from *E. coli* K1 [20] and *E. coli* K92 [26], where maximum levels of both enzymatic activities appear during the logarithmic phase of growth. However, in our case the quantity of enzyme generated by *P. haemolytica* A2 was higher (three orders of magnitude) than that found for other microorganism sources. In view of this result, it is possible that CMP-Neu5Ac synthetase might not be the limiting protein in PA biosynthesis by *P. haemolytica* A2. Study of the kinetic parameters revealed

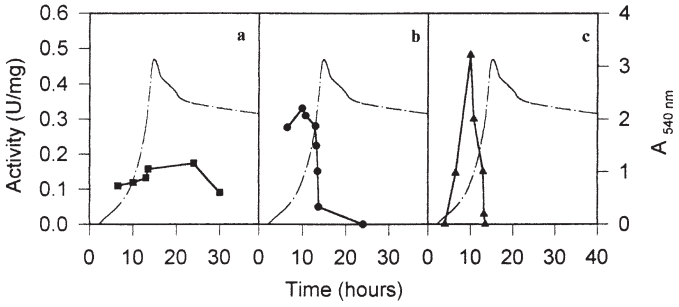


Figure 3. Time course of Neu5Ac lyase (a) (■); CMP-Neu5Ac synthetase (b) (●); and sialyltransferase (c) (▲) enzymes from *P. haemolytica* A2 grown at 37°C in BHIB medium cellular growth (-•-).

that CMP-Neu5Ac synthetase activity follows hyperbolic behavior for CTP and Neu5Ac (Figure 5). However, when higher CTP concentrations were employed (more that 20 mM) enzyme activity decreased. This effect, which has also been detected in CMP-Neu5Ac synthetase from other sources [33], may regulate the synthesis of CMP-Neu5Ac to prevent its accumulation, hence the extreme toxicity of this product at high concentrations [20]. The values calculated were 0.5 and 1.85 mM for CTP and Neu5Ac, respectively, and these lie in the range of those observed for CMP-Neu5Ac synthetase from *N. meningitidis* [34], calf brain [35], rat liver [33], and pig submaxillary gland [36]. However, they are lower (one order of magnitude) than those observed for other bacteria such as *E. coli* K1 and *E. coli* K92 (Gonzalez-Clemente, C., unpublished results).

Finally, we detected the presence of a sialyltransferase activity that catalyzes the incorporation of sialic acid from CMP-Neu5Ac into polymeric products. This enzyme was also detected as from the early logarithmic phase of growth (Figure 3C) and reached maximum levels at 10 h, the quantity of protein thereafter decreasing rapidly and no activity being detected as from 15 h. This rate of synthesis is very similar to that observed for *E. coli* K1 [37] and *E. coli* K92 (Ferrero, M.A. unpublished work) sialyltransferases. Comparison of the maximum levels attained revealed that the enzyme activity of *P. haemolytica* A2 is up to two and three magnitude orders lower that the levels observed for *E. coli* K1 and *E. coli* K92 sialyltransferases, respectively. This suggests that the polymerization process is an important regulatory point in PA biosynthesis in *P. haemolytica* A2. Sialyltransferase activity followed a hyperbolic type of behavior for CMP-Neu5Ac (Figure 6), and the Km calculated was 250 μM, similar to the value obtained for other bacterial sources that also produce polysialic acid [37] (Ferrero, M.A., unpublished results).

Analysis of the time course of the appearance of the enzymes directly involved in PA synthesis by *P. haemolytica* ***(Neu5Ac lyase, CMP-Neu5Ac synthetase, and sialyltransferase) revealed that in all cases maximum activity levels occur before 30 h of cellular growth (Figure 3), although maximum PA production was observed after 80 h of incubation (Figure 1). Similarly, the situation with*** *E. coli* K1 [21] results suggest that *P. haemolytica* actively synthesizes PA during the logarithmic phase of growth and that polymer release into the broth takes place mainly when cellular growth is stopped.

