Uptake of *N*-acetyl-D-mannosamine: an essential intermediate in polysialic acid biosynthesis by *Escherichia coli* K92

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Abstract The *N*-acetyl-D-mannosamine (ManNAc) transport system of *Escherichia coli* K92 was studied when this bacterium was grown in a chemically defined medium containing ManNAc as carbon source. Kinetic measurements were carried out in vivo at 37°C in 25 mM phosphate buffer, pH 7.5. Under these conditions, the uptake rate was linear for at least 15 min and the calculated K_m for ManNAc was 280 μM. The transport system was strongly inhibited by sodium arsenate (97%), potassium cyanide (84%) and 2,4-dinitrophenol (88%) added at final concentrations of 1 mM (each). Analysis of bacterial ManNAc phosphotransferase activity revealed in vitro ManNAc phosphorylation activity only when phosphoenolpyruvate was present. These results strongly support the notion that ManNAc uptake depends on a specific phosphotransferase system. Study of specificities showed that *N*-acetylglucosamine and mannosamine specifically inhibited the transport of ManNAc in this bacterium. Analysis of expression revealed that the ManNAc transport system was induced by ManNAc, glucosamine, galactosamine, mannosamine and mannose but not by *N*-acetylglucosamine or *N*-acetylgalactosamine. Moreover, ManNAc permease was subject to glucose repression and cAMP stimulation. Full induction of the ManNAc transport system required the simultaneous presence of both cAMP and ManNAc.

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

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] that has been shown to be a pathogenic determinant in *Neisseria meningitidis, Escherichia coli, Pasteurella haemolytica, Moraxella nonliquefaciens* and several strains of *Salmonella*. Bacterial PA shows biochemical and epitope similarities with certain eukaryotic cell glycoconjugates, such as the neural cell adhesion molecule (N-CAM) [3]. Regarding the analysis of PA production, we have developed different chemically defined media in which the production of bacterial capsular PA is optimal and in which its biosynthesis is strictly regulated by the cell growth temperature [2,4,5]. In order to develop a system that would permit the regulation of polymer synthesis, we have recently observed that when *N*-acetyl-D-mannosamine (ManNAc), an essential precursor of NeuAc [6–8], is used for the growth of *E. coli*, PA production is strongly affected [9]. Moreover, analysis of bacterial growth in ManNAc revealed the presence of intracellular ManNAc 6-phosphate (MaNAc-6-P) [9]. This observation, together with the fact that the sugar-phosphate derivative is not a substrate for NeuAc synthesis [8,9], suggests that the uptake of ManNAc by *E. coli* K92 occurs via a specific phosphotransferase system [9]. Here, we present for the first time the biochemical characteristics of this transport system, confirming the existence of a specific phosphorylation mechanism involved in the transport of this sugar.

2. Materials and methods

2.1. Strain, culture media and growth conditions

E. coli K92 (ATCC 35860) was maintained on trypticase soy agar (Difco) and grown at 37°C in xylose-asparagine (Xyl-Asn) medium, which is ideal for PA production [4,9]. In experiments addressing the expression of ManNAc transport the carbon source (Xyl) was replaced by other sugars.

2.2. Amino sugar uptake in whole cells

Cells grown in ManNAc-Asn medium or in the required media were harvested after 19 h of incubation ($A_{540\,nm} = 1.0$), washed twice with sterile distilled water, and resuspended in 25 mM sodium-potassium phosphate buffer (pH 7.5). 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 per min. Then, 0.41 μmol/ml of ManNAc (containing 10 nmol [^{14}C]ManNAc: 18 mCi/mmol) or GlcNAc (containing 2 nmol [^{14}C]GlcNAc: 57 mCi/mmol) was added. Aliquots of 1 ml were taken from the uptake mixture after 5 min 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 radiolabeled amino sugar. ManNAc and GlcNAc uptakes are given in units (nmol of ManNAc or GlcNAc incorporated/min per ml of cellular suspension at $A_{540\,nm} = 0.5$).

2.3. Determinations of the ManNAc transport system half-life

Cells were grown in ManNAc-Asn medium and at 19 h protein synthesis was stopped by adding 50 μg of chloramphenicol per ml. From this time up to 70 h ManNAc transport was measured at different times.

