Transport of *N*-acetyl-D-mannosamine and *N*-acetyl-D-glucosamine in *Escherichia coli* K1: effect on capsular polysialic acid production

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Received 10 August 2001; revised 17 December 2001; accepted 17 December 2001

First published online 4 January 2002

Edited by Hans-Dieter Klenk

Abstract ***N*-Acetyl-D-mannosamine (ManNAc) and *N*-acetyl-D-glucosamine (GlcNAc) are the essential precursors of *N*-acetylneuraminic acid (NeuAc), the specific monomer of polysialic acid (PA), a bacterial pathogenic determinant. *Escherichia coli* K1 uses both amino sugars as carbon sources and uptake takes place through the mannose phosphotransferase system transporter, a phosphoenolpyruvate-dependent phosphotransferase system that shows a broad range of specificity. Glucose, mannose, fructose, and glucosamine strongly inhibited the transport of these amino-acetylated sugars and GlcNAc and ManNAc strongly affected ManNAc and GlcNAc uptake, respectively. The ManNAc and the GlcNAc phosphorylation that occurs during uptake affected NeuAc synthesis in vitro. These findings account for the low in vivo PA production observed when *E. coli* K1 uses ManNAc or GlcNAc as a carbon source for growth.**

Key words: *N*-Acetylmannosamine; *N*-Acetylglucosamine; Phosphotransferase system; Sialic acid

1. Introduction

Amino sugars are major components or precursors of the peptidoglycan and the outer membrane lipopolysaccharide layer as well as of the capsular polysaccharides of Gram-negative bacteria. Thus, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmannosamine (ManNAc) are the essential precursors of *N*-acetylneuraminic acid (NeuAc), the only monomer of bacterial capsular polysialic acid (PA) [1,2]. 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 [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 bacteraemia 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 analysed [10–12] and the PA biosynthesis pathway has been established [5–8]. Regarding the analysis of PA production, we have recently observed that when ManNAc is used for the growth of *E. coli* K92, PA production is strongly affected [12]. Study of the ManNAc transport system in this bacterium revealed for the first time the existence of a specific and sugar-inducible phosphotransferase system (ManNAc PTS) involved in the uptake of this sugar [13]. We now present the results obtained in transport studies of ManNAc and GlcNAc in *E. coli* K1, another strain that produces capsular polysialic acid as a virulence factor involved in its pathogenic capacity. The effect caused by these amino sugars on PA capsular polymer production is also discussed.

2. Materials and methods

2.1. Strain, culture media and growth conditions

E. coli K1 (ATCC 13027) 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 [14]. Incubations were carried out on a rotary shaker (250 rpm) at 37°C for different times using the chemically defined medium xylose/proline (Xyl–Pro), which is ideal for PA production [14]. When necessary, the carbon source was replaced by others, the C concentration being maintained constant.

2.2. PA, xylose and ManNAc determinations

The sialyl polymer produced by *E. coli* K1 was analysed using the Svennerholm [15] methodology, described elsewhere [14]. Residual xylose and ManNAc present in the media were determined as previously described [12,14].

2.3. Cell-free extracts and enzymatic assays

Cell-free extracts of *E. coli* K1 grown up to $A_{540\,nm} = 2.0$ were obtained as previously described [7,10] and the homogenates were centrifuged at $200\,000 \times g$ for 60 min at 4°C. Supernatants were concentrated by ammonium sulphate precipitation (80% saturation), resuspended and applied to a column (2.5 cm × 76 cm) of Sephacryl S-200 equilibrated and eluted with 50 mM Tris–HCl, pH 8.0, containing 25 mM $MgCl_2$ and 1 mM dithiothreitol (TMD buffer) as previously described [16]. Phosphatase-free fractions containing NeuAc lyase or CMP-NeuAc synthetase activities were used to study the effect of GlcNAc-6-phosphate according to reported methodologies [9,16].

Finally, pellets from the original centrifugations (membrane-rich fractions) were washed and resuspended in TMD buffer and used to measure sialyltransferase activity [14]. One unit was defined as the amount of enzyme that synthesised 1 pmol of product ([1-^{14}C]NeuAc

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Abbreviations: ManNAc, *N*-acetyl-D-mannosamine; Xyl, xylose; Pro, proline; PA, polysialic acid; NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetyl-D-glucosamine; PTS, phosphotransferase system

0014-5793/02/$22.00
PII: S0014-5793(01)03318-X

binding to the acceptor) per minute at 35°C under the assay conditions.

