Regulation of capsular polysialic acid biosynthesis by *N*-acetyl-D-mannosamine, an intermediate of sialic acid metabolism

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Abstract ***N*-Acetyl-D-mannosamine (ManNAc) is a specific substrate for the synthesis of *N*-acetylneuraminic acid, the essential precursor of bacterial capsular polysialic acid (PA). When *Escherichia coli* K92 used ManNAc as a carbon source, we observed a dramatic reduction (up to 90%) in in vivo PA production. Experiments in which the carbon source was changed revealed that the maximal inhibitory effect occurred when this sugar was present in the medium before the logarithmic phase of bacterial growth had started. Enzymatic analysis revealed that high concentrations of ManNAc-6-phosphate inhibit NeuAc lyase, the enzyme that synthesizes NeuAc for PA biosynthesis in *E. coli*. These results indicate that ManNAc-6-phosphate is able to regulate NeuAc lyase activity and modulate the PA synthesis.**

Key words: *N*-Acetyl-D-mannosamine; Polysialic acid; Capsular polysaccharide

1. Introduction

Polysialic acid (PA) is a bacterial capsular homopolysaccharide of *N*-acetylneuraminic acid (NeuAc) with α-(2,8) and/or α-(2,9) ketosidic linkages [1–3]. This polymer has been shown to be a pathogenic determinant in the capsular antigens of *Neisseria meningitidis*, *Escherichia coli*, *Pasteurella haemolytica*, *Moraxella nonliquefaciens* and several strains of *Salmonella*. Bacterial PA shows biochemical and epitope resemblances to certain eukaryotic cell glycoconjugates such as cell adhesion molecules (N-CAM) [3]. This similarity protects it from host-bacteria killing and is at least partially responsible for many cases of neonatal meningitis and a large proportion of cases of bacteremia and urinary tract infections caused by these microorganisms [4]. The structure, production and biosynthesis of bacterial PA have therefore received considerable attention [5–11]. The relevance of NeuAc linkages to spatial polymer structures has been analyzed [10–12] and the PA biosynthesis pathway has been established [5–8]. Regarding the analysis of PA production, we have developed different chemically defined media capable of supporting the growth of *E. coli* K1 [2] and *E. coli* K92 [10], in which the production of capsular PA is optimal. Using these media, we have purified PA and have studied its immunogenic capacity [13]. We have also observed that PA biosynthesis is strictly regulated by cell growth temperature [9,10]. When *E. coli* grows in these media at temperatures below 20°C (restrictive temperatures), PA production is negligible [2,10]. Enzymatic analysis has shown that CMP-NeuAc synthetase, the enzyme that activates NeuAc to CMP-NeuAc in the biosynthesis of the polymer, is responsible for this effect [9]. We now describe the effect of *N*-acetyl-D-mannosamine (ManNAc), an essential precursor of NeuAc [14,15], on *E. coli* PA production when the sugar is used as a carbon source for growth. Finally, we analyze and discuss the molecular significance of this physiological effect.

2. Materials and methods

2.1. Chemicals

NeuAc, ManNAc, mannosamine, resorcinol, Dowex 1 (×8; 100–200 mesh) pyruvate, D-xylose, L-asparagine, and CTP were purchased from Sigma (St. Louis, MO, USA). CMP-[4,5,6,7,8,9-^{14}C]NeuAc (256 mCi/mmol) was from DuPont NEN (Hertfordshire, UK). Sephacryl S-200 and Sephadex G-25 (PD-10) were purchased from Pharmacia (Sweden). Other reagents used were of analytical quality.

2.2. Culture media and growth conditions

E. coli K92 (ATCC 35860) was obtained from the American Type Culture Collection. The strain was maintained on Trypticase soy agar (Difco) and slants grown for 8 h at 37°C were used for seeding liquid media as previously described [2]. Incubations were carried out in a rotary shaker (250 rpm) at 37°C for different times using two chemically defined media: (1) a xylose/asparagine medium known to be ideal for PA production [10] and (2) a ManNac/asparagine medium identical to the above but where the xylose (8.4 g/l) had been replaced by a similar concentration of ManNAc (7.8 g/l).

