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# Purification and characterization of GlcNAc-6-P 2-epimerase from *Escherichia coli* K92

Miguel Ángel Ferrero✉, Honorina Martínez-Blanco, Federico Felino Lopez-Velasco, Carlos Ezquerro-Sáenz, Nicolas Navasa, Sofia Lozano and Leandro B. Rodríguez-Aparicio✉

*Departamento de Biología Molecular, Universidad de León, Campus de Vegazana, León, Spain*

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**_N_-Acetylmannosamine (ManNAc) is the first committed intermediate in sialic acid metabolism. Thus, the mechanisms that control intracellular ManNAc levels are important regulators of sialic acid production. In prokaryotic organisms, UDP-_N_-acetylglucosamine (GlcNAc) 2-epimerase and GlcNAc-6-P 2-epimerase are two enzymes capable of generating ManNAc from UDP-GlcNAc and GlcNAc-6-P, respectively. We have purified for the first time native GlcNAc-6-P 2-epimerase from bacterial source to apparent homogeneity (1200 fold) using Butyl-agarose, DEAE-FPLC and Mannose-6-P-agarose chromatography. By SDS/PAGE the pure enzyme showed a molecular mass of 38.4±0.2 kDa. The maximum activity was achieved at pH 7.8 and 37ºC. Under these conditions, the $K_m$ calculated for GlcNAc-6-P was 1.5 mM. The 2-epimerase activity was activated by $Na^+$ and inhibited by mannose-6-P but not mannose-1-P. Genetic analysis revealed high homology with bacterial isomerases. GlcNAc-6-P 2-epimerase from *E. coli* K92 is a ManNAc-inducible protein and is detected from the early logarithmic phase of growth. Our results indicate that, unlike UDP-GlcNAc 2-epimerase, which promotes the biosynthesis of sialic acid, GlcNAc-6-P 2-epimerase plays a catabolic role. When *E. coli* grows using ManNAc as a carbon source, this enzyme converts the intracellular ManNAc-6-P generated into GlcNAc-6-P, diverting the metabolic flux of ManNAc to GlcNAc.**

**Keywords**: *N*-acetylglucosamine, sialic acid, capsular polysialic acid, 2-epimerase

## INTRODUCTION

*N*-Acetylneuraminic acid (NeuAc) is an acidic sugar present in prokaryotic (capsular polysaccharides and lipopolysaccharides) and eukaryotic (glycoproteins and glycolipids) organisms and plays an essential role in many biological recognition processes (Reuter *et al*., 1982; Rodriguez-Aparicio *et al*., 1992; Rutishauser, 1993; Revilla-Nuin *et al*., 1998a; Maru *et al*., 2002; Bork *et al*., 2005; Tanner, 2005).

The biosynthetic pathway of NeuAc has been extensively studied and in both eukaryotic and prokaryotic cells *N*-acetylmannosamine (ManNAc) is the essential sugar precursor (Corfiel & Schauer, 1982; Van Rinsum *et al*., 1983; Rodriguez-Aparicio *et al*., 1992; Vann *et al*., 1993; Maru *et al*., 2002; Chou *et al*., 2003; Tanner, 2005; Viswanathan *et al*., 2005). In this biosynthetic process, at least three different amino sugar 2'-epimerases have been implicated in ManNAc metabolism: UDP-*N*-acetylglucosamine (GlcNAc) 2-epimerase (EC 5.1.3.14); GlcNAc 2-epimerase (EC 5.1.3.8), and GlcNAc 6-phosphate 2-epimerase (EC 5.1.3.9). In eukaryotic cells, UDP-GlcNAc 2-epimerase and GlcNAc 2-epimerase have been implicated in the synthesis and catabolism of sialic acid, respectively (Hinderlich *et al*., 1997; Stasche *et al*., 1997; Luchansky *et al*., 2003). In both cases, the enzymes have been extensively studied and their biological significance established (Maru *et al*., 1996; Hinderlich *et al*., 1998; Itoh *et al*., 2000;

✉**Corresponding authors**: Leandro B. Rodríguez-Aparicio and Miguel Ángel Ferrero, Departamento de Biología Molecular, Universidad de León, Campus de Vegazana, 24007 León, Spain; tel.: (34 98) 729 1212 1227; fax: (34 98) 729 1226; e-mail: leandro.redriguez@unileon.es; ma.ferrero@unileon.es

**Abbreviations**: Asn, asparagine; GlcNAc, *N*-acetyl-D-glucosamine; GlcNAc-6-P, *N*-acetyl-D-glucosamine 6-phosphate; ManNAc, *N*-acetyl-D-mannosamine; NeuAc, *N*-acetylneuraminic acid; PA, polysialic acid; PTS, phosphotransferase transport system; Xyl, xylose.

Blume *et al.*, 2004; Noguchi *et al.*, 2004). Prokaryotic organisms lack GlcNAc 2-epimerase activity (Plumbridge & Vimr, 1999) and UDP-GlcNAc 2-epimerase and GlcNAc-6-phosphate 2-epimerase have been related to the synthesis and catabolism of sialic acid, respectively (Plumbridge & Vimr, 1999; Walters *et al.*, 1999; Ringenberg *et al.*, 2003; Murkin *et al.*, 2004; Vann *et al.*, 2004). Bacterial UDP-GlcNAc 2-epimerase is a protein encoded by the *neuC* gene, which catalyzes the conversion through hydrolysis of UDP-GlcNAc into ManNAc and UDP in the first step of the both sialic acid biosynthesis pathways (Ringenberg *et al.*, 2003; Murkin *et al.*, 2004; Vann *et al.*, 2004). GlcNAc-6-P 2-epimerase catalyzes the isomerization of GlcNAc-6-P to ManNAc-6-P (Grosh & Roseman, 1965; Rodríguez-Aparicio *et al.*, 1999). In this case, the activity of the enzyme has been specifically detected in different bacterial sources (Grosh & Roseman, 1965; Rodríguez-Aparicio *et al.*, 1999) and *siaA* from *Neisseria meningitides* B and *nanE* from *Escherichia coli* have been proposed as the putative encoding genes (Plumbridge & Vimr, 1999; Petersen *et al.*, 2000; Kalivoda *et al.*, 2003). Here we describe for the first time the purification and characterization of the GlcNAc-6-P 2-epimerase present in *E. coli* K92. Its physiological role in the regulation of the intracellular pool of free sialic acid and the modulation of capsular polysialic acid biosynthesis are also discussed.

## MATERIALS AND METHODS

**Chemicals.** *N*-acetyl-D-neuraminic acid (NeuAc), *N*-acetyl-D-mannosamine (ManNAc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-glucosamine-6-phosphate, mannose-1-phosphate, mannose-6-phosphate, pyruvate, D-xylose (Xyl), L-asparagine (Asn), bovine serum albumin (BSA), 2-thiobarbituric acid, periodic acid, streptomycin sulphate, alkaline phosphatase type III from *Escherichia coli*, NeuAc lyase from *Clostridium perfringens,* Butyl-agarose and Mannose-6-phosphate-agarose were purchased from Sigma Chemical Co. Sephacryl S-200, Sephadex G-25 (PD-10) and [1-$^{14}$C]-pyruvic acid (28 mCi/mmol) were from GE-Healthcare. Other reagents used were of analytical quality.

**Microorganisms, plasmids and growth conditions.** Bacterial strains were maintained on Trypticase Soy Agar (Difco) and slants grown for 8–12 h at 37ºC were used for seeding liquid media. *E. coli* K92 (ATCC 35860) was grown in Xyl-Asn or ManNAc-Asn liquid media, as previously described (González-Clemente *et al.*, 1990; Revilla-Nuín *et al.*, 1998b). *E. coli* BL21 (DE3) and DH5$\alpha$ were grown in Luria–Bertani (LB) (Sambrook *et al.*, 1989) medium at 37°C. Recombinant *E. coli* BL21 (DE3) and DH5$\alpha$ cells were cultured in the same medium supplemented with kanamycin (25 to 50 μg/ml). The plasmid used in this work was pET-42b(+) (Novagen). Cellular incubations were carried out on a rotary shaker at 37ºC for the time required.