2.4. Assay of bacterial ManNAc phosphotransferase activity

E. coli K92 was grown in ManNAc-Asn medium up to $A_{540\,nm} = 1.0$. The membrane extract precipitate (enzyme II-rich fraction [11]), the ultracentrifugation supernatant (enzyme I- and HPr-rich fraction [11]) and ManNAc phosphotransferase activity were obtained and assayed using the methodology described by Curtis and Epstein for the characterization of the glucose phosphotransferase and mannose phosphotransferase from *E. coli* [11]. Protein was measured by the method of Bradford [12] using BSA as standard.

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Abbreviations: ManNAc, *N*-acetyl-D-mannosamine; ManNAc-6-P, *N*-acetyl-D-mannosamine 6-phosphate; PEP, phosphoenolpyruvate; PA, polysialic acid; NeuAc, *N*-acetylneuraminic acid; GlcNAc, *N*-acetyl-D-glucosamine; Asn, asparagine; Glc, glucose; Xyl, xylose; N-CAM, neural cell adhesion molecule; GalNAc, *N*-acetyl-D-galactosamine; cAMP, cyclic AMP; PTS, phosphotransferase system; HPr, heat-stable protein of PTS; BSA, bovine serum albumin

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2.5. Induction experiments on the ManNAc and GlcNAc transport system

E. coli K92 was grown in Xyl-Asn medium up to $A_{540\,nm} = 1.0$. Bacteria were collected by centrifugation (10 000 × *g* 10 min), washed twice with 25 mM phosphate buffer and resuspended in a fresh medium containing Asn and one of the following sugars as carbon source: glucose, glycerol, xylose, galactose, mannose, glucosamine, *N*-acetylglucosamine or *N*-acetylgalactosamine. After cellular transfer, transport analysis was monitored at intervals. All procedures were carried out under sterile conditions. The bacterium was unable to grow when the carbon source in the medium was galactosamine or mannosamine.

3. Results and discussion

3.1. Time course of the appearance of the ManNAc uptake system

Time course analysis using cells grown in Xyl-Asn medium (ideal for PA production) revealed a very low level of ManNAc uptake activity along growth (Fig. 1). However, when the carbon source was ManNAc (ManNAc-Asn medium), a strong increase in ManNAc transport was observed during the first hours of growth, this reaching maximal levels at 19 h ($A_{540\,nm} = 1.0$). Thereafter, uptake decreased continuously (Fig. 1). These results indicate that maximal uptake activity takes place during the early exponential phase of cellular growth and suggest that ManNAc transport is a specific and substrate-inducible system.

3.2. Characteristics of ManNAc transport

Analysis of the optimal physicochemical parameters of the *E. coli* K92 ManNAc transport system showed that maximal uptake was attained at 37°C in 25 mM phosphate buffer (pH 7.5) and that ManNAc incorporation was linear for at least 15 min (Fig. 2). Under these conditions, and for 5 min, initial uptake rates were measured at different ManNAc concentrations; the K_m for ManNAc was 280 μM (Fig. 2d). The half-life of permease was 16 h (data not shown), suggesting that no substantial changes had occurred in the ManNAc transport system during this period and that the protein(s) involved in this uptake had a turnover rate similar to those of other related transport systems, such as NeuAc uptake by *E. coli* K1 [10].

Analysis of the energy requirements of the *E. coli* K92 ManNAc transport system revealed that the presence of metabolic inhibitors [13–15] such as arsenate, cyanide, and 2,4-dinitrophenol (1 mM) strongly inhibited the amino sugar uptake (97, 84 and 88%, respectively). These results suggest that ManNAc uptake in live *E. coli* K92 cells takes place via an active transport system that is dependent on an energized membrane or on a high-energy phosphate intermediate [16,17].

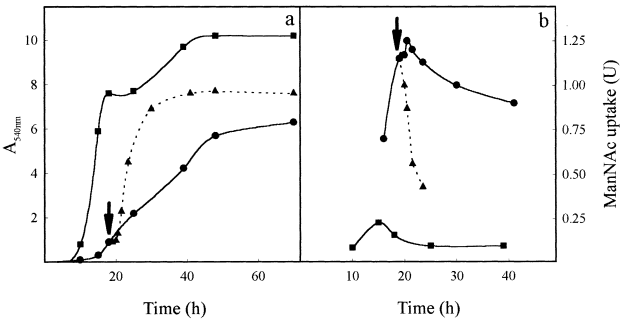

Fig. 1. Growth of *E. coli* K92 (a) and time course of appearance of ManNAc transport (b) when this bacterium was grown at 37°C in Xyl-Asn (■) or ManNAc-Asn (●) media. Dotted lines indicate cellular growth (a) and ManNAc transport (b) when cells grown in ManNAc-Asn medium up to $A_{540\,nm} = 1.0$ were collected and transferred to fresh Glc-Asn medium. Arrows indicate the time of transfer.