2.3. PA determinations

The sialyl polymer produced by *E. coli* K92 was analyzed by the Svennerholm [16] methodology, described elsewhere [2].

2.4. Cell-free extracts and enzymic assays

Cell-free extracts of *E. coli* K92 obtained as previously described [7,10] were ultracentrifuged at 200 000×*g*, 60 min at 4°C. Supernatants were concentrated by ammonium sulfate precipitation (80% saturation), resuspended and desalted by passage through a Sephadex G-25 (PD 10) column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 25 mM $MgCl_2$ and 1 mM DTT (TMD buffer). The eluate was used for the quantification of NeuAc lyase (synthesis direction) and CMP-NeuAc synthetase activities according to reported methodologies [9,14]. One unit was defined as the amount of enzyme that synthesizes 1 nmol of product (NeuAc or CMP-NeuAc) per minute under the assay conditions.

When the effect of ManNAc-6-phosphate was analyzed, the precipitated supernatants were resuspended and applied to a column (2.5 cm×76 cm) of Sephacryl S-200 equilibrated and eluted with TMD buffer, as previously described [14]. Phosphatase-free fractions containing NeuAc lyase or CMP-NeuAc synthetase activities were used for this study.

Finally, precipitates from the original ultracentrifugations (mem-

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Abbreviations: PA, polysialic acid; NeuAc or sialic acid, *N*-acetyl-D-neuraminic acid; N-CAM, cell adhesion molecule; ManNAc, *N*-acetyl-D-mannosamine; GlcNAc, *N*-acetyl-D-glucosamine; CTP, cytidine 5′-triphosphate; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; DTT, dithiothreitol; Pyr, pyruvate; BSA, bovine serum albumin

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Table 1
Evaluation of NeuAc lyase, CMP-NeuAc synthetase and sialyltransferase activities when *E. coli* K92 was grown at 37°C in Xyl-Asn and ManNAc-Asn media

Medium	Specific activity[a]		
	NeuAc lyase	CMP-NeuAc synthetase	Sialyltransferase
Xylose-Asn	55 ± 3.1	0.030 ± 0.005	24 ± 3.6
ManNAc-Asn	32 ± 2.6	0.020 ± 0.010	18 ± 2.5

[a]Values are given as means ± S.E.M. ($n = 4$).

brane-rich fractions) were washed and resuspended in TMD buffer and used to measure sialyltransferase activity [2]. Incubation mixtures contained in a final volume of 30 μl: 50 mM Tris-HCl (pH 8.0), 40 mM NH_4Cl, 10 mM $BaCl_2$, 10 mM DTT, 2.4 μM (256 mCi/mmol) CMP-[^{14}C]NeuAc (72 pmol, 563 dpm per pmol) and 10 μl of protein extract. One unit was defined as the amount of enzyme that synthesizes 1 pmol of product 1 ([^{14}C]NeuAc binding to the acceptor) per minute at 35°C under the assay conditions.

Specific activities were expressed as units/mg protein. Protein was measured by the method of Bradford [17] using BSA as standard.

2.5. Analysis of intracellular ManNAc and ManNac-6-phosphate

Cells from 62.5 ml of the bacterial culture grown in the above media to A_{540}=2.0 were collected by centrifugation of 10 000 × *g* for 10 min at 4°C and washed twice with sterile distilled water. Cells were mixed and extracted by gentle stirring with ethanol as previously described [18]. Bacterial cells were removed by centrifugation (10 000 × *g*, 10 min) and the supernatants were lyophilized. To measure the presence of ManNAc-6-phosphate, the lyophilized samples were resuspended in water and chromatographed using a Dowex 1 (×8, 100–200 mesh; formate form) column according to the methodology described by Van Rinsum et al. [19] and the sugar phosphate eluted was lyophilized again. Finally, the presence of ManNAc and ManNAc-6-phosphate was analyzed by cellulose thin-layer chromatography [20,21] and the amount of product was determined by the colorimetric method of Spirak and Roseman [22] using commercial ManNAc as standard. Authentic ManNAc and ManNAc-6-phosphate were also quantified by coupling to NeuAc synthesis using commercial (Sigma) NeuAc lyase [15]. For enzymatic evaluation of the phosphate sugar it was necessary to perform dephosphorylation beforehand by incubation with commercial (Boehringer) alkaline phosphatase (1 U at 37°C during 30 min).

