NeuA O-acetylesterase activity is specific for CMP-activated O-acetyl sialic acid in Streptococcus suis serotype 2

Lili Song [a], Hui Zhou [a], Xuehui Cai [b], Chunyang Li [c], Jingnan Liang [c], Cheng Jin [a,*]

[a] Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Harbin 0001, China
[c] Core Facility of Equipment, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

ARTICLE INFO

ABSTRACT

Several bacteria causing meningitis, such as Escherichia coli K1, Streptococcus suis, Neisseria meningitidis, and group B Streptococci (GBS), produce sialic acid (Neu5Ac)-containing capsular polysaccharide (CPS). Biosynthesis of the Neu5Ac-containing CPS requires CMP-Neu5Ac as substrate, which is synthesized by CMP-Neu5Ac synthetase from CTP and Neu5Ac. In E. coli or GBS, the NeuA protein encoded by the neuA gene has been known encoding a bifunctional enzyme that possesses both CMP-Neu5Ac synthetase and O-acetylesterase activity. In this report, we found that the S. suis NeuA (SsNeuA) was also a bifunctional CMP-Neu5Ac synthetase/O-acetylesterase. Biochemical analyses revealed that the SsNeuA strictly de-O-acetylated CMP-O-acetyl-Neu5Ac, whereas the E. coli NeuA (EcNeuA) preferentially de-O-acetylated CMP-O-acetyl-Neu5Ac. E. coli devoid of NeuA O-acetylesterase activity was unable to produce capsule and only CMP-Neu5Ac synthetase activity of the EcNeuA or SsNeuA could not restore its ability to produce capsule. These results suggest that the O-acetylesterase is essential for the synthesis of capsular Neu5Ac in E. coli, probably in S. suis and GBS as well. Our findings are key to understanding the biosynthesis of capsular Neu5Ac in E. coli, S. suis and GBS.

1. Introduction

Streptococcus suis is a major cause of meningitis, sepsis and arthritis in piglets and a zoonotic agent. Among the 35 serotypes described to date, S. suis serotype 2 is the one most frequently recovered from diseased animals [1]. As an emerging zoonotic agent, S. suis has been isolated from an increasing number of human cases of meningitis, endocarditis, and toxic shock-like syndrome [2,3]. In 2005, an unprecedented outbreak in China resulted in >200 human cases that were directly linked to a concurrent outbreak of S. suis infection in pigs. Of these human cases, 20% were fatal [2]. Since then, the number of human S. suis cases reported in the literature has increased significantly over the past few years. In a review article published in 2009, >700 human S. suis cases were reported, with most cases originating in Southeast Asia [4].

Although S. suis can cause human meningitis, little is known how S. suis invades the host and crosses the blood brain barrier. The only factor identified thus far as playing a critical role in pathogenesis is capsular polysaccharide (CPS) [4–7]. The CPS of S. suis serotype 2 is composed of glucose, galactose, N-acetylglucosamine,

rhamnose, and sialic acid (N-acetylneuraminic acid, Neu5Ac) in a ratio of 1:3:1:1:1 [8]. Neu5Ac-containing CPS is also known as a major virulence factor in several other bacteria causing meningitis, such as Escherichia coli K1 [9], Neisseria meningitidis [10], and group B Streptococci (GBS, or Streptococcus agalactiae) [11]. Furthermore, a significant portion of the native capsular Neu5Ac is O-acetylated in E. coli, N. meningitidis and GBS [12–14]. It is believed that modification of capsular Neu5Ac by O-acetylation correlates with increased virulence in patients with bacteremia and also affects eukaryotic cell binding, invasion and resistance to innate immunity [15–17]. Although levels of capsular O-acetyl-Neu5Ac are thought to be controlled by Neu5Ac-specific O-acetyltransferases and O-acetylesterases with the different activity levels [18,19], its mechanism remains unclear.