**GlcNAc-6-P 2-epimerase assays.** GlcNAc-6-P 2-epimerase activity was evaluated using an assay coupled to NeuAc synthesis, as previously described (Rodríguez-Aparicio *et al.*, 1999). Briefly, the incubation mixture contained the following in a final volume of 50 μl: 0.125 M Tris/HCl pH 7.5, 5 mM GlcNAc-6-phosphate; 10 mM $MgSO_4$, 10 mM NaCl and protein extract. After incubation at 37ºC for 30 min, the reaction was stopped by cooling on ice. The enzymatically generated ManNAc-6-phosphate was dephosphorylated to ManNAc and coupled to NeuAc synthesis by simultaneous addition of 1 U of alkaline phosphatase, 8.75 mM of pyruvate and 0.05 U of NeuAc lyase from *C. perfringens* (Sigma). The final volume of this reaction was 60 μl and after incubation at 37ºC for 30 min (or the required time) the NeuAc synthesized was quantified colorimetrically according to the 2-thiobarbituric acid methodology (Warren, 1959) described elsewhere (Rodríguez-Aparicio *et al.*, 1999).

GlcNAc-6-phosphate 2-epimerase activity was also evaluated by a radiometric assay using commercial radioactive pyruvate (1 μCi). In this case, the NeuAc generated was chromatographed and evaluated by scintillation counting (Rodríguez-Aparicio *et al.*, 1987). In all cases, reaction mixtures without GlcNAc-6-P or with boiled protein extracts (95ºC for 10 min) were used as controls.

One unit of each enzyme was defined as the amount of 2-epimerase that synthesizes 1 nmol of ManNAc-6-phosphate/min at 37ºC under each assay condition. Specific activity (SA) was expressed as units/milligram of protein.

For enzymatic quantification, in all cases control 2-epimerase reaction assays containing heat-denatured protein extracts were used as blanks. In the colorimetric reactions, we also used reaction mixtures without substrate (GlcNAc-6-P or pyruvate) as controls.

**6-P-Gluconolactonase assay.** 6-P-Gluconolactonase activity was evaluated using the Kupor and Fraenkel assay (1969) as described by Thomason *et al.* (2004).

Proteins were measured by the method of Bradford (1976), using BSA as standard.

**Polysialic acid determinations.** The sialyl-polymer produced by *E. coli* K92 was analyzed by the Svennerholm methodology (1958) described elsewhere (Rodríguez-Aparicio *et al.*, 1988).

**Purification of GlcNAc-6-P 2-epimerase.** *E. coli* was grown in the ManNAc-Asn-defined chemical medium up to $OD_{540}$ = 2.5. Bacteria were

collected by centrifugation (10000×*g*, 10 min at 2°C), washed twice with saline solution, and resuspended in 50 mM Tris/HCl buffer, pH 7.6. Cells (20 g wet mass/100 ml of buffer) were subjected to four rounds of sonication (30 s of sonication followed by 30 s on ice in a model B12 Branson:Sonifier) and the homogenate was centrifuged at 17000×*g*, 10 min at 2°C. The pellet was discarded and the supernatant fluid was treated with 0.75% streptomycin sulphate to remove the nucleic acids. After centrifugation (17000×*g*, 10 min at 2°C), the supernatant was treated with ammonium sulphate. The protein fraction precipitating at 45% (containing 96% of GlcNAc-6-P 2-epimerase activity) was collected by centrifugation (17000×*g*, 15 min at 2°C). The pellet was then dissloved in 50 mM Tris/HCl (pH 7.5), and passed through a Sephadex G-25 (PD-10) column to eliminate the excess of ammonium sulphate. Ammonium sulphate was added to the desalted extract to 35% saturation and applied to a column (10×2 cm) of Butyl-agarose (from Sigma) equilibrated with the same buffer containing ammonium sulphate (25% satn.). The proteins were eluted with 30 ml of a linear gradient of ammonium sulphate (25–0% satn.) in 50 mM Tris/HCl buffer, pH 7.5. Fractions containing maximal enzyme activity were pooled, desalted by passing through a PD-10 column, and subjected to DEAE-FPLC chromatography. Two protein pool fractions from the Butyl-agarose chromatography were introduced into an FPLC system (Waters-Millipore) equipped with an anion exchange column (Waters Protein Pack DEAE 8HR 1000 A 8 μm: 10 x 100 mm) equilibrated with 50 mM Tris/HCl buffer, pH 7.5. After a wash with the same buffer, the proteins were eluted with a 0 to 0.18 M NaCl linear gradient and fractions (1 ml) were collected and assayed for GlcNAc-6-P 2-epimerase activity. Fractions containing maximal enzyme activity were equilibrated against Tris/HCl buffer 10 mM, pH 8.0, desalted (PD-10 column chromatography) and introduced on a mannose 6-phosphate-agarose column (10× 5 mm) equilibrated with 10 mM Tris/HCl buffer, pH 8.0. After a wash with 5 ml of the same buffer, GlcNAc-6-P 2-epimerase was eluted with 2 ml of 25 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl. Fractions of 0.5 ml that contained the maximum enzyme activity were tested in a 12% SDS/PAGE system.

Using this procedure the GlcNAc-6-P 2-epimerase of *E. coli* was purified 1200 fold (see Table 1).

**Electrophoretic analyses.** SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (Laemmli, 1970) was performed in 12% slab gels under reducing conditions. Phosphorylase *b* ($M_r$ 94000), bovine serum albumin ($M_r$ 67000), ovalbumin ($M_r$ 43000), carbonic anhydrase ($M_r$ 30000), soybean trypsin inhibitor ($M_r$ 20100) and α-lactalbumin ($M_r$ 14000) were used as relative molecular mass standards.

Agarose gels were between 0.8% and 1.0% according to the procedure of Sambrook *et al.* (1989). The DNA markers used to estimate the size of unknown DNA were 1kb DNA Plus Ladder (Invitrogen) and Lambda DNA/*Hin*d III (Promega).

**Amino acid sequencing.** The purified enzyme was desalted by HPLC on a Vydac reverse phase 5-μm C-4 column (type 214TP54, 0.46×25 cm) using a 30 min linear gradient (1 ml/min) from 20 to 95% acetonitrile (0.1% trifluoroacetic acid present). The protein was dissolved in 30 μl of 0.15% trifluoroacetic acid and loaded onto the sequencer. Amino acid sequencing was carried out as described elsewhere (Hewick *et al.*, 1981).

**Tryptic digestion and mass spectrometry analyses of protein.** A gel piece from SDS/PAGE containing purified GlcNAc-6-P 2-epimerase was carefully excised and washed twice in water. Protein was reduced in-gel, alkylated and digested with trypsin according to Shevchenko *et al.* (2000). Briefly, the gel piece was washed twice with water, shrunk for 15 min with 100% acetonitrile and dried in a Savant SpeedVac for 30 min. Then, the sample was reduced with 10 mM dithioerythritol in 25 mM ammonium bicarbonate for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, the samples were digested with 12.5 ng/μl sequencing grade trypsin (from Roche Molecular Biochemicals) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C. After digestion, the supernatant was collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml α-cyano-4-hydroxy-transcinnamic acid matrix (from Sigma) in 50% acetonitrile was added to the dried peptide digest and allowed to air-dry again at room temperature. MALDI-TOF MS analyses were performed on a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) at the Genomics and Proteomics Centre, Complutense University of Madrid (Spain), operated in positive reflector mode, with an accelerating voltage of 20000 V. All mass spectra were calibrated internally using peptides from the autodigestion of trypsin. Analysis by MALDI-TOF/TOF mass spectrometry affords peptide mass fingerprints and the peptides observed can be collated and represented as a list of monoisotopic molecular masses. For protein identification, the non-redundant NCBI database was searched using MASCOT 1.9 (matrixscience.com) through the Global Protein Server v3.5 from Applied Biosystems. The search parameters were: carbamidomethyl cysteine as the fixed modification and oxidized methionine as the variable modification, a peptide mass tolerance 100 p.p.m. and 1 missed trypsin cleavage site.