2.6. Preparation of ManNAc-6-phosphate

ManNAc-6-phosphate was prepared from mannosamine in succession by phosphorylation with hexokinase (Boehringer Mannheim, Mannheim, Germany) and *N*-acetylation [21,23]. Sugar phosphate purification was accomplished using a Dowex 1 (×8; 100–200 mesh; formate form) column, as above, analyzed by cellulose thin-layer chromatography [20,21], and quantified by the colorimetric method of Spirak and Roseman [22]. Only the fractions of ManNAc-6-phosphate synthesized that were more than 95% pure were used.

3. Results and discussion

Bacterial capsular PA biosynthesis begins with the formation of NeuAc through an enzymatic system other than that observed in eukaryotic cells and which seems to be species-specific (for a review, see [1,3,14,15]). In this sense, the synthesis of sialic acid by condensation of ManNAc with phosphoenolpyruvate (NeuAc synthase activity) has been observed in *N. meningitidis* and *E. coli* [24,25] whereas the synthesis of NeuAc by direct condensation of ManNAc with pyruvate (NeuAc-lyase activity) has only been observed in *E. coli* strains [14,15]. Nevertheless, in all cases ManNAc is the common precursor to NeuAc synthesis. Accordingly, when the bacteria are grown using ManNAc as the carbon source polysialic acid production is likely to be affected.

Previous results have shown that when *E. coli* K92 is grown in a chemically defined medium containing D-xylose and L-asparagine as the only carbon and nitrogen sources (Xyl-Asn medium), PA production reaches maximum values (∼450 μg/ml) [27]. Using the same medium, here we analyzed the effect of the replacement of D-xylose by ManNAc (ManNAc-Asn medium) on cellular growth and PA production. As shown in Fig. 1, when *E. coli* K92 was incubated in ManNAc-Asn medium growth was slow and PA production only reached 42 μg/ml. Since no substantial variations in pH were observed during bacterial growth in either medium (data not shown), the dramatic reduction in PA production observed (90% compared to Xyl-Asn medium) suggests that ManNAc affects the polymer metabolism of *E. coli* K92. Moreover, when cells grown in Xyl-Asn medium were transferred to fresh ManNAc-Asn medium, we observed that the effect on PA production was strictly dependent on the time of transference and only took place when this was performed during the earliest stages of growth (Fig. 1). Furthermore, when cells incubated in ManNAc-Asn medium were transferred to Xyl-Asn medium, the rate of growth and the final titer of PA accumulated in the broth was strictly dependent on the time of transference (Fig. 2). The effect caused by ManNAc was only partially reversed when bacteria were transferred to Xyl-Asn medium before 24 h, which is when the logarithmic growth phase occurs in ManNAc-Asn medium. The facts that cells incubated in ManNAc-Asn medium for more than 24 h behaved as though ManNAc were still present (Fig. 2) and that the maximal effect on PA synthesis took place when transference to ManNAc-Asn medium was carried out before 8 h of growth, when no logarithmic phase of growth had yet been initiated (Fig. 1) and no polymer syn-

Table 2
Effect of ManNAc and ManNAc-6-phosphate addition on NeuAc lyase, CMP-NeuAc synthetase and sialyltransferase activities obtained from *E. coli* K92 grown at 37°C

Reaction mixture	% Activity[a]		
	NeuAc lyase	CMP-NeuAc synthetase	Sialyltransferase
Control	100	100	100
+ManNAc (10 mM)	–	98 ± 1.2	104 ± 6.2
+ManNAc-6-P (10 mM)	80 ± 2.6	102 ± 2.1	103 ± 4.2

[a]Values are given as means ± S.E.M. ($n = 4$).