Neu5Ac is corporate into the CPS by sialyltransferase, which requires cytidine-5-monophosphate (CMP)-Neu5Ac as the obligate donor in both pro- and eukaryotic cells. CMP-Neu5Ac synthetase (EC2.7.7.43) is responsible for activation of free Neu5Ac to produce the unique nucleotide sugar CMP-Neu5Ac. Given the importance of Neu5Ac in the modulation of virulence in pathogenic bacteria, the enzyme involved in Neu5Ac activation would be a potential target for drug development. Our previous work reveals that E. coli K1 CMP-Neu5Ac synthetase (EcNeuA) encoded by the neuA gene is a bifunctional enzyme that possesses both CMP-Neu5Ac synthetase

and acetylhydrolase activity [20]. Subsequent work by Steenbergen et al. confirms that the native substrate of the *Ec*NeuA C-terminal acetylhydrolase domain is *O*-acetyl-Neu5Ac and thought to regulate *O*-acetylation of the CPS by de-*O*-acetylation of the *O*-acetyl-Neu5Ac or CMP-activated *O*-acetyl-Neu5Ac [18]. Meanwhile a homologous bifunctional NeuA is also found in GBS [21]. The GBS NeuA (*Sa*NeuA) de-*O*-acetylates free *O*-acetyl-Neu5Ac and this activity is enhanced by CTP and Mg^{2+}, the substrate and co-factor of the N-terminal CMP-Neu5Ac synthetase domain respectively. It is proposed that *E. coli* and GBS modulate their capsular *O*-acetylation via two alternate enzymatic pathways: de-*O*-acetylation of Neu5,9Ac$_2$, followed by CMP-activation of Neu5Ac; or, activation of Neu5,9Ac$_2$, then de-*O*-acetylation of CMP-Neu5,9Ac$_2$ [22]. The latter pathway is thought to be a minor pathway to regulate capsular *O*-acetylation in *E. coli* and its function is unknown yet [18].

In this report, we found that the *S. suis neuA* (*SsneuA*) also encodes a bifunctional CMP-Neu5Ac synthetase/*O*-acetylesterase. A combined biochemical and genetic approach was applied to compare the properties of the *S. suis* and *E. coli* *O*-acetylesterase. A live bacterial model was used to study the phenotypes resulting from genetic deletion of the *neuA* and the complemented mutant strains with the *Ec*NeuA esterase domain or *Ss*NeuA esterase domain. Our results demonstrate that the *Ss*NeuA exclusively acts on CMP-*O*-acetyl-Neu5Ac while the *Ec*NeuA and *Sa*NeuA preferentially acts on CMP-*O*-acetyl-Neu5Ac either *in vivo* or *in vitro*, a unique mechanism controlling synthesis of the capsular is proposed in these bacteria causing meningitis. Furthermore, NeuA *O*-acetylesterase activity is required for synthesis of capsular Neu5Ac in *E. coli*.

2. Materials and methods

2.1. Construction of the mutant NeuAs

All mutants of the CMP-Neu5Ac synthetase (NeuA) were generated by PCR with pyrobest DNA polymerase (Takara) using the *S. suis* DNA (Harbin Veterinary Research Institute, Chinese Academy of Agriculture Sciences, Harbin 150001, China.) according to our previous work [20]. The amplified fragments were inserted into the corresponding sites of pET-15b and confirmed by DNA sequencing. Site-directed mutagenesis of the *Ss*NeuA Serine 258 to alanine in pET-b-*Ss*NeuA plasmid and *Ec*NeuA Serine 257 to alanine in pET-b-*Ec*NeuA plasmid were accomplished by GeneTailor™ Site-Directed Mutagensis System (Invitrogen). The expected mutations were also confirmed by DNA sequencing. Primers used in our investigation are summarized in Supplementary Table 1.

2.2. Protein expression and purification

The recombinant plasmid was transformed into *E. coli* BL21(DE3). The resulting transformant was grown at 37 °C in LB broth containing 100 µg/ml ampicillin. When the A$_{600}$ value reaches 0.6, the culture was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 10 h at 25 °C. The cells were harvested and the expressed protein was isolated and purified by Ni Sepharose 6 Fast Flow.