**Table 1. Purification of GlcNAc-6-P 2-epimerase from *E. coli* K92**

| Treatment | Volume (ml) | Protein (mg) | Enzyme* activity (units) | Specific activity (units/mg) | Yield of recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Crude extract | 100 | 128 | 3.20 | 0.025 | 100 | 1 |
| Streptomycin sulphate (0.75%) | 98 | 112 | 3.02 | 0.026 | 94 | 1 |
| Ammonium sulphate precipitation (45%) | 9 | 22 | 2.09 | 0.130 | 90 | 5 |
| Butyl-agarose | 10 (×2)[a] | 3 (×2)[a] | 1.80 (×2)[a] | 0.60 | 56 | 24 |
| DEAE-FPLC | 4 | 0.16 | 1.2 | 7.50 | 19 | 300 |
| Mannose-6-P-agarose | 2 | 0.02 | 0.6 | 30.00 | 10 | 1200 |

*One unit is the amount of enzyme which synthesizes 1 nmol of ManNAc-6-P per 30 min at 37ºC. [a]For DEAE-FPLC chromatography two protein pool fractions from Butyl-agarose were used (see Material and Methods section).

In the protein identification, the probability scores were greater than the score fixed as significant, with a p-value of less than 0.05.

**PCR analyses and DNA sequencing.** The GlcNAc-6-P 2-epimerase gene was amplified from chromosomal DNA of *E. coli* K92. Amplification reactions were carried out in a Perkins Elmer DNA Thermal Cycler 2400 with primers engineered to include desired restriction sites for cloning into pET-42b(+) expression vector (Novagen). The primers used were GTCAGTTGCGAATTCCAAAGGAGC (forward) and GGAGAGACTGCAGCGGGTAAATCAG (reverse), which contained an *Eco*RI and a *Pst*I site, respectively (underlined). PCR reactions were performed under standard conditions.

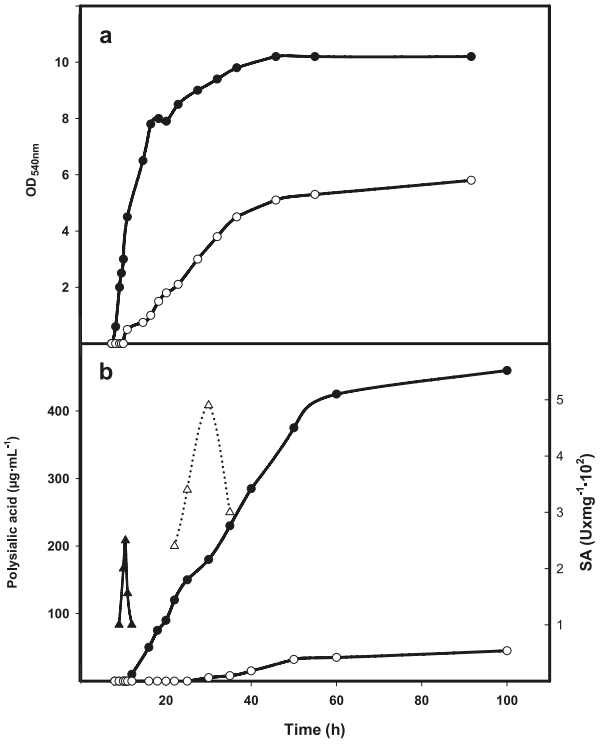


**Figure 1. Time course of the appearance of GlcNAc-6-P 2-epimerase.**
a) Growth of *E. coli* K92 (●, ○); b) Polysialic acid production (●, ○) and time-course of formation of GlcNAc-6-P 2-epimerase (▲, Δ) when this bacterium was grown at 37ºC in Xyl-Asn (●, ▲) or ManNAc-Asn (○, Δ) medium. SA: specific activity.

Specific primers were annealed to chromosomal DNA and incubated with DNA polymerase (Biotools B&M Labs. S.A.) or *Pfu* DNA polymerase from *Pyrococcus furiosus* (Promega) over 35 cycles (30 s at 24ºC, 63 s 55ºC and 120 s at 72ºC). PCR amplification products were analyzed by horizontal agarose gel electrophoresis.

After amplification, the PCR product was digested with *Eco*RI and *Pst*I, and the resulting fragment was cloned into the corresponding sites of the pET-42b(+) expression vector from Novagen according to the manufacturer's guidelines. The resulting plasmid, pETepim, was transformed into *E. coli* BL21(DE3) (Novagen) and DH5α.

The plasmid sequence was verified and the insert deemed to be free of PCR errors by sequencing at the Laboratorio of Técnicas Instrumentales (University of León, León, Spain) using DYEnamic™ ET Dye Terminator Kit (Amersham Biosciences Part of GE Healthcare) according to the Sanger method (Sanger *et al.*, 1977).

## RESULTS AND DISCUSSION

### Time course of the appearance of GlcNAc-6-P 2-epimerase during culture

The GlcNAc-6-P 2-epimerase from *E. coli* K92 began to be synthesized from the early logarithmic phase of growth when this bacterium was incubated at 37ºC in both Xyl/Asn, an ideal medium for polysialic acid (PA) production (González-Clemente *et al.*,

1990), and ManNAc/Asn, which is ideal for induction of the specific ManNAc transport system (Revilla-Nuín *et al.*, 1999). The level of the enzyme increased linearly during the early logarithmic phase (Fig. 1a), a maximum being reached after 10–11 h (in Xyl-Asn medium) or 29–31 h (ManNAc-Asn medium) of incubation. Thereafter it decreased continuously. This kind of kinetic behaviour, which parallels that of bacterial growth, is very similar to that observed for NeuAc lyase (Ferrero *et al.*, 1996), CMP-synthetase (González-Clemente *et al.*, 1989) and sialyltransferase (Ortíz *et al.*, 1989), other enzymes directly involved in the metabolism of PA by *E. coli.*

A comparative analysis of such kinetic behaviour revealed that the level of the enzyme was significatively higher (1.9 fold) when *E. coli* K92 was grown in ManNAc-Asn medium. However, as previously described (Revilla-Nuín *et al.*, 1998b), a dramatic decrease in PA production was recorded (90%) (see Fig. 1b). These results confirm the notion that ManNAc is involved in the regulation of capsular polysialic acid biosynthesis (Revilla-Nuín *et al.*, 1998b) and suggest that GlcNAc-6-P 2-epimerase from *E. coli* K92 is a ManNAc-inducible enzyme. Moreover, the fact that ManNAc could be the molecule that induces the ManNAc-phosphotransferase transport system (ManNAc-PTS) in this bacterium (Revilla-Nuín *et al.*, 1999), implicates GlcNAc-6-P 2-epimerase in the metabolism of ManNAc and GlcNAc when these sugars are used as carbon sources (see below).