To study whether a phosphotransferase system (PTS) might be involved in ManNAc transport in *E. coli* K92, we explored the effect of each of the protein components of the phosphotransferase system on ManNAc phosphorylation. As shown in Table 1, when ManNAc phosphorylation activity was assayed using soluble bacterial extracts (fractions rich in enzyme I and HPr but not in the specific enzyme II protein; see Section 2), a very low level of ManNAc phosphorylation was observed in both the presence and absence of phosphoenolpyruvate (PEP). Similar results were obtained when the bacterial extracts used were from the membrane fraction (rich in enzyme II). Only when the activity analysis was carried out in the presence of both protein fractions containing PEP was a significant degree of ManNAc phosphorylation detected (Table 1). These results clearly demonstrate that ManNAc phosphorylation in *E. coli* K92 is a PEP-dependent mechanism. The facts that both protein extracts (soluble and particulate membranous fractions) are necessary for ManNAc-6-P formation and that high intracellular levels of this amino sugar phosphate are present when the cells were grown in ManNAc-Asn medium [9] justify the existence of a specific phosphotransferase system directly involved in the transport of this molecule.

The specificity of the ManNAc transport system was explored by adding several different sugars and sugar derivatives to the uptake mixture. Analysis of the specificity of the ManNAc transport system showed that monosaccharides (glucose, fructose, mannose, galactose, xylose, arabinose, ribose and sorbose), disaccharides (lactose, melibiose, maltose or sucrose) and sugar derivatives (galactitol, mannitol and sorbitol), or the *N*-acetyl derivatives *N*-acetylgalactosamine, *N*-acetylneuraminic acid and *N*-acetylglucosaminitol or the hexosamines

Table 1
ManNAc phosphorylation activity of extract fractions from *E. coli* K92

Phosphorylation reaction mixture	ManNAc phosphorylated (pmol)[b]
SC[a]+0.25 μg soluble extract (enzyme I- and HPr-rich fraction)	0.22 ± 0.08
SC+0.25 μg membrane extract (enzyme II-rich fraction)	0.35 ± 0.13
SC+0.25 μg soluble extract+0.25 μg membrane extract	0.92 ± 0.18
SC+0.25 μg soluble extract+PEP (12.5 mM)	2.3 ± 0.43
SC+0.25 μg membrane extract+PEP (12.5 mM)	3.5 ± 0.83
SC+0.25 μg soluble extract+0.25 μg membrane extract+PEP (12.5 mM)	98.0 ± 4.8

[a]SC = standard cocktail contained 10 mM KF, 0.05 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6) and 0.41 mM ManNAc (containing 10 μM [^{14}C] ManNAc) as described [11].
[b]Activity is expressed as pmol of phosphorylated ManNAc generated after 30 min of incubation at 37°C. Values are given as means ± S.E.M. ($n = 4$).

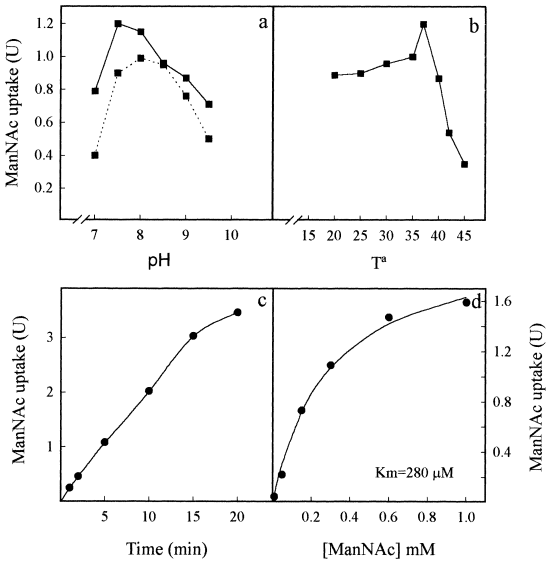

Fig. 2. Effect of pH (a), temperature (b), incubation time (c) and substrate concentration (d) on the ManNAc transport system. Continuous and dotted lines indicate the use of 25 mM phosphate and Tris-HCl buffers, respectively.