Specific activities are expressed as U/mg protein as previously described [12]. Protein was measured by the method of Bradford [17] using bovine serum albumin as standard.

2.4. Amino sugar uptake in whole cells

Cells grown in ManNAc-Pro medium or in the required media were harvested after 19 h incubation ($A_{540\,nm} = 1.0$), washed twice with sterile distilled water, and resuspended in 25 mM sodium–potassium phosphate buffer (pH 7.5 or 6.5 for ManNAc or GlcNAc transport experiments, respectively). Cell concentrations were adjusted to an $A_{540\,nm}$ of 0.5, placed in 25 ml Erlenmeyer flasks and preincubated at 37°C for 5 min in a thermostatically controlled bath at 160 strokes/min. Then, 400 nmol/ml of ManNAc and 10 nmol of [^{14}C]ManNAc (18 mCi/mmol from ICN Pharmaceuticals, USA) or 408 nmol/ml of GlcNAc and of 2 nmol of [^{14}C]GlcNAc (57 mCi/mmol from Amersham International, UK) were added. Aliquots of 1 ml were taken from the uptake mixture after 1 or 5 min (for GlcNAc or ManNAc transport, respectively) of incubation and the radioactivity incorporated was quantified as previously described [10] using Ecoscint A (National Diagnostic, UK) as scintillation fluid. Effectors were tested by adding these 2–3 min before the radiolabelled amino sugar. ManNAc uptakes are given in units (nmol of ManNAc or GlcNAc incorporated/min per ml of cellular suspension at $A_{540\,nm} = 0.5$).

3. Results and discussion

Previous results have shown that when *E. coli* K1 is grown in a chemically defined medium containing D-xylose and L-proline as the only carbon and nitrogen sources (Xyl–Pro medium), PA production reaches maximum values (1350 μg/ml) [14]. Using this medium, we analysed the effect of the replacement of D-xylose and proline by ManNAc medium on cellular growth and PA production. As shown in Fig. 1, when *E. coli* K1 was incubated in ManNAc medium cellular growth was slow and PA production only reached 130 μg/ml. Since no substantial variations in pH were observed during cellular growth (data not shown), the dramatic reduction recorded in PA production (90% with respect to Xyl–Pro medium) suggests that ManNAc affects the polymer metabolism of this bacterium. As in *E. coli* K92 [12,13], differences in the enzymatic activity of some proteins involved in polymer synthesis could explain the in vivo PA depletion observed in this microorganism.

Studies on ManNAc transport have revealed that when *E. coli* is grown using ManNAc, the uptake of this amino sugar occurs through a phosphotransferase mechanism that involves the phosphorylation of the substrate as a 6-phosphate derivative [1,13]. Therefore, when ManNAc is used as a carbon and nitrogen source high intracellular concentrations of this phosphorylated amino sugar can be present. Previous results have demonstrated that high concentrations of ManNAc-6-phosphate inhibit the NeuAc lyase from *E. coli* K1 [12]. The decrease in PA production observed when this bacterium grows using ManNAc (Fig. 1) could be a direct consequence of the inhibition caused by the high levels of phosphorylated ManNAc on polysialic acid synthesis.

Until now, two phosphotransferase systems have been implicated in ManNAc uptake: in *E. coli* K12, a non-PA-producing bacterium, it has been described that this amino sugar is taken up by the Man PTS (ManXYZ-encoded PTS), a transporter with broad substrate specificity [1], whereas in the PA-producing *E. coli* K92 it has been reported that the permease responsible for the transport of this amino sugar is the specific and inducible ManNAc PTS [13].