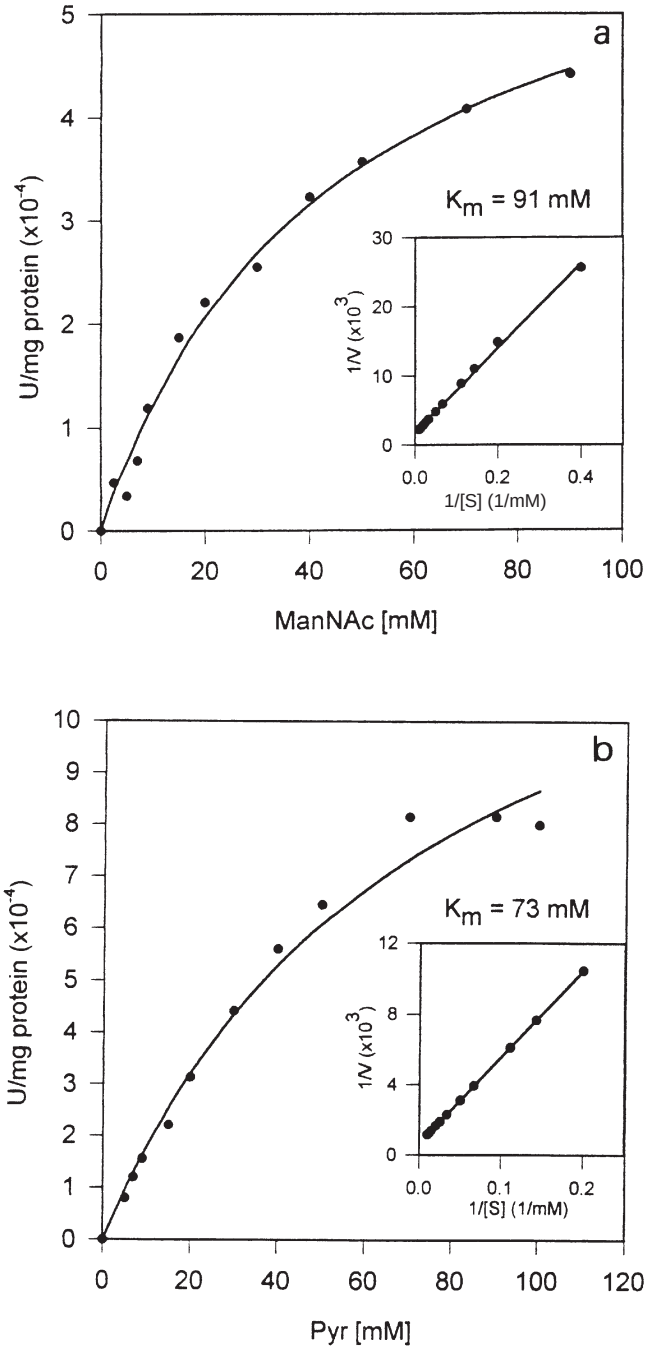
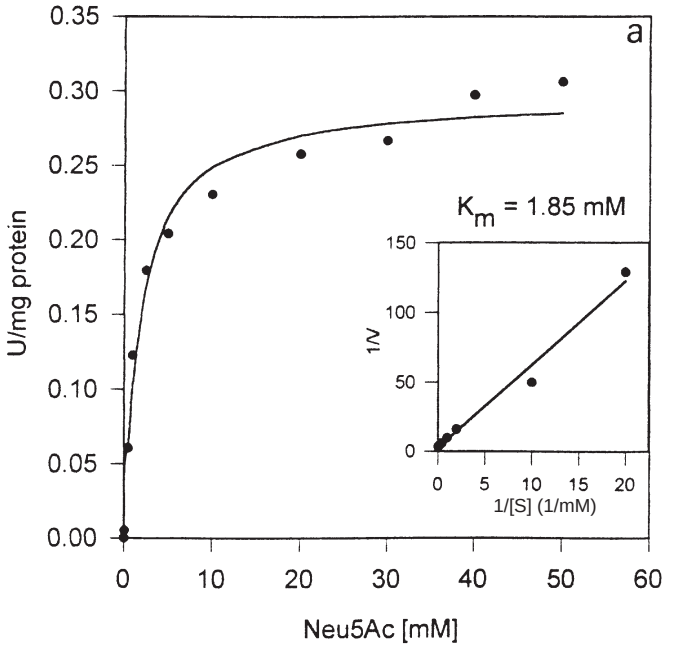


Figure 4. Neu5Ac lyase substrate analysis. Determination of Km for ManNAc (a) and Pyr (b). Each substrate was assayed under saturating conditions for the others. Kinetic parameters were calculated using the "Enzfitter" (Elsevier) nonlinear regression data analysis program.