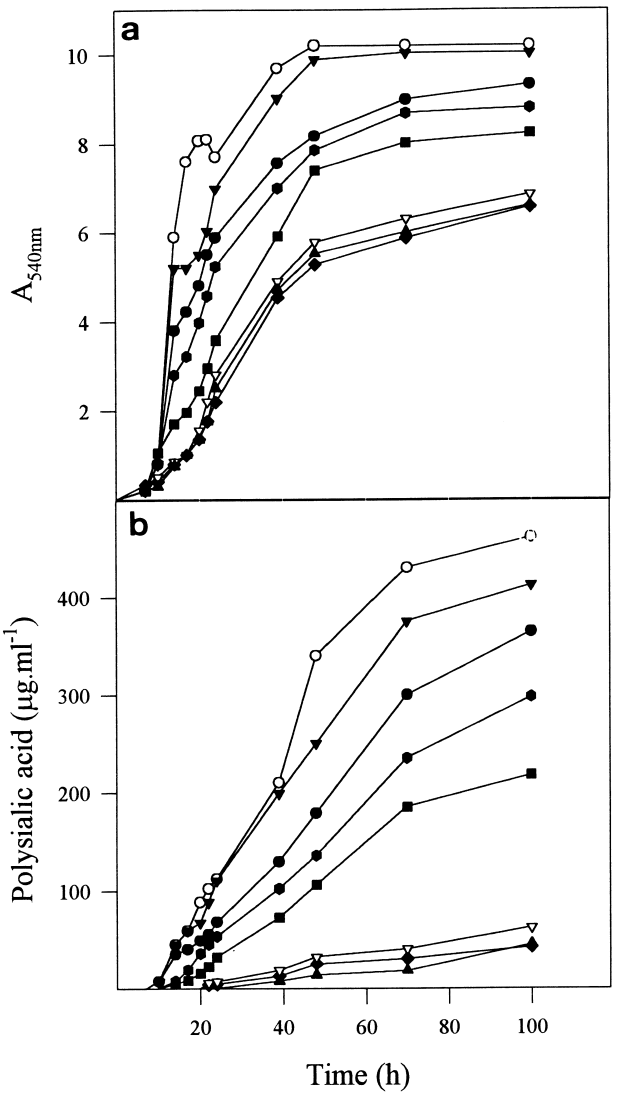

Fig. 1. Effect of the transfer of *E. coli* K92 culture from Xyl-Asn medium to ManNAc-Asn medium. (a) Bacterial growth and (b) PA production when transfer was made at: time 0 h (filled diamonds); 6 h (filled upward triangles); 7 h (open downward triangles); 10 h (filled squares); 13 h (filled hexagons); 14 h (filled circles), 15 h (filled downward triangles) and without transference (open circles). Each value is the mean of quadruplicate experiments.