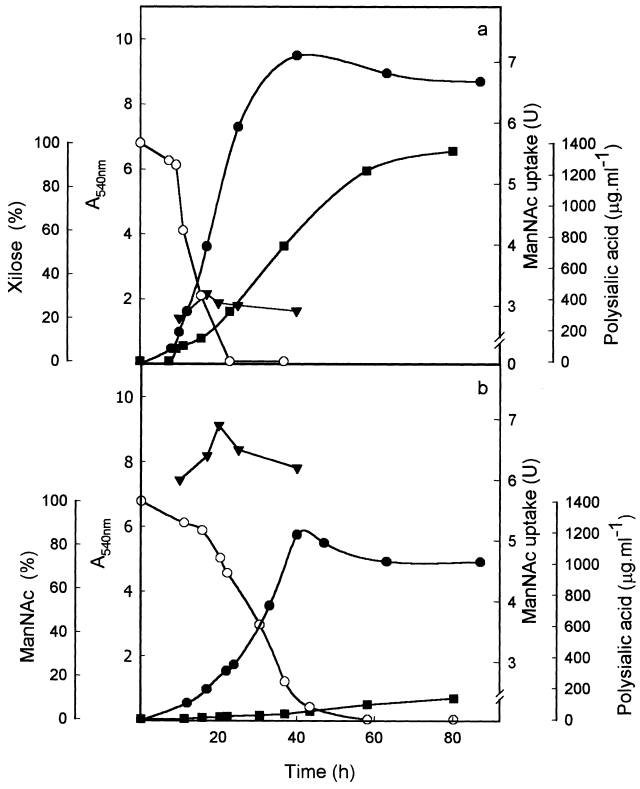

Fig. 1. Growth of *E. coli* K1 (●) and time of appearance of ManNAc transport (▼) when this bacterium was grown at 37°C in Xyl–Pro (a) or ManNAc (b) media. (■) PA production; (○) residual xylose and ManNAc, respectively.

Analysis of ManNAc transport using cells of *E. coli* K1 grown in Xyl–Pro medium (ideal for PA production [14]) revealed a residual capacity of ManNAc uptake (Fig. 1). Nevertheless, a strong increase (230%) in the transport of the amino sugar was observed when the carbon source was ManNAc (ManNAc medium) (Fig. 1), suggesting that *E. coli* K1 has a substrate-inducible system for ManNAc uptake. Study of the characteristics of the ManNAc transporter in this bacterium revealed that maximum uptake was attained at 37°C in 25 mM phosphate Na–K buffer (pH 6.5) and the K_m for ManNAc was 8 mM (Fig. 2). Analysis of the energy requirements revealed that the presence of metabolic inhibitors such as arsenate, cyanide, sodium azide and 2,4-dinitrophenol (5 mM) strongly inhibited the uptake of the amino sugar (80, 80, 90 and 85%, respectively). These results suggest that ManNAc transport in live *E. coli* K1 cells takes place via an active transport system that, as in *E. coli* K92 [13], may be dependent on a high-energy phosphate intermediate. Nevertheless, the low ManNAc uptake affinity observed in *E. coli* K1 (Fig. 2) with respect to *E. coli* K92 [13] (the K_m was 28-fold higher), reflected in its reduced ManNAc transport capacity (it only showed 2.5% of that observed in *E. coli* K92, [13]), indicates that in these bacteria the ManNAc transport system differs significantly.

Study of the specificity of the ManNAc transport system in *E. coli* K1 revealed that addition to the uptake mixture of glucose, fructose, mannose, glucosamine, mannosamine and GlcNAc (5 mM), but not other sugars, caused a marked inhibition in ManNAc transport (70, 60, 70, 60, 65 and 85%, respectively). This wide diversity in the carbohydrates that