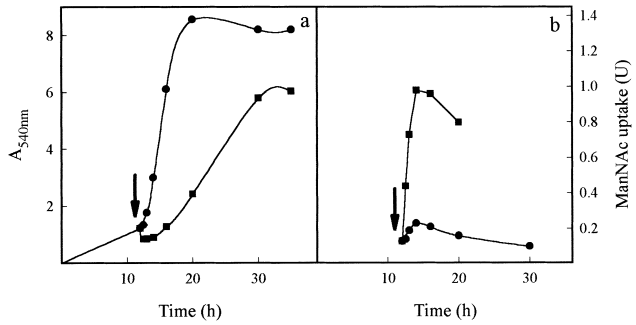

Fig. 3. Effect of ManNAc as carbon source on the cellular growth (a) and ManNAc transport rate (b). Cells were grown in Xyl-Asn medium up to $A_{540\,nm} = 1.0$ and were then collected and transferred to fresh ManNAc-Asn (■) or Xyl-Asn (●). Arrows indicate the time of transfer.

glucosamine and galactosamine did not affect ManNAc transport, even at concentrations of 5 mM. However, the addition of *N*-acetylglucosamine (GlcNAc) or mannosamine (50 μM) to the uptake mixture caused a marked inhibition in ManNAc transport (90 and 50%, respectively), suggesting that ManNAc, GlcNAc and mannosamine can be taken up by a common carrier. ManNAc is a molecule that occurs rarely as a sugar intermediate in carbohydrate metabolism and only a few bacteria, such as *E. coli* K92, use ManNAc to synthesize their capsular polysaccharides [1,4,7,18]. In this context, it would be expected that the transport of ManNAc would occur through an inducible GlcNAc PTS. Kinetic analysis of the inhibition caused by mannosamine and GlcNAc revealed that the effect of mannosamine corresponds to a competitive model with a $K_i = 21$ μM, while inhibition due to GlcNAc corresponds to a non-competitive model ($K_i = 65$ μM). The non-competitive inhibition caused by GlcNAc on the transport of ManNAc, together with the absence of inhibition by ManNAc when this amino sugar was tested as an effector on the *E. coli* K92 GlcNAc transport system, even when high concentrations (5 mM) were used (data not shown), precludes a common system for the transport of both *N*-acetylamino sugars. Moreover, the low K_i value observed when GlcNAc was used as effector suggests that ManNAc transport activity is regulated by minimal variations in the GlcNAc concentration and indicates that GlcNAc is preferentially used as carbon source for this bacterium.

3.3. Expression of the ManNAc transport system

Analysis of ManNAc uptake using cells grown in Xyl-Asn and ManNAc-Asn media (Fig. 1) suggests that the process occurs via an inducible system. To confirm this hypothesis, we studied ManNAc uptake when cells grown in Xyl-Asn medium were transferred to a fresh ManNAc-Asn medium. As shown in Fig. 3, following the transfer we observed a rapid increase in ManNAc transport, which reached levels similar to those of cells grown in ManNAc-Asn medium after 3 h incubation. These results indicate that the ManNAc transport system in *E. coli* K92 is induced by the action of ManNAc. Similar experiments revealed that when GlcNAc and GalNAc were used as carbon sources, ManNAc transport was not induced. However, when glucosamine, mannosamine, galactosamine and mannose were employed, ManNAc transport increased, although to a lesser extent than ManNAc cell growth (Table 2). In these cells, ManNAc uptake followed a similar kind of behavior to that observed for the bacterial transport induced by the presence of ManNAc. In all cases, uptake was inhibited by GlcNAc and mannosamine and was affected by the presence of cyanide and arsenate (data not

Table 2
Uptake of ManNAc and GlcNAc in *E. coli* K92 when grown in different carbon sources

Culture medium	ManNAc uptake (pmol/min)[a]	GlcNAc uptake (pmol/min)[a]
Xylose-asparagine	190 ± 12	610 ± 40
Glucose-asparagine	250 ± 19	770 ± 50
Glycerol-asparagine	300 ± 17	1080 ± 80
Mannose-asparagine	580 ± 27	1250 ± 90
Galactose-asparagine	670 ± 41	1700 ± 110
Glucosamine-asparagine	960 ± 54	2560 ± 210
Mannosamine-asparagine	730 ± 62	1330 ± 100
Galactosamine-asparagine	420 ± 33	1010 ± 90
N-Acetylglucosamine-asparagine	270 ± 17	3750 ± 280
N-Acetylmannosamine-asparagine	125 ± 10	4570 ± 310
N-Acetylgalactosamine-asparagine	210 ± 13	2890 ± 210