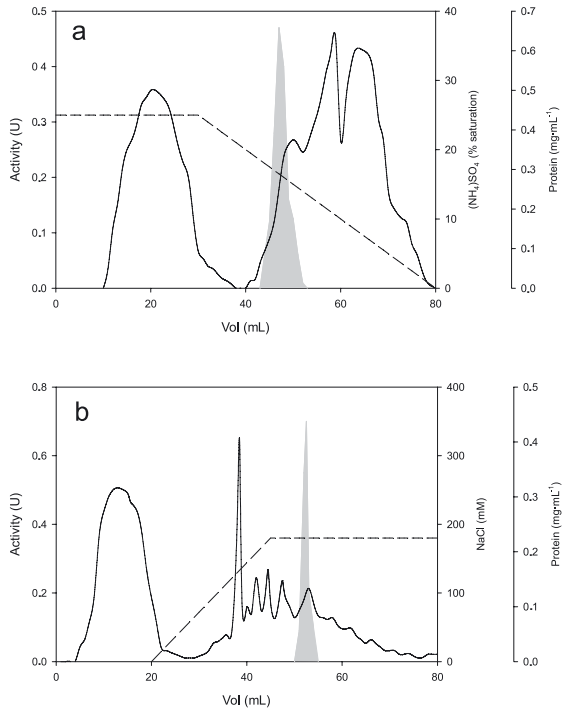


**Figure 2. Elution profile of GlcNAc-6-P 2-epimerase from *E. coli* K92.**
Butyl-agarose (a) and DEAE-FPLC (b) columns.

**Enzyme purification**

A pure and active GlcNAc-6-P 2-epimerase preparation was obtained from *E. coli* K92 extracts growth in ManNAc-Asn medium. The purification steps included Butyl-agarose chromatography and anion exchange on DEAE-FPLC (Fig. 2) followed by affinity chromatography on mannose-6-P-agarose. The purification scheme is summarized in Table 1. In the purification process it was necessary to pool two protein fractions from the Butyl-agarose elution procedure to perform the DEAE-FPLC chromatography. Moreover, exhaustive washes in all chromatographic procedures used were crucial for obtaining good purification results. Although after DEAE-chromatography we observed a high level of purification, it was necessary to use the affinity column (mannose-6P-agarose) to obtain a single, sharp electrophoretic (SDS/PAGE) protein band (Fig. 3). Under these conditions (Table 1), the final preparation was 1200-fold enriched in enzyme activity, with an overall recovery of 10%. Moreover, the use of other gel-filtration, ionic and hydrophobic chromatographic techniques gave no further increases in the specific activity yield of this enzyme (results not show).

**Molecular mass determinations**

Analysis of pure GlcNAc-6-P 2-epimerase from *E. coli* K92 by SDS/12%-PAGE revealed a single band with a molecular mass of 38.4±0.2 kDa (Fig. 3). This result differs from the expected for a pro-

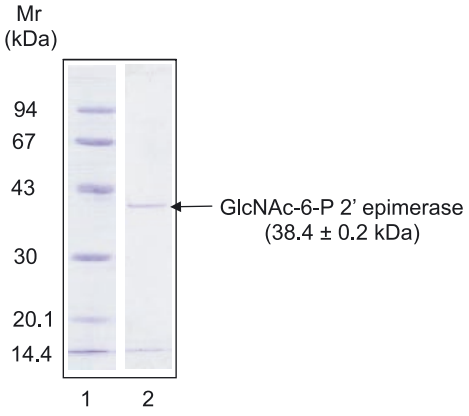


**Figure 3. Electrophoretic mobility of purified GlcNAc-6-P 2-epimerase from *E. coli* K92.**
Molecular mass standard proteins (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa) (lane 1), and a purified sample of enzyme from Mannose-6-P-agarose chromatography (lane 2) were run on SDS/12%-PAGE. Proteins were stained with Coomassie Blue R-250.

**Table 2. Amino-acid sequences of tryptic peptides obtained from purified *E. coli* K92 GlcNAc-6-P 2-epimerase.**

| Peptide | Molecular mass | | Sequence |
|---|---|---|---|
| | (expt) | (calc) | |
| 1 | 1397.72 | 1397.75 | YLYVGVRPEFR |
| 2 | 1325.72 | 1325.75 | TLWVPALKQDR |
| 3 | 3053.35 | 3053.48 | ICLFTVSDDGHLVAQDPAEVTTVEGAGPR |
| 4 | 1350.64 | 1350.67 | WAADIHITPDGR |
| 5 | 933.39 | 933.41 | HLYACDR |
| 6 | 1288.58 | 1288.60 | EGFQPTETQPR |
| 7 | 1803.89 | 1803.93 | GFNVDHSGKYLIAAGQK |
| 8 | 2174.06 | 2174.11 | SHHISVYEIVGEQGLLHEK |
| 9 | 1642.76 | 1642.79 | YAVGQGPMWVVVNAH |

tein encoded by *nanE,* the gene whose product has 28 kDa and which in *E. coli* has been related to GlcNAc-6-P 2-epimerase activity (Plumbridge & Vimr, 1999; Kalivoda *et al.*, 2003). The electrophoretic mass observed is more similar to the protein encoded by *siaA* (40–41 kDa), the gene that has been proposed to code for GlcNAc-6-P 2-epimerase in *Neissera meningitidis* (Petersen *et al.*, 2000). Different transcriptional origins or post-translational modifications could account for these molecular mass differences.

The molecular mass of native GlcNAc-6-P 2-epimerase from *E. coli* K92 was determined by gel-filtration chromatography in Shephacryl S-200 (using 10 mM Tris/HCl, pH 8.0, as buffer) to be 76±2 kDa. This finding suggests that the active epimerase from *E. coli* K92 is a dimeric protein with two similar chains.

### Physicochemical characteristics and kinetic properties

To establish the best conditions for the enzymatic assays, we studied the effect of pH and temperature on the GlcNAc-6-P 2-epimerase reac-

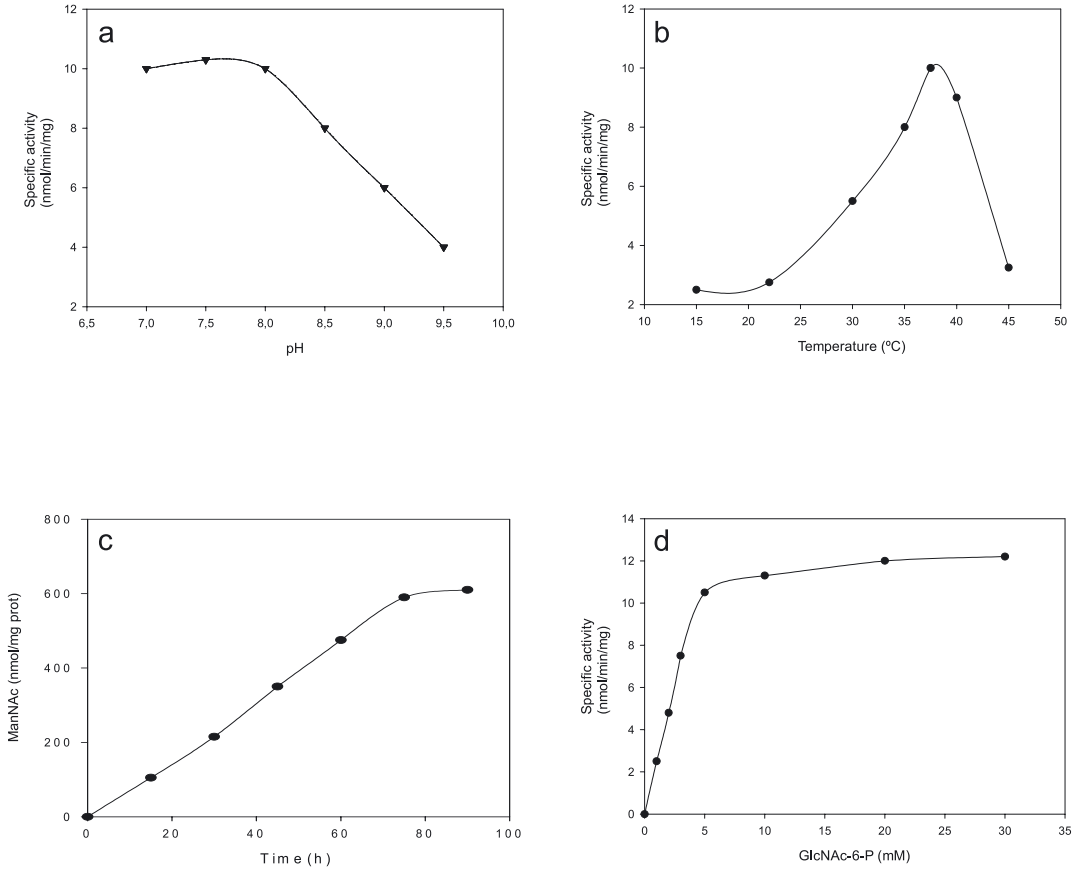


**Figure 4. Physicochemical characteristics and kinetic properties of GlcNAc-6-P 2-epimerase from *E. coli* K92.** Effects of pH (Tris/HCl) (a), temperature (b), time (c) and substrate concentration (d) on the GlcNAc-6-P 2-epimerase activity from *E. coli* K92.