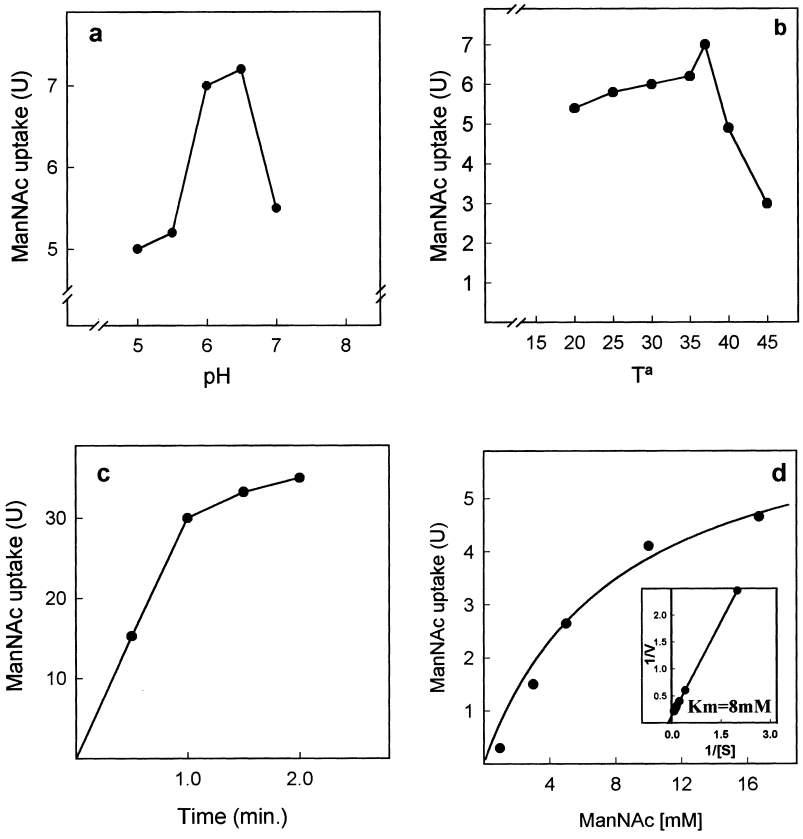

Fig. 2. Effect of pH (a), temperature (°C) (b), incubation time (c) and substrate concentration (d) on the ManNAc transport system.

affect ManNAc transport excludes the specific ManNAc PTS transporter described in *E. coli* K92 [13] as being responsible for the uptake of ManNAc in *E. coli* K1 and suggests that, in this case, the amino sugar is taken up by a system(s) with a broad range of specificity. The fact that these sugar inhibitors can be efficiently taken up by the Man PTS transporter [1,18] supports the possibility that in *E. coli* K1, as has also been described in *E. coli* K12 [1], ManNAc would be taken up by this multispecific system. Nevertheless, the high inhibition caused by GlcNAc does not preclude the possibility that the specific GlcNAc PTS (Nag PTS) [19,20] might also be involved in *E. coli* K1 ManNAc transport.

Studies on the expression of the ManNAc transport system revealed that when *E. coli* K1 was grown using ManNAc, GlcNAc or Man as carbon sources, ManNAc uptake was higher than that observed when the sugar was xylose (Table 1). These results indicate that the ManNAc transport system in this bacterium is inducible. Moreover, if two transport mechanisms are involved in ManNAc transport (the Man PTS and Nag PTS), the higher induction observed when ManNAc was used as the carbon source (Table 1) suggests that in the presence of this amino sugar both systems are induced and that when GlcNAc or Man is the source only the Nag PTS or Man PTS is induced, respectively. Kinetic analysis of ManNAc transport using cells grown in GlcNAc (where the Nag PTS would be induced) or Man (where the system induced would be the Man PTS) revealed that the K_m and V_{max} parameters calculated for the transport of this sugar were similar to those obtained with cells grown in ManNAc (data not shown). These results indicate that the transport system involved in the uptake of ManNAc is the same when *E. coli* K1 uses ManNAc, GlcNAc or Man as carbon source, and suggest that the induced permease also takes up Man and GlcNAc. To establish whether this transporter was the Nag PTS, we studied the effect of different sugars on the transport of GlcNAc and ManNAc using cells grown in these amino sugars. As shown in Table 2, we observed that regardless of the carbon source used for *E. coli* K1 growth, the sugars that are transported by the Man PTS, such as mannose, glucose, fructose and glucosamine, strongly inhibited the uptake of both amino sugar derivatives. Furthermore, the presence of GlcNAc or ManNAc in the uptake reaction strongly affected the transport of ManNAc or GlcNAc, respectively. These results clearly indicate that ManNAc and GlcNAc enter *E. coli* K1 by the same transport system, demonstrating, as has been described in *E. coli* K12 for ManNAc uptake [1], that the multispecific Man PTS is responsible for the uptake: in this

Table 1
Effect of carbon source on ManNAc transport

Culture medium[a]	ManNAc uptake (%)
ManNAc	214
GlcNAc	137
Mannose-proline[b]	170
Xyl–Pro	100

[a]Cells grown in Xyl–Pro medium up to $A_{540\,nm} = 1$ were collected by centrifugation (10 000 × *g*, 10 min), washed twice with 25 mM phosphate buffer, and resuspended in a fresh medium containing similar carbon concentrations of ManNAc, GlcNAc or mannose-proline. Transport was measured after 4 h of incubation at 37°C in the new medium (see Section 2).

[b]Since the bacterium was unable to grow when the carbon source was mannose, the medium using this sugar was supplemented with proline.