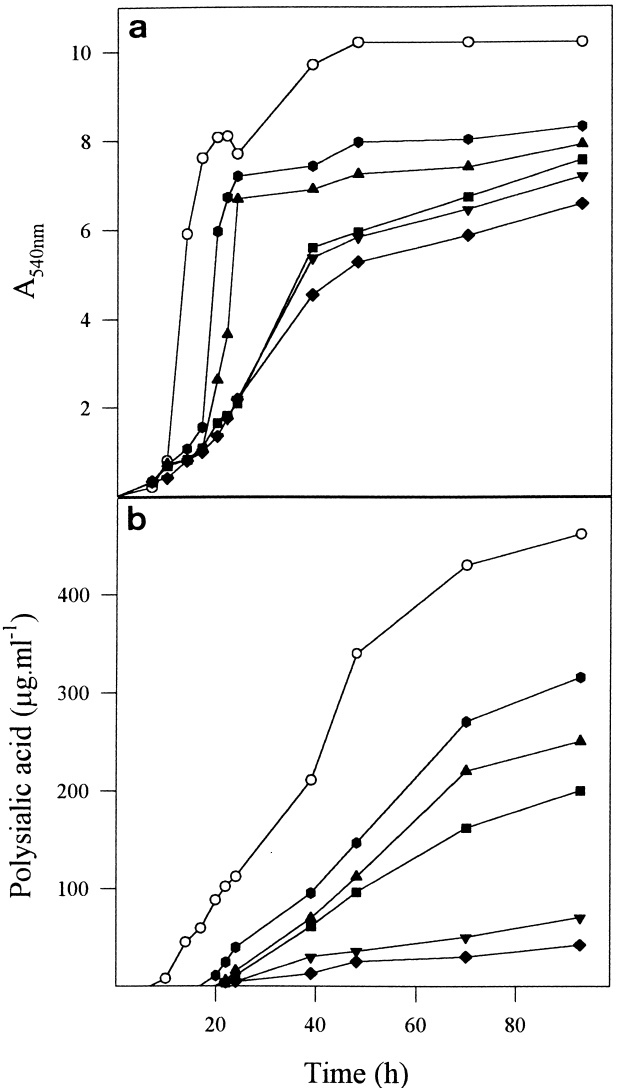

Fig. 2. Effect of the transfer of *E. coli* K92 culture from ManNAc-Asn medium to Xyl-Asn medium. (a) Bacterial growth and (b) PA production when the transfer was made at: time 0 h (open circles); 10 h (filled hexagons); 17 h (filled upward triangles); 24 h (filled squares); 30 h (filled downward triangles) and without transference (filled diamonds). Each value is the mean of quadruplicate experiments.

thesis was occurring [2,7,10], suggest that when *E. coli* K92 is grown using ManNAc as the carbon source the PA biosynthetic pathway is affected directly. Such an effect could be explained in terms of a change in the enzymatic activity of some proteins involved in polymer synthesis due to differences in quantity or to the direct action of ManNAc or ManNAc intermediates.

In this bacterium, PA biosynthesis involves the formation of sialic acid by the action of NeuAc lyase [14,15], the activation of NeuAc through binding to CTP (CMP-NeuAc synthetase activity) [26,27] and the polymerization of NeuAc by its incorporation from CMP-NeuAc into an endogenous acceptor and transfer of the native chain to a final acceptor, thus allowing the polymer to grow [3,7,28]. These latter reactions are catalyzed by a membrane-bound sialyltransferase complex [1,8,28].

Table 3
Effect of ManNAc-6-phosphate on NeuAc lyase activity (synthesis direction) from different sources

Reaction mixture	NeuAc lyase activities[a] from:			
	E. coli K92	*E. coli* K1	*E. coli* (Sigma)	*C. perfringens* (Sigma)
Control	100	100	100	100
+ManNAc-6-P (10 mM)	80 ± 2.6	82 ± 3.0	78 ± 1.2	112 ± 6.0

[a]Values are given as means ± S.E.M. ($n = 4$).

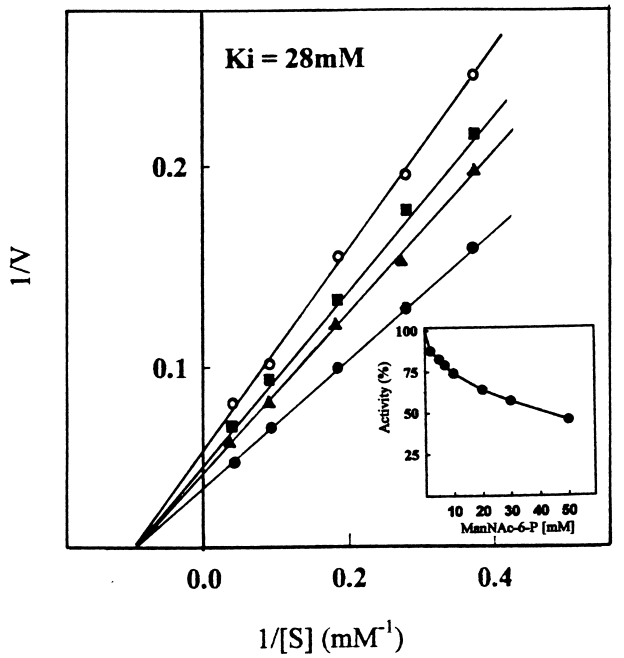

Fig. 3. Effect of ManNAc-6-phosphate on the direction of synthesis of NeuAc lyase activity from *E. coli* K92. Kinetic determination of K_i value for ManNAc-6-phosphate. (●) control; (▲) plus 7 mM ManNAc-6-phosphate; (■) plus 10 mM ManNAc-6-phosphate; (○) plus 20 mM ManNAc-6-phosphate. Inset shows the inhibition in NeuAc lyase activity (%) caused by different concentrations of ManNAc-6-phosphate when 10 mM of ManNAc as substrate was used. Each value is the mean of quadruplicate experiments.