**Table 3. Comparative amino-acid homologies study of tryptic peptides obtained from purified *E. coli* K92 GlcNAc-6-P 2-epimerase with non-redundant GenBank CDS Database using BlastP server (NCBI)***

| Protein (Da) | Description | Ident. % | Reference | Accession |
|---|---|---|---|---|
| 36677 | Probable isomerase [imported] — *Escherichia coli* (strain O157:H7, substrain EDL933) | 100 | Perna *et al.*, 2001 | gi\|25497761 |
| 36624 | Putative isomerase [*Shigella flexneri* 2a str. 301] | 100 | Jin *et al.*, 2002 | gi\|56479762 |
| 36152 | Putative isomerase [*Shigella sonnei* Ss046] | 100 | Yang *et al.*, 2005 | gi\|73854783 gi:74311306 |
| 36167 | Putative isomerase [*Shigella boydii* Sb227] | 100 | Yang *et al.*, 2005 | gi\|81244626 gi:82543215 |
| 36177 | Putative isomerase [*Shigella dysenteriae* Sd197] | 100 | Yang *et al.*, 2005 | gi\|81241396 gi:82777248 |
| 36177 | 6-Phosphogluconolactonase [*Escherichia coli* K12] | 100 | Thomason *et al.*, 2004; Blattner *et al.*, 1997; Riley *et al.*, 2006 | gi\|16128735 |

*Only the ORFs that show 100% identity.

tion. The highest enzymatic activity was observed at pH 7.8 and a temperature of 37°C (Fig. 4). These results are similar to those previously described for GlcNAc-6-P 2-epimerase from *E. coli* K1 using partially purified protein extracts (Rodríguez-Aparicio *et al.*, 1999) and are in the optimum range of PA production (González-Clemente *et al.*, 1990).

A kinetic analysis revealed that under these conditions product generation by GlcNAc-6-P 2-epimerase was linear up to 60 min (Fig. 4). Moreover, the enzyme activity displayed a hyperbolic type of behaviour for GlcNAc-6-P (Fig. 4); the $K_m$ calculated for the substrate was 1.5 mM. This $K_m$ value, similar to that described for the *E. coli* K1 enzyme (Rodríguez-Aparicio *et al.*, 1999), is in the range of other proteins involved in PA metabolism (Grosh & Roseman, 1965; Ortíz *et al.*, 1989; Ferrero *et al.*, 1996; Bravo *et al.*, 2001).

**Effect of cations and other molecules**

The effect of different cations (5 mM chloride form) on GlcNAc-6-P 2-epimerase activity from *E. coli* K92 was studied. Whereas the addition of monovalent cations such as $K^+$, $Li^+$ and $Rb^+$ to the reaction mixture had no effect, the presence of $Na^+$ caused a significant increase in epimerase activity, which was optimum at 10 mM (145%). This positive effect accounts for the presence of the $Na^+$ cation in the reaction mixture (see Material and Methods).

However, the addition of divalent cations to the reaction mixtures, such as $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ (5 mM) strongly inhibited the enzyme activity (by 95, 70, 85, 72, 53 and 65%). The inhibition caused by the heavy metal ions suggests that, as in other proteins (Martínez-Blanco *et al.*, 1990; Ferrero *et al.*, 1996; Walters *et al.*, 1999; Bravo *et al.*, 2001; Solana *et al.*, 2001), the presence of SH groups is essential for catalytic activity. This suggestion is also supported by the strong inhibition (96%) of epimerase activity caused by *p*-chloromercuribenzoate (1 mM) when added to the epimerase reaction mixture. The significant inhibition caused by $Mg^{2+}$, $Mn^{2+}$ and $Co^{2+}$ on the GlcNAc-6-P 2-epimerase activity may occur through a similar effect and could be related to the specific characteristics of the epimerase reaction. As previously described (Rodríguez-Aparicio *et al.*, 1992), the specific effect caused by $Mg^{2+}$ on CMP-sialic acid synthetase from rat liver (another enzyme involved in eukaryotic sialic acid metabolism) can be mimicked by $Mn^{2+}$ and $Co^{2+}$. Moreover, the inhibitory effect caused by these cations can be related to the strong decrease in PA production detected (80%) when *E. coli* is grown in the presence of 10 mM $Mn^{2+}$ (Ferrero *et al.*, 1996). Finally, we observed that GlcNAc-6-P 2-epimerase from *E. coli* K92 was inhibited by ammonium sulphate, the salt used during the enzyme purification (see above). A concentration of 1.0 M (25% satn.) caused a 64% inhibition. This effect, although reversible by desalting the enzyme, requires careful elimination of the ammonium sulphate present in the samples to achieve a good quantification of 2-epimerase activity.

**Substrate specificity and effect of different sugars and sugar derivatives**

GlcNAc-6-P 2-epimerase from *E. coli* K92 showed high specificity for GlcNAc-6-P since this phosphoamino sugar could not be replaced by GlcNAc or UDP-GlcNAc (not shown), another two intimately related amino sugars that can be used as substrates for the enzymatic synthesis of ManNAc (see Introduction). Furthermore, when we used boiled protein extracts in the reaction mixtures no epimerase activity was observed with any of these sugars, indicating that under our assay conditions no enzymatic hydrolysis or epimerization takes place. When other sugars and sugar derivatives were added to the reaction mixture in the presence of GlcNAc-6-P, we observed 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, GlcNAc, *N*-acetylneuraminic acid and *N*-acetylglucosaminitol, or the hexosamines glucosamine and galactosamine did not affect GlcNAc-6-P 2-epimerase activity, even at a concentration of 5 mM. However, the addition of mannose-6-P, but not mannose-1-P, to the reaction mixture caused a marked inhibition of enzyme activity. A concentration of mannose-6-P of 5 mM, similar to that of GlcNAc-6-P used in the epimerase reaction assays (see Materials and Methods), caused a 50% inhibition. These results suggest that *in vivo* GlcNAc-6-P 2-epimerase activity may be specifically regulated by variations in the mannose-6-P intracellular pool.

**GlcNAc-6-P 2-epimerase and sialic acid metabolism**

As shown in Fig. 1, when *E. coli* K92 used ManNAc as a carbon source the amount of GlcNAc-6-P 2-epimerase increased up to 1.9 fold. This indicates that our enzyme is directly involved in the intracellular turnover of ManNAc, the amino sugar precursor of sialic acid for capsular PA synthesis.

