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# Genetic engineering of *Escherichia coli* for the economical production of sialylated oligosaccharides

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ABSTRACT

We have previously described a microbiological process for the conversion of lactose into 3′sialyllactose and other ganglioside sugars by living *Escherichia coli* cells expressing the appropriate recombinant glycosyltransferase genes. In this system the activated sialic acid donor (CMP-Neu5Ac) was generated from exogenous sialic acid, which was transported into the cells by the permease NanT. Since sialic acid is an expensive compound, a more economical process has now been developed by genetically engineering *E. coli* K12 to be capable of generating CMP-Neu5Ac using its own internal metabolism. Mutant strains devoid of Neu5Ac aldolase and of ManNAc kinase were shown to efficiently produce 3′sialyllactose by coexpressing the α-2,3-sialyltransferase gene from *Neisseria meningitidis* with the *neuC*, *neuB* and *neuA Campylobacter jejuni* genes encoding *N*-acetylglucosamine-6-phosphate-epimerase, sialic acid synthase and CMP-Neu5Ac synthetase, respectively. A sialyllactose concentration of 25 g $l^{-1}$ was obtained in long-term high cell density culture with a continuous lactose feed. This high concentration and low cost of fermentation medium should make possible to use sialylated oligosaccharides in new fields such as the food industry.



## 1. Introduction

*N*-Acetylneuraminic acid (Neu5Ac) is frequently found as a terminal sugar in cell surface complex carbohydrates and plays a major role in many biological processes such as cellular adhesion and the binding of toxins and viruses (Varki, 1993). In particular Neu5Ac is a major component of the carbohydrate portion of gangliosides, which are notably abundant in brain tissues and are involved in several pathologies (Zhang and Kiechle, 2004). Free sialylated oligosaccharides are found at high concentrations in human milk and are known to have both anti-infective and immunostimulating properties (Boehm and Stahl, 2007). They are also believed to increase the brain ganglioside and glycoprotein sialic acid concentration and favor the brain maturation of breastfed infants by serving as an exogenous source of sialic acid (Wang et al., 2003).

Due to their important biological functions, sialylated structures have attracted considerable interest and many methods have been developed for the synthesis of sialylated oligosaccharides. Since chemical synthesis are not practical because of the multiple protection and deprotection steps involved, great effort has been put into enzymatic and biotechnological methods. The development of efficient systems for the enzymatic synthesis of sialylated oligosaccharides has been possible through the identification of bacterial sialyltransferase genes which are well expressed in *Escherichia coli* and the design of multiple enzymatic systems for the synthesis of CMP-Neu5Ac (Gilbert et al., 1998). It was later shown that the cost of synthesis could be significantly reduced by using permeabilized (Endo et al., 2000) or living (Priem et al., 2002) whole *E. coli* cells. In the latter approach, lactose, which was used as exogenous acceptor, was internalized by the LacY permease and was sialylated by recombinant glycosyltransferase, using CMP-Neu5Ac, which was constantly regenerated by the enzymatic machinery of the living cells. Since the only *E. coli* strains that naturally produce CMP-Neu5Ac are pathogenic strains that cannot be used in biotechnological processes, a pathway for the synthesis of CMP-Neu5Ac had to be imported into *E. coli* strain K12 derivatives used for the production of sialylated oligosaccharides. Taking advantage of the fact that *E. coli* is able to catabolize Neu5Ac and possesses a sialic acid permease, an anabolic pathway for the synthesis of CMP-NeuAc from exogenous Neu5Ac was engineered by over-expressing the *neuA* gene for CMP-Neu5Ac synthase and by disrupting the NanA aldolase, which catalyzes the conversion of Neu5Ac into ManNAc and pyruvate. This system was first used for the production of 3′sialyllactose as illustrated in Fig. 1A and was later extended to the production of the carbohydrate portion of the gangliosides GM2