To confirm whether any of the above hypotheses were correct, we analyzed each of these enzymatic activities. As shown in Table 1, all the enzymes were detected in cell-free extracts of *E. coli* K92 grown in both Xyl-Asn and ManNAc-Asn media. However, although the bacteria were incubated in the respective media up to $A_{540} = 2.0$, a growth time during which PA is known to be actively synthesized [2,7,10], the amounts of enzyme in extracts of cells from ManNAc-Asn medium were found to be low (60–75%). These differences (Table 1) account for the variations observed in the rate of cellular growth in both media but not the higher degree of inhibition observed in PA production when *E. coli* K92 was grown in ManNAc-Asn medium (Figs. 1 and 2).

Study of the transport system of ManNAc in *E. coli* K92 has revealed that the uptake of this amino sugar occurs by a specific phosphotransferase system where the substrate is phosphorylated as 6-phosphate derivative (Revilla-Nuin, B., Reglero, A., Ferrero, M.A. and Rodríguez-Aparicio, L.B., manuscript in preparation). Therefore, to analyze whether the effect of ManNAc PA production might result from the inhibition of any of these enzymatic activities, we added ManNAc and ManNAc-6-phosphate to the reaction mixtures using phosphatase-free extracts (see Section 2). As shown in Table 2, the presence of these sugars did not elicit any significant effect on either CMP-NeuAc synthetase or sialyltransferase activity. However, in the case of NeuAc lyase activity a 10 mM concentration of ManNAc-6-phosphate (an amount similar to the substrate ManNAc used in control reactions, see Section 2) caused a 20% inhibition (Table 3). This effect was proportional to the ManNAc-6-phosphate concentration, showing a non-competitive model with a K_i of 28 mM (Fig. 3). Moreover, high concentrations of ManNAc-6-phosphate also affected NeuAc lyase activity from other sources such as the enzymes from commercial *E. coli* (from Sigma) and from *E. coli* K1 (another bacterium that produces PA [2]) but not the commercial protein (Sigma) from *Clostridium perfringens* (an enzyme considered to be very different from that present in *E. coli* [14,15,29]).

To relate the specific in vitro effect of ManNAc-6-phosphate on NeuAc lyase activity with the in vivo PA production observed when ManNAc is used as a carbon source by *E. coli* K92, we analyzed the presence of bacterial intracellular ManNAc and its 6-phosphate derivative (see Section 2). When *E. coli* K92 was grown using xylose as the carbon source (Xyl-Asn medium) we did not detect the intracellular presence of either ManNAc or ManNAc-6-phosphate. However, when ManNAc was used as the cellular carbon source (ManNAc-Asn medium), both sugars were found intracellularly and the observed ManNAc-6-phosphate level was three times higher than that of ManNAc (1830 and 516 nmol, respectively). Assuming that the volume of cell water is 2.7 μl/mg dry weight [30], it can be readily calculated that the intracellular concentration of ManNAc-6-phosphate is about 7 mM. This concentration and the presence of a low intracellular amount of ManNAc (equivalent to 2 mM, a value four-fold lower than that observed for ManNAc Km in NeuAc lyase [15]) imply the in vivo possibility of a significant inhibition of NeuAc lyase activity and account for the effect of ManNAc on PA production when it is used as the carbon source. In fact, in vitro analysis of these conditions showed that this enzymatic activity was inhibited up to 70% by the presence 7 mM of ManNAc-6-phosphate when 2 mM ManNAc was used as substrate (a concentration five times lower than that used in standard analysis, see Fig. 3). These results clearly demonstrate that the effect of ManNAc on in vitro PA production is directly related to the inhibition of NeuAc lyase by intracellular ManNAc-6-phosphate. The analysis of the enzyme expression and the study of the ManNAc transport system in *E. coli* K92 should enable us to confirm the importance of this sugar and the key function of NeuAc lyase in the regulation of PA metabolism. Further research in this sense is currently in progress.

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