Different authors have reported that the ManNAc used by *E. coli* for sialic and polysialic acid biosynthesis is generated by UDP-GlcNAc 2-epimerase (Ringenberg *et al.*, 2003; Vann *et al.*, 2004; Tanner, 2005) and that the phosphorylated form of ManNAc is not involved in this process (Ringenberg *et al.*, 2003). Moreover, the existence in *E. coli* K92 of a ManNAc-inducible GlcNAc-6-P 2-epimerase and the fact that when this bacterium grew using ManNAc as a carbon source a dramatic decrease in PA production took place (Fig. 1) suggest that, in this bacterium, two different proteins could be involved in sialic acid metabolism: UDP-GlcNAc 2-epimerase for the biosynthetic function and the GlcNAc-6-P 2-epimerase described in this work exerting a catabolic role. Thus, when *E. coli* K92 uptakes ManNAc from the growth medium, the specific and ManNAc-inducible PTS generates intracellular ManNAc-6-P (Revilla-Nuín *et al.*, 1998b), which is used by the GlcNAc-6-P 2-epimerase to generate GlcNAc-6-P. Accordingly, the existence of this enzyme, now characterized, may permit this bacterium to use extracellular ManNAc as a carbon source (Revilla-Nuín *et al.*, 1998b; 1999; 2002; Ringenberg *et al.*, 2003). Furthermore, it is very likely that the catabolism of ManNAc would take place *via* GlcNAc since, as described previously (Revilla-Nuín *et al.*, 1998b; 1999; Ezquerro-Sáenz *et al.*, 2006), when *E. coli* K92 uses ManNAc, the intracellular pool of ManNAc-6-P and GlcNAc-6-P is increased. Also in favour of this hypothesis is the fact that GlcN-6-P deaminase, an enzyme of the GlcNAc metabolism, is also induced by ManNAc (Biswas *et al.*, 1979). Finally, ManNAc induces both ManNAc-PTS (Revilla-Nuín *et al.*, 1999) and GlcNAc-6-P 2-epimerase expression (Fig. 1), suggesting that, as in ManNAc catabolism, the genes that encode these proteins form part of a regulon that responds to the same regulation signals.

**Genetic study**

Different authors have proposed *nanE* as a putative bacterial gene that codes for GlcNAc-6-P 2-epimerase (Plumbridge & Vimr, 1999; Walters *et al.*, 1999; Kalivoda *et al.*, 2003; Ringenberg *et al.*, 2003). To establish whether GlcNAc-6-P 2-epimerase from *E. coli* K92 is indeed codified for this gene, we analyzed the amino-acid sequence of the purified protein. Unfortunately, attempts to sequence the purified GlcNAc-6-P 2-epimerase by N-terminal sequence analysis failed because of amino-terminal blockage. We therefore undertook sequencing by tryptic digestion and mass spectrometry analysis (see Materials and Methods). Table 2 shows the amino-acid sequences of nine peptides obtained from purified GlcNAc-6-P 2-epimerase by tryptic digestion that were compared in a search in a non-redundant NCBI protein sequence database.

A comparative study of these tryptic peptide sequences with a non-redundant GenBank CDS Database (NCBI-BLAST) revealed the existence of a high homology with putative isomerases from different sources (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Jin *et al.*, 2002; Yang *et al.*, 2005) and the *pgl* gene of *E. coli* K12 (Thomason *et al.*, 2004) although, surprisingly, not with the epimerases previously related (see above). As shown in Table 3 and Fig. 6, all tryptic peptides obtained showed a complete sequence homology with the recently identified *pgl* gene from *E. coli* K12 and isomerases from different

*E. coli* strains and *Shigella* species. Moreover, in all cases the molecular mass calculated for the respective expected protein products (36–36.7 kDa) were similar to that of the GlcNAc-6-P 2-epimerase purified by us (Table 3 and Fig. 5). However, non-homology was observed when we compared these tryptic peptides sequences with the protein products of *nanE* and *siaA*, the two different genes that have been proposed to encode GlcNAc-6-P 2-epimerase in bacteria (Plumbridge & Vimr, 1999; Walters *et al.*, 1999; Petersen *et al.*, 2000; Kalivoda *et al.*, 2003; Ringenberg *et al.*, 2003) (not shown).

To establish the identity of the gene that codes for the GlcNAc-6-P 2-epimerase from *E. coli* K92, and using the information obtained from the comparative sequencing studies, we designed different primers that permitted us to amplify, by PCR, a fragment of 1 kbp from *E. coli* K92 genomic DNA (see Materials and Methods). Cloning and sequencing of this fragment revealed an ORF of 996 nucleotides encoding 331 amino acids (Fig. 5), whose

```
tacggtacggtggtggctcggtattgagggacgcgggtccatggatatcggggatccgaat
tccaaaggagcattcatgaagcaaacagtttatatcgccagccctgagagccagcaaatt    45
            M  K  Q  T  V  Y  I  A  S  P  E  S  Q  Q  I          15
cacgtctggaatctgaatcatgaaggcgcactgacgctgacacaggttgtcgatgtgccg   105
 H  V  W  N  L  N  H  E  G  A  L  T  L  T  Q  V  V  D  V  P    35
gggcaggtgcagccgatggtggtcagcccggacaaacgttatctctatgttggtgttcgc   165
 G  Q  V  Q  P  M  V  V  S  P  D  K  R  Y  L  Y  V  G  V  R    55
cctgagtttcgcgtcctggcgtatcgtatcgccccggacgatggcgcactgacctttgcc   225
 P  E  F  R  V  L  A  Y  R  I  A  P  D  D  G  A  L  T  F  A    75
gcagagtctgcgctgccgggtagtccgacgcatatttccaccgatcaccaggggcagttt   285
 A  E  S  A  L  P  G  S  P  T  H  I  S  T  D  H  Q  G  Q  F    95
gtctttgtaggttcttacaatgcgggtaacgtgagcgtaacgcgtctggaagatggcctg   345
 V  F  V  G  S  Y  N  A  G  N  V  S  V  T  R  L  E  D  G  L   115
ccagtgggcgtcgtcgatgtggtcgaggggctggacggttgccattccgccaatatctca   405
 P  V  G  V  V  D  V  V  E  G  L  D  G  C  H  S  A  N  I  S   135
ccggacaaccgtacgctgtgggttccggcattaaagcaggatcgcatttgcctgtttacg   465
 P  D  N  R  T  L  W  V  P  A  L  K  Q  D  R  I  C  L  F  T   155
gtcagcgatgatggtcatctcgtggcgcaggaccctgcggaagtgaccaccgttgaaggg   525
 V  S  D  D  G  H  L  V  A  Q  D  P  A  E  V  T  T  V  E  G   175
gccggcccgcgtcatatggtattccatccaaacgaacaatatgcgtattgcgtcaatgag   585
 A  G  P  R  H  M  V  F  H  P  N  E  Q  Y  A  Y  C  V  N  E   195
ttaaacagctcagtggatgtctgggaactgaaagatccgcacggtaatatcgaatgtgtc   645
 L  N  S  V  D  V  W  E  L  K  D  P  H  G  N  I  E  C  V      215
cagacgctggatatgatgccggaaaacttctccgacacccgttgggcggctgatgttcat   705
 Q  T  L  D  M  M  P  E  N  F  S  D  T  R  W  A  A  D  V  H   235
atcaccccggatggtcgccatttatacgcctgcgaccgtaccgccagcctgattaccgtt   765
 I  T  P  D  G  R  H  L  Y  A  C  D  R  T  A  S  L  I  T  V   255
ttcagcgtttcggaagatggcagcgtgttgagtaaagaaggcttccagccaacggaaacc   825
 F  S  V  S  E  D  G  S  V  L  S  K  E  G  F  Q  P  T  E  T   275
cagccgcgcggcttcaatgttgatcgcagcggcaagtatctgattgccgccgggcaaaaa   900
 Q  P  R  G  F  N  V  D  R  S  G  K  Y  L  I  A  A  G  Q  K   295
tctcaccacacctcggtatacgaaattgttggcgagcaggggctactgcatgaaaaaggc   960
 S  H  H  T  S  V  Y  E  I  V  G  E  Q  G  L  L  H  E  K  G   315
cgctatgcggtcgggcagggaccaatgtgggtggtggttaacgcacactaaccgctgatt   996
 R  Y  A  V  G  Q  G  P  M  W  V  V  V  N  A  H  -             332
tacccgctgcagtctctccctgcgccggtgtattaacctatctcctgtaacgcgtgtctct
ggcgttcgacgatattggtccacaaat
```

**Figure 5. The nucleotide and translated sequence of the PCR-amplified fragment from *E. coli* K92 genomic DNA.** The upper row shows the nucleotide sequence and the second row shows the amino-acid sequence. The nucleotides underlined show the PCR amplification primers (the reverse primer corresponding to the inverse and complementary chain) and the bold nucleotides corresponding to *Eco*RI and *Pst*I restriction sites. The underlined amino acids show the tryptic peptide sequences of the protein from *E. coli* K92.

calculated mass (38.4±0.2 kDa) was similar to that obtained by electrophoretic analysis of the GlcNAc-6-P 2-epimerase protein purified by us (Fig. 3). A comparison of the amino-acid sequence of this protein with those found in the GenBank CDS Database using BlastP (NCBI) program revealed very high homology with the isomerases from *E. coli* (strain O157:H7) (100%), different *Shigella* species (98–99%) and the 6-phosphogluconolactonase (*pgl* gene) from *E. coli* K12 (Fig. 6). However, no isomerase (evaluated as GlcNAc-6-P 2-epimerase) or 6-phosphogluconolactonase activities were detected in protein extracts from *E. coli* BL21 (DE3) and DH5α transformed with pETepim and induced with IPTG (see Materials and Methods). Moreover, and similar to the comparative tryptic studies, a low degree of amino acid homology was observed with the products of *nanE* and *siaA* (12% and 11%, respectively) and even with the related UDP-GlcNAc 2-epimerase encoded by the *neuC* gene (10%), the enzyme that generates ManNAc in polysialic acid biosynthesis pathways (Ringenberg *et al.*, 2003; Murkin *et al.*, 2004; Vann *et al.*, 2004). Nevertheless, the fact that by gel-filtration chromatography the native protein (detected by epimerase activity) had a molecular mass of 74–78 kDa — twofold the 38 kDa obtained from SDS/PAGE (denaturing conditions) — precludes the possibility that in the protein amino-acid analysis, a contaminant protein could have been sequenced. If the epimerase purified by us were a product of *nanE*, the expected molecular mass should be 2×28 kDa. However, the molecular mass determined for our native protein was 74–78 kDa. The results obtained from tryptic digestion and peptide sequence analysis revealed the existence of a unique polypeptide chain sequence that showed very high homology with putative isomerases from different bacterial strains. At this juncture, it is also surprising that this protein sequence exhibited high homology with the gene that encodes 6-phosphogluconolactonase in *E. coli* K12 (Table 3 and Fig. 6) i.e., a protein involved in the pentose-P pathway (Thomason *et al.*, 2004). This homology again throws doubt on our results that relate the GlcNAc-6-P 2-epimerase with this genetic sequence. However, as reported by Thomason and coworkers in the same work (2004), this genetic sequence product could correspond to a bifunctional protein and, as in *Thermoanaerobacter tengcongensis* and *Vibrio vulnificus* CMCP6, could show lactonase and isomerase activity. Although *nanE* has been described in *E. coli* K12 and, by molecular genetic studies, it has been related to GlcNAc-6-P 2-epimerase (Ringenberg *et al.*, 2003), ManNAc is metabolized by this bacterium very slowly and a previous mutation is required to derepress the gene (Plumbridge & Vimr, 1999). Nevertheless, *E. coli* K92 grows very efficiently using ManNAc as a carbon source. As shown in Fig. 1, the use of

```
6-P-gluc [E coli K12]          MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Isom. ybhE [E. coli O157:H7]   MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Isom. [Sh. dysenteriae Sd197]  MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Isom. [Sh. sonnei Ss046]       MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Isom. [Sh. boydii Sb227]       MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Isom. [Sh. flexneri 2a]        MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
Epim. [E. coli K92             MKQTVYIASPESQQ-----IHVWNLNHEG-----ALTLTQVVDVPGQVQP 40
SiaA [N. meningitidis]         MKRILCITGTRADFGKLKPLLAYIENHPDLELHLIVTGMHMMKTYGRTYK 50
NeuC [E. coli]                 MKKILYVTGSRAEYGIVRRLLTMLRETPEIQLDLAVTGMHCDNAYGNTIH 50
NanE [E. coli K12]             --------------------------------------------------

6-P-gluc [E coli K12]          MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESALPG---SPTHI 87
Isom. ybhE [E. coli O157:H7]   MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAEXALPG---SPTHI 87
Isom. [Sh. dysenteriae Sd197]  MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESALPG---SPTHI 87
Isom. [Sh. sonnei Ss046]       MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESALPG---SPTHI 87
Isom. [Sh. boydii Sb227]       MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESALPG---SPTHI 87
Isom. [Sh. flexneri 2a]        MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESVLPG---SPTHI 87
Epim. [E. coli K92]            MVVSPDKRYLYVGVRPEFRVLAYRIAPDDGALTFAAESALPG---SPTHI 87
SiaA [N. meningitidis]         EVTRENYQHTYLFSNQIQGEPMGAVLGNTITFISRLSDEIEP---DMVMI 97
NeuC [E.coli]                  IIEQDNFNIIKVVDININTTSHTHILHSMSVCLNSFGDFFSNNTYDAVMV 100
NanE [E. coli K12]             --------------MSLLAQLDQKIAANGGLIVSCQPVPDSP-------- 28
                                                 :  .

6-P-gluc [E coli K12]          STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Isom. ybhE [E. coli O157:H7]   STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Isom. [Sh. dysenteriae Sd197]  STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Isom. [Sh. sonnei Ss046]       STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Isom. [Sh. boydii Sb227]       STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Isom. [Sh. flexneri 2a]        STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
Epim. [E. coli K92]            STDHQGQFVFVGSYNAGNVSVTRLEDGL-PVGVVD-----VVEGLDGCHS 131
SiaA [N. meningitidis]         HGDRLEALAGAAVGALSSRLVCHIEGGE-LSGTVDDSIRHSISKLSHIHL 146
NeuC [E.coli]                  LGDRYEIFSVAIAASMHNIPLIHIHGGEKTLANYDEFIRHSITKMSKLHL 150
NanE [E. coli K12]             LDKPEIVAAMALAAEQAGAVAIRIEGVA------------NLQATR---- 62
                                 .       .      .    ::..              :

6-P-gluc [E coli K12]          ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
Isom. ybhE [E. coli O157:H7]   ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
Isom. [Sh. dysenteriae Sd197]  ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
Isom. [Sh. sonnei Ss046]|      ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
Isom. [Shigella boydii Sb227]  ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTIEG------ 175
Isom. [Sh. flexneri 2a]        ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
Epim. [E. coli K92]            ANISPDNRTLWVPALKQDRICLFTVSDDGHLVAQDPAEVTTVEG------ 175
SiaA [N. meningitidis]         VANEQAVTRLVQMGEKRKHIHIIGSPDLDVMASSTLPSLEEVKEYYGLPY 196
NeuC [E.coli]                  TSTEEYKKRVIQLGEKPGSVFNIGSLGAENALSLHLPNKQELELKYGSLL 200