2.3. Activity and protein assay

The CMP-Neu5Ac synthetase activity was assayed using the thiobarbituric acid method [23]. The *O*-acetylesterase activity was assayed by using pNP-Ac as a substrate as described by Yu et al. [21]. Protein concentrations were determined by the method described by Bradford [24]. Bovine serum albumin was used as a standard.

2.4. DMB derivatization and HPLC analysis of the NeuA esterase

Purified enzyme was incubated with Neu5Acs (Prozyme) in 100 mM Tris, pH 7.2, in presence or absence of 5.5 mM CTP and 20 mM MgCl$_2$ at 37 °C for 90 min. Neu5Acs were derived with Signal™ DMB Labeling Kit according to the manufacturer's instruction (Prozyme). Derived Neu5Acs were resolved on a reverse phase Agilent HC-C18 column (4.6-mm internal diameter, 25 cm, 55 µm) and 40-min isocratic elution in 9% acetonitrile and 7% methanol in water at a flow rate of 0.9 ml/min (Agilent 1100 Series HPLC).

2.5. Construction of E. coli mutants and complemented strains

The *neuA*-KO strain was produced constructed as described by Datsenko and Wanner [25] and confirmed by Southern blot. Complemented strains were constructed by transformation of plasmids bearing the *EcneuA*, *SsneuA*, and other mutant genes that were obtained in this study and our previous work [20] into the *neuA*-KO strain, respectively.

2.6. Quantitative analysis of capsular and intracellular Neu5Acs

Capsular Neu5Acs were released from phosphate-buffered saline-washed *E. coli* by 2 N acetic acid. Intracellular Neu5Acs were enriched by precipitation of the cell lysate with 70% ethanol overnight at −20 °C. Neu5Acs were fluorescently labeled with Signal™ DMB Labeling Kit (Prozyme) according to the manufacturer's instruction and resolved by reverse phase HPLC as described above. Peaks were assigned based on retention times of standard Neu5Acs.

3. Results and discussion

3.1. Confirmation of the SsNeuA as a bifunctional protein

Alignment of amino acid sequence of the *S. suis* NeuA (*Ss*NeuA) with its counterpart in *E. coli* or GBS reveals that *Ss*NeuA shares a similarity of 41% and 62% with the *Ec*nNeuA and *Sa*NeuA, respectively. As the case in *E. coli* or GBS, the highly conserved residues present in the active site, substrate-binding pocket and dimerization domain required for CMP-Neu5Ac synthetase (CS) can be found within the N-terminal half (1–210 amino acid residues) of the *Ss*NeuA, while a signature consensus sequence for serine esterase, Gly–Xaa–Ser–Xaa–Gly, is found within the C-terminal half, suggesting that *Ss*NeuA is a bifunctional protein.

The recombinant *Ss*NeuA expressed in *E. coli* was able to produce CMP-NeuAc and degrade pNP-Ac. After purification with Ni Sepharose™ 6 Fast Flow (Table 1), the increase of purification fold of the *O*-acetylesterase activity was found to be almost the same as that of the CS activity. Both esterase and CS activity could be detected when the protein was purified to homogeneity at the final step (Supplementary Fig. 1a). These results clearly confirm that *O*-acetylesterase activity is associated with the *Ss*NeuA protein.

The mutant *Ss*NeuAs terminated at amino acid position of 227, 232, 233, 241, 247, 267, 283, 293, 312, 322, 338, 355 and 377 were inactive in CS activity, even removal of 33 amino acid residues at C-terminal of the *Ss*NeuA led to a complete loss of CS activity, suggesting that CS activity requires a full-length of the *Ss*NeuA. Ser258 is a putative catalytic residue in the consensus sequence of Gly–Xaa–Ser–Xaa–Gly for the *Ss*NeuA esterase, mutation of Ser258 to Ala completely diminished the esterase activity associated with the *Ss*NeuA, while the mutant protein (*Ss*NeuA$_{S258A}$) still remained its CS activity. This finding confirms that CS activity structurally depends on a complete C-terminal domain, which is different from the NeuA in *E. coli*. *Ec*NeuA can be separated into the CS and esterase active polypeptides [20].