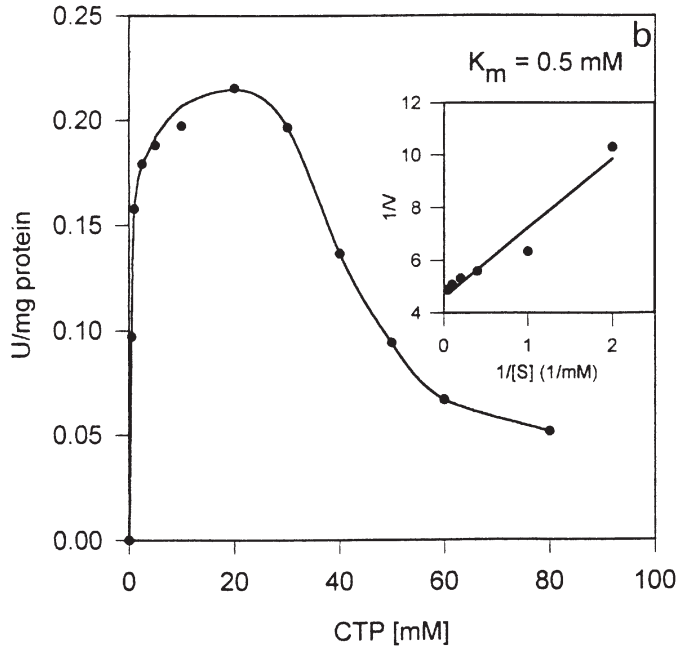


Figure 5. CMP-Neu5Ac synthetase substrate analysis. Determination of Km for Neu5Ac (a) and CTP (b). Each substrate was assayed under saturation conditions for the others. Kinetic parameters were calculated using the "Enzfitter" (Elsevier) nonlinear regression data analysis program.

The results offered here demonstrate the key importance of different physical parameters such as temperature and aeration in the growth and optimal PA production in *P. haemolytica* A2. Moreover, they point to the involvement of an enzymatic system responsible for PA biosynthesis in

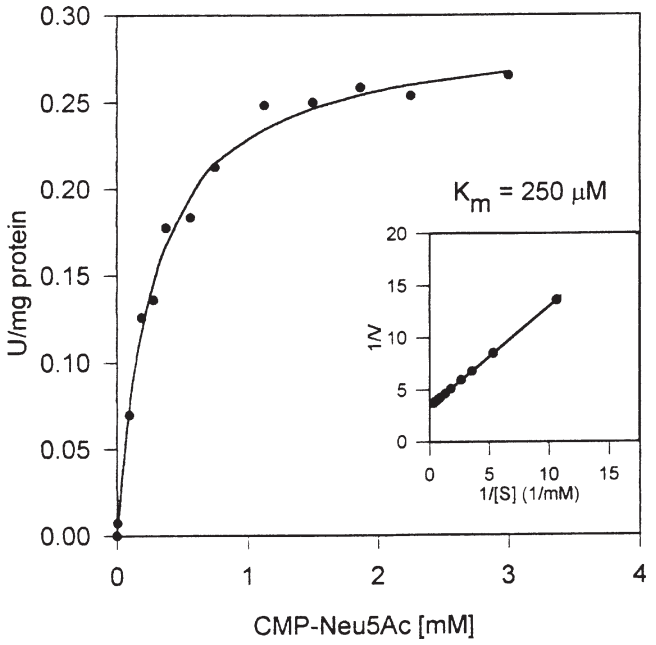


Figure 6. Sialyltransferase substrate analysis. Determination of Km for CMP-Neu5Ac. Kinetic parameters were calculated using the "Enzfitter" (Elsevier) nonlinear regression data analysis program.

P. haemolytica A2 similar to that used by other prokaryotic organisms, such as *E. coli* K1 and *E. coli* K92, in which the sequential participation of Neu5Ac lyase, CMP-Neu5Ac synthetase, and sialyltransferase has been reported [19–20].

A better understanding of each of the enzymes directly involved in the biosynthesis of capsular polymer by *P. haemolytica* through their purification, characterization, and gene analysis should permit studies about the basic aspects of PA production and may eventually lead to therapeutic approaches for controlling the biosynthesis of this pathogenic determinant, with the consequent economic effects. Further research on this topic is currently in progress.

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