NanE [E. coli K12]             AVVSVPIIGIVKRDLEDSPVRITAYIEDVDALAQAGADIIAIDG------ 106
                               .  .     :     :   :            :    ..   :.

6-P-gluc [E coli K12]          AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Isom. ybhE [E. coli O157:H7]   AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Isom. [Sh. dysenteriae Sd197]  AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Isom. [Sh. sonnei Ss046]|      AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Isom. [Shigella boydii Sb227]  AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Isom. [Sh. flexneri 2a]        AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
Epim. [E. coli K92]            AGPRHMVFHPNEQYAYCVNELNSS--------------VDVWELKDPHGN 211
SiaA [N. meningitidis]         ENYGISMFHPVTTEAHLMPQYAAQYFKALELSG--QNIISIYPNNDTGTE 244
NeuC_[E.coli]                  KRYFVVVFHPETLSTQSVNDQIDELLSAISFFKNTHDFIFIGSNADTGSD 250
NanE [E. coli K12]             ----TDRPRPVPVETLLARIHHHG-------------LLAMTDCSTPEDG 139
                                      :*    :                       : :     .

6-P-gluc [E coli K12]          IECVQTLDMMPE-NFSDT-RWAADIHITPDGRHLYACDRTASLITVFSVS 259
Isom. ybhE [E. coli O157:H7]   IECVQTLDMMPE-NFSDT-RWAADIHITPDGRHLYACDRTASLITVFSVS 259
Isom. [Sh. dysenteriae Sd197]  IECVQTLDMMPE-NFSDT-RWAADIHITPDGRHLYACDRTASLITVFSVS 259
Isom. [Sh. sonnei Ss046]       IECVQTLDMMPE-NFSDT-CWAADIHITPDGRHLYACDRTASLITVFSVS 259

Isom. [Sh. boydii Sb227]       IECVQTLDMMPE-NFSDT-RWAADIHITPDGRHLYACDRTASLITVFSVS 259
Isom. [Sh. flexneri 2a]        IECVQTLDMMPE-NFSDT-RWAADIHITPDGRHLYACDRTASLITVFSVS 259
Epim. [E. coli K92]            IECVQTLDMMPE-NFSDT-RWAADVHITPDGRHLYACDRTASLITVFSVS 259
SiaA [N. meningitidis]         SILQELLKYQSD-KFIAFPSIRFEYFLVLLKHAKFMVGNSSAGIREAPLY 293
NeuC [E.coli]                  IIQRKVKYFCKEYKFRYLISIRSEDYLAMIKYSCGLIGNSSSGLIEVPSL 300
NanE [E. coli K12]             LACQKLGAEIIG---------------------TTLSGYTTPETPEEPDL 168
                                  :                               . ::.     .

6-P-gluc [E coli K12]          EDGSVLSKEGFQPTETQPRGFNVDHSGKYLIAAG---QKSHHISVYEIVG 306
Isom. ybhE [E. coli O157:H7]   EDGSVLSKEGFQPTETQPRGFNIDHRGKYLIAAG---QKSHHISVYEIVG 306
Isom. [Sh. dysenteriae Sd197]  EDGSVLSKEGFQPTETQPRGFNVDHSGKYLIAAG---QKSHHISVYEIVG 306
Isom. [Sh. sonnei Ss046]       EDGSVLSKEGFQPTETQPRGFNVDHSGKYLIVAG---QKSHHISVYEIVG 306
Isom. [Sh. boydii Sb227]       EDGSVLSKEGFQPTETQPRGFNVDHSGKYLIAAG---QKSHLISVYEIVG 306
Isom. [Sh. flexneri 2a]        EDGSVLSKEGFQPTETQPRGFNVDYSGKYLIAAG---QKSHHISVYEIVG 306
Epim. [E. coli K92]            EDGSVLSKEGFQPTETQPRGFNVDRSGKYLIAAG---QKSHHTSVYEIVG 306
SiaA [N. meningitidis]         GVPSIDVGTRQNNRHMGKSIIHTDYETKNIFDAI---QQACSLGKFEADD 340
NeuC [E.coli]                  KVATINIGDRQKGRVRGASVIDVPVEKNAIVRGINISQDEKFISVVQSSS 350
NanE [E. coli K12]             ALVKTLSDAG--CRVIAEGRYNTPAQAADAMRHG---------------- 200
                                  .                 .       .

6-P-gluc [E coli K12]          -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Isom. ybhE [E. coli O157:H7]   -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Isom. [Sh. dysenteriae Sd197]  -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Isom. [Sh. sonnei Ss046]       -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Isom. [Sh. boydii Sb227]       -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Isom. [Sh. flexneri 2a]        -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
Epim. [E. coli K92]            -----EQGLLHEKGRYAVGQGPMWVVVNAH---------------- 331
SiaA [N. meningitidis]         TFNGGDTRTSTERFAEVINNPETWNVSAQKRFIDLNL---------- 377
NeuC_[E.coli]                  -----NPYFKENALINAVRIIKDFIKSKNKDYKDFYDIPECTTSYD 391
NanE [E. coli K12]             -----AWAVTVGSAITRLEHICQWYNTAMKKAVL------------ 229
                                                 :     :     :
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Xyl or ManNAc as a source of carbon did not elicit significant delays in growth, suggesting that unlike *E. coli* K12, in *E. coli* K92 the genetic derepression through mutation is not necessary for the growth of this bacterium on ManNAc. Moreover, as previously described (Plumbridge & Vimr, 1999), for *E. coli* K12 to grow using ManNAc it is also necessary that the multispecific transport system that uptakes this amino sugar in this bacterium (ManXYZ) should be derepressed by an *mlc* mutation. In *E. coli* K92 this previous gene derepression is not necessary since in this bacterium the uptake of ManNAc is carried out by a different transport system that is very specific, induced by ManNAc-6-P, and much more efficient than the ManXYZ transporter (Revilla-Nuín *et al.*, 1999; 2002). All these metabolic differences support the idea that, as proposed, the protein purified by us would be the putative isomerase reported (Table 3) and that it would be responsible for the GlcNAc-6-P 2-epimerase activity in *E. coli* K92. Although in *E. coli* K92 we have observed the presence of *nanE* (we detected it by PCR analysis, results not shown), it is possible that this gene might be repressed, as in *E. coli* K12, and that both the specific ManNAc-PTS transport system and the epimerase related in this work could be responsible for the efficient growth of this bacterium when it uses ManNAc as a carbon source. Thus, *E. coli* K92 has evolved by developing a new strategy that permits good cellular growth using ManNAc as a carbon source without having to depend on previous mutations. Expression studies by mRNA quantification and by direct reaction of the respective protein products with specific antibodies, analysis of enzymatic activities, and the use of knockout bacteria in these genes should provide key data to confirm these hypotheses and to establish the exact role these genes play in bacterial amino sugar metabolism. Further research in this topic is currently in progress.

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**Figure 6. Protein sequence alignment (CLUSTALW) of GlcNAc-6-P 2-epimerase of *E. coli* K92.**
Alignment with other related bacterial epimerase proteins involved in ManNAc metabolism (NeuC, SiaA and NanE) and isomerases from *E. coli* strain O157:H7 (Hayashi *et al.*, 2001; Perna *et al.*, 2001), *Sh. dysenteriae* Sd197 (Yang *et al.*, 2005), *S. sonnei* Ss046 (Yang *et al.*, 2005), *S. boydii* Sb227 (Jin *et al.*, 2002), *S. flexneri* 2a (Yang *et al.*, 2005) and 6-phosphogluconolactonase of *E. coli* K12 (Blattner *et al.*, 1997; Thomason *et al.*, 2004; Riley *et al.*, 2006). NeuC: UDP-*N*-acetylglucosamine 2-epimerase from *E. coli* (Vann *et al.*, 2004); NanE: predicted *N*-acetylmannosamine-6-P epimerase from *E. coli* (Walters *et al.*, 1999); SiaA: *N*-acetylmannosamine-6-P 2-epimerase from *N. meningitidis* serogroup B (Petersen *et al.*, 2000). Isom.: putative isomerase. Epim.: epimerase. 6-P-gluc.: 6-phosphogluconolactonase. Identical amino acids (*), conservative amino acid changes (:), amino acid related (.), and lack of similarity (blank space) are indicated.

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