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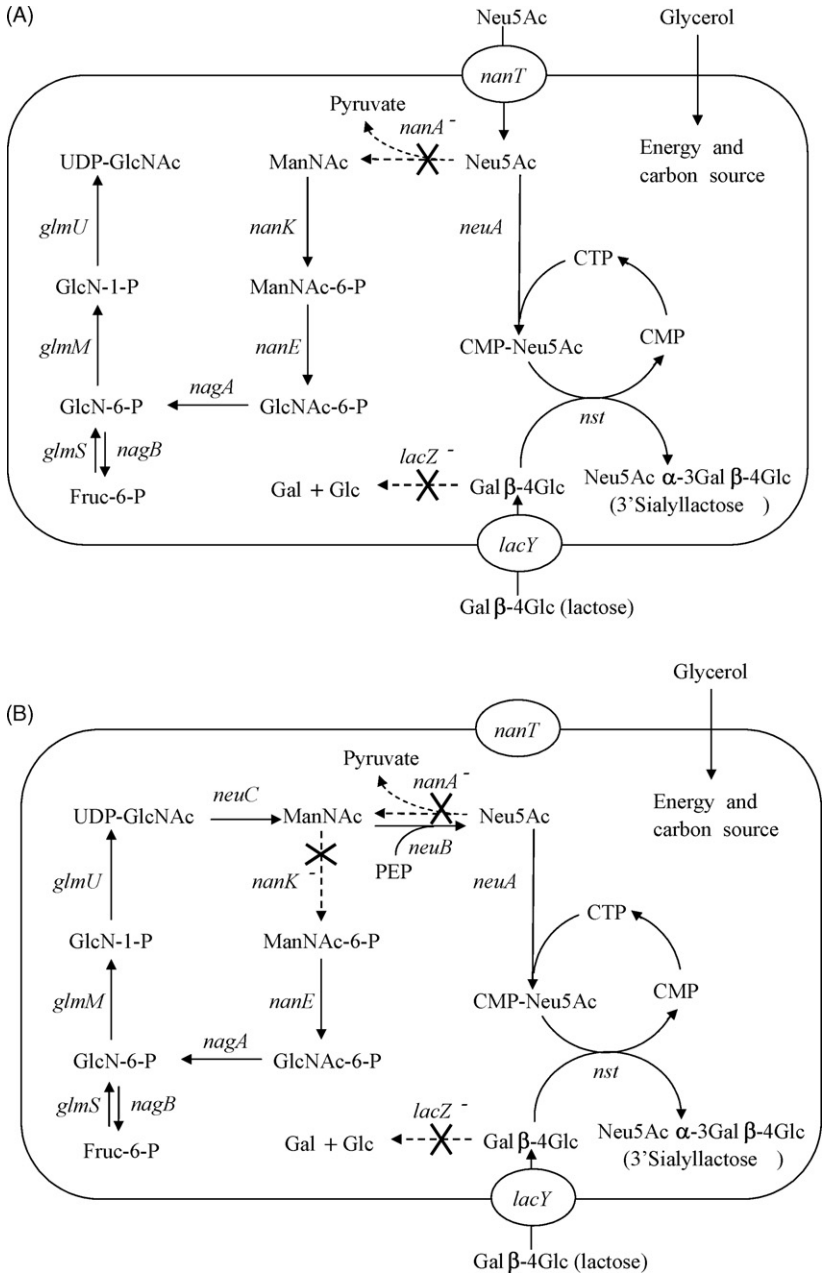


**Fig. 1.** Engineered metabolic pathways for the production of 3′sialyllactose with (**A**) an exogenous supply of Neu5Ac (Priem et al., 2002) or (**B**) with an endogenous synthesis of Neu5Ac from UDP-GlcNAc. Over-expressed heterologous genes are in bold. Discontinued arrows represent the enzymatic activities that have been eliminated.

and GM1 by additionally expressing the appropriate glycosyltransferase genes (Antoine et al., 2003). Polysialylated oligosaccharides (GD3 and GT3 sugars) were also produced by this method, using the *Campylobacter cstII* gene that encodes a bifunctional α-2,3- and α-2,8-sialyltransferase (Antoine et al., 2005).

Sialic acid, used as precursor for the synthesis of sialylated oligosaccharide, can be purified from natural sources such as milk and egg yolk (Koketsu et al., 1992), but the yields are low and the procedure is not suitable for large-scale production. Sialic acid is thus generally prepared by enzymatic synthesis by the sialic acid aldolase, using *N*-acetylmannosamine (ManNAc) and pyruvate as substrates. To reduce the cost, ManNAc can be prepared by chemical or enzymatic epimerization of *N*-acetylglucosamine, which is a cheaper substrate than ManNAc (Lee et al., 2004; Maru et al., 1998). In spite of these improvements, sialic acid is still relatively expensive and its cost can hamper the development of applications that require large amounts of sialylated oligosaccharides. We have thus investigated the possibility of producing sialylated oligosaccharides without an exogenous supply of Neu5Ac. In both *E. coli* K1 and *N. meningitidis*, Neu5Ac is synthesized in two enzymatic steps from UDP-GlcNAc. The first step, which is the conversion of UDP-GlcNAc into MAnNAc via a 2-acetamidoglucal intermediate (Vann et al., 2004), is catalyzed by the UDP-GlcNAc 2 epimerase encoded by the *neuC* gene. The second step is the condensation of ManNAc and phosphoenolpyruvate into Neu5Ac and is catalyzed by the sialic acid synthase encoded by the *neuB* gene (Vann et al., 1997).

In this paper we demonstrate that sialylated oligosaccharides can be economically produced by bacterial fermentation by showing that *E. coli* K12, co-expressing the three *nanABC* genes with the α-2,3-sialyltransferase genes can efficiently produce 3′sialyllactose, providing that the ManNAc kinase and Neu5Ac aldolase activities have been eliminated.