Table 1
Purification of recombinant SsNeuA.

Purification step	Total protein (mg)	Total activity (U)		Specific activity (U/mg)		Purification (fold)	
		CS	Esterase	CS	Esterase	CS	Esterase
Cytosol	75.99	1.54	10.73	0.02	0.14	1	1
Ni Sepharose 6 Fast Flow	2.87	0.85	5.31	0.296	1.85	14.81	13.21

Under the assay condition described under Section 2, one unit of the CS activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of CMP-Neu5Ac per minute, while one unit of the *O*-acetylesterase is defined as the amount of enzyme that catalyzes the formation of 1 μmol of pNP per minute. CS, CMP-Neu5Ac synthetase; esterase, *O*-acetylesterase.

Meanwhile, the mutant SsNeuAs truncated from 166 to 233 amino acids at the N-terminal were active for pNP-Ac and their esterase activities were almost the same as the wild-type, only with one exception of the SsNeuA$_{167-410}$ (Supplementary Table 2). These results demonstrate that esterase domain is composed of 177 amino acid residues at C-terminal of the SsNeuA and its activity does not structurally depend on the CS domain.

3.2. Characterization of the bifunctional SsNeuA

Since the minimal active domain of the esterase was found to be SsNeuA$_{234-410}$, the purified native SsNeuA and SsNeuA$_{234-410}$ were characterized (Supplementary Fig. 1). The SsNeuA exhibited its highest synthetase activity at 30–37 °C in a buffer of pH7–10 (Fig. 1A and B). After incubation at 40 °C for 1 h, the SsNeuA still remained 70% of its synthetase activity (Fig. 1C). Its km for Neu5Ac was determined as 1.9 mM.

As for esterase activity toward pNP-Ac, both SsNeuA and SsNeuA$_{234-410}$ showed the highest activity at 25 °C in a buffer of pH8–8.5 (Fig. 1D and E). The km of the SsNeuA and SsNeuA$_{234-410}$ toward pNP-Ac was determined as 10 mM and 7.2 mM, respectively. After incubation at 40 °C for min, the SsNeuA$_{234-410}$ still remained 50% of its esterase activity, prolonged incubation for 1 h

led to 90% loss of esterase activity. While the SsNeuA only remained 10% of its esterase activity at 40 °C after 5 min (Fig. 1F). It appears that, although the SsNeuA$_{234-410}$ exhibits properties similar to those of the native SsNeuA, the esterase domain alone is more stable than the one associated with the native SsNeuA.

It has been shown that C-terminal hydrolase domain of the *Ec*-NeuA is resistant to trypsin [20]. In this study, we also treated the SsNeuA with trypsin. As shown in Supplementary Fig. 2a, the SsNeuA could be degraded by trypsin to yield two polypeptides, 29 kDa and 17 kDa. When the degraded products were recovered and subjected to N-terminal sequencing, it turns out that trypsin cleavage site was the peptide bond between 163 and 164 or 164 and 165 residue (Supplementary Fig. 2b). Thus, the 29-kDa of product was a peptide composed of 163–410 or 164–410, while the 17-kDa of peptide was composed of 1–163 or 1–164 residues at the N-terminal. In *E. coli*, the NeuA synthetase domain is completely degraded by trypsin, only the C-terminal esterase domain is resistant to trypsin, however, this trypsin-resistant fragment is inactive [20]. Unlike its counterpart in *E. coli*, the SsNeuA was cleaved into two fragments, in which the 29-kDa protein recovered from SDS–PAGE still remained its *O*-acetylesterase activity and gave a specific activity of 1.8 U/mg, which is the same as that of the SsNeuA before proteolysis. Although its physiological consequence is unclear yet,