Table 2
Effect of different sugars on the uptake of ManNAc and GlcNAc from cells grown using ManNAc or GlcNAc as carbon source

Effector (5 mM)	Culture medium			
	ManNAc		GlcNAc	
	ManNAc transport (%)	GlcNAc transport (%)	ManNAc transport (%)	GlcNAc transport (%)
Control[a]	100	100	100	100
Mannose	28	5	8	12
Glucose	28	3	7	17
Fructose	39	5	21	10
Glucosamine	37	6	27	31
N-Acetylglucosamine	25	–	18	–
N-Acetylmannosamine	–	14	–	55

[a]100% uptake corresponds to transport without effector.

case of both amino sugars. Thus, in *E. coli* K1 the sole presence of the Man PTS transporter leads these acetylamino sugars to become phosphorylated as 6-phosphate derivatives through the same PTS action.

Previous results have demonstrated that ManNAc 6-phosphate is an inhibitor of the ManNAc lyase from *E. coli* K1 [12], an enzyme involved in the synthesis of NeuAc, the essential monomer of capsular polysialic acid [2]. When this bacterium takes up ManNAc, the action of the Man PTS leads to high intracellular concentrations of this 6-phosphoamino sugar being present. In this state, the synthesis of polysialic acid is depleted, accounting for the low capsular polymer production that we observed when *E. coli* K1 used ManNAc as the only carbon and nitrogen source (Fig. 1).

When *E. coli* K1 was grown using GlcNAc, the Man PTS action also led to high intracellular concentrations of GlcNAc-6-phosphate. As shown in Fig. 3, GlcNAc is a good carbon source for *E. coli* K1 and using this carbohydrate the bacterium grows to $A_{540\,nm} = 12$ but only produces up to 400 μg/ml of polysialic acid. Analysis of PA-specific production (SP = μg of PA/mg cell dry weight) showed that in this medium the SP was 42, a value similar to that obtained when ManNAc was used as the carbon source (SP = 38) but significantly lower than that calculated for cells grown in the Xyl–Pro medium (SP = 254) (Fig. 1). These results suggest that like the effect of ManNAc-6-phosphate, when *E. coli* K1 is grown using GlcNAc as the carbon source a high intracellular GlcNAc-6-phosphate level will be present, and that this phosphoamino sugar could also affect the PA biosynthetic

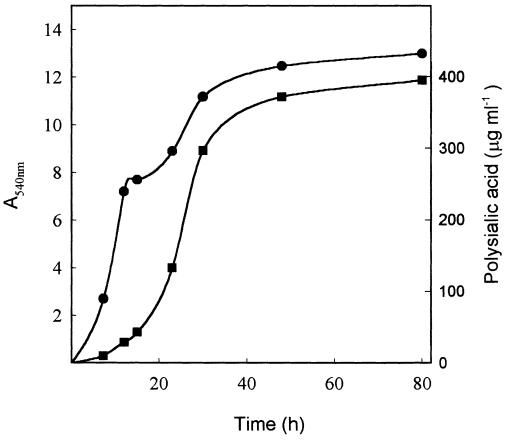

Fig. 3. Growth of *E. coli* K1 (●) and PA production (■) when this bacterium was grown at 37°C in GlcNAc medium.

pathway. In fact, analysis of the possible effect of GlcNAc-6-phosphate revealed that the presence of 10 mM of this sugar phosphoderivative (from Sigma, St. Louis, MO, USA) 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 GlcNAc-6-phosphate (an amount similar to the substrate ManNAc used in control reactions, see Section 2) caused 25% inhibition (from 1250 to 937 units, see Section 2).

These results also explain the low PA production observed when *E. coli* K1 uses GlcNAc as the carbon source and clearly confirm that variations in the concentration of these amino sugar phosphates can specifically regulate PA metabolism.

Finally, our findings suggest that the ManNAc PTS described in *E. coli* K92 and the Nag PTS from *E. coli* K12 involved in the specific transport of ManNAc and GlcNAc respectively [13,19,20] are not present in *E. coli* K1. Molecular analysis of the permease genes should provide key data to confirm this hypothesis and to establish whether in *E. coli* K1 the genes that encode the specific ManNAc and GlcNAc permeases described in *E. coli* K92 and *E. coli* K12, respectively, are present but are not expressed, or whether only the ManXYZ genes are responsible for the transport of *N*-acetylamino sugars. Further research in this direction is currently in progress.

Acknowledgements: We are gratefully indebted to R. Sánchez Barbero for her participation in the elaboration of the manuscript. I.G.B. is the recipient of a postgraduate fellowship of the Fundación Ramón Areces. This work was supported by Grants from the Dirección General de Investigación Científica y Técnica (PB 96-0161) and the Junta de Castilla y León (Ref. LE34/00B and LE35/00B).

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