Cells were grown in Xyl-Asn medium up to $A_{540\,nm} = 1.0$ and at this time were transferred to a new medium containing Asn and one of the following sugars as carbon source: glucose, xylose, glycerol, mannose, galactose, glucosamine, mannosamine, galactosamine, GlcNAc, ManNAc or GalNAc (see Section 2). Transport was measured after 3 h of growth.
[a]Values are given as means ± S.E.M. ($n = 4$).

shown). These results suggest that a common metabolic intermediate of these sugars and ManNAc could be the true permease inducer. However, although mannosamine and glucosamine are good inducers of ManNAc transport (Table 2), they are not used by *E. coli* K92 as a carbon source (see Section 2). This, together with the lack of ManNAc uptake induction capacity (Table 2) shown by GlcNAc and GalNAc, suggests that glucosamine, mannosamine, galactosamine and mannose are gratuitous inducers of this system and that ManNAc transport is specifically induced by ManNAc or an intimately related intermediate, such as ManNAc-6-P. Regarding this, previous results have shown that in *E. coli* K92, ManNAc-6-P is not a substrate for NeuAc formation and that at high concentrations NeuAc synthesis is blocked [9]. Thus, when this bacterium is grown in ManNAc, the metabolism of the ManNAc-6-P formed involves the necessary epimerization to GlcNAc-6-P through the action of GlcNAc-6-P 2′-epimerase [19], suggesting the presence of high intracellular levels of both amino sugar phosphates. However, when GlcNAc is taken up, the GlcNAc-6-P formed is directly incorporated into the cell wall and outer membrane and/or is directly metabolized by the action of GlcNAc-6-P deacylase. Accordingly, a low intracellular level of ManNAc-6-P would be expected. The facts that the ManNAc transport system was not induced by GlcNAc and that ManNAc was also an inducer of the GlcNAc PTS (Table 2) support this possibility and suggest that, like the GlcNAc PTS [20], ManNAc-6-P is the true inducer of the ManNAc transport system.

Study of the expression of the ManNAc transport system also showed that when the bacteria were grown using xylose or glucose as carbon source (Xyl-Asn and Glc-Asn media, respectively), the level of ManNAc uptake was minimal and lower than that obtained using cells grown in glycerol (Table 2). Moreover, when cells grown in ManNAc-Asn medium were transferred to a new medium containing glucose as carbon source (Glc-Asn medium), ManNAc uptake was rapidly and strongly decreased (Fig. 1). These results indicate that the expression of the ManNAc transport system of *E. coli* K92 is also subject to glucose repression and suggest that the effect of glucose could be a consequence of modifications in intracellular cAMP levels [21,22].

To confirm this possibility, we analyzed the effect of cAMP on the expression of the ManNAc transport system. The presence of cAMP in the growth culture caused a significant increase in the ManNAc transport rate of cells grown in both Glc-Asn and ManNAc-Asn media (Table 3). Since exogenously added cAMP would increase internal cAMP concentrations, these results suggest that a functional cAMP-CAP complex would stimulate the expression of the ManNAc transport system. Together with the inductive effect of ManNAc, this indicates that full expression of the ManNAc transport system in *E. coli* K92 requires the simultaneous presence of both cAMP and ManNAc. In this way, the ManNAc phosphotransferase system that we have demonstrated and characterized in *E. coli* K92 must be specifically induced by the substrate and the activity of the cAMP-CAP complex would be able to modulate the relative levels of expression. Molecular analysis of the permease gene should provide key data to confirm this hypothesis and to establish whether the ManNAc transport system is part of a regulon that includes other gene(s) encoding the enzyme(s) specifically involved in ManNAc metabolism. In this sense, we have observed that the expression of the GlcNAc-6-P 2′-epimerase gene of *E. coli* K92 is also increased by the presence of ManNAc (unpublished results). Knowledge of the exact mechanism(s) responsible for regulating the metabolism of ManNAc is essential for the development of therapeutic approaches for the control of NeuAc synthesis, and hence PA production, in these pathogenic bacteria. Further research on this topic is currently in progress.

Table 3
Effect of cyclic AMP on ManNAc transport rate

Medium	Uptake (%)
Glucose-Asn	16
Glucose-Asn+cAMP	30
ManNAc-Asn	100
ManNAc-Asn+cAMP	128

Cells of *E. coli* K92 were grown in Glc-Asn medium up to $A_{540\ nm} = 1.0$ and at this time were transferred to a new Glc-Asn or ManNAc-Asn medium in the presence or absence of cAMP (10 mM). Transport was measured after 3 h of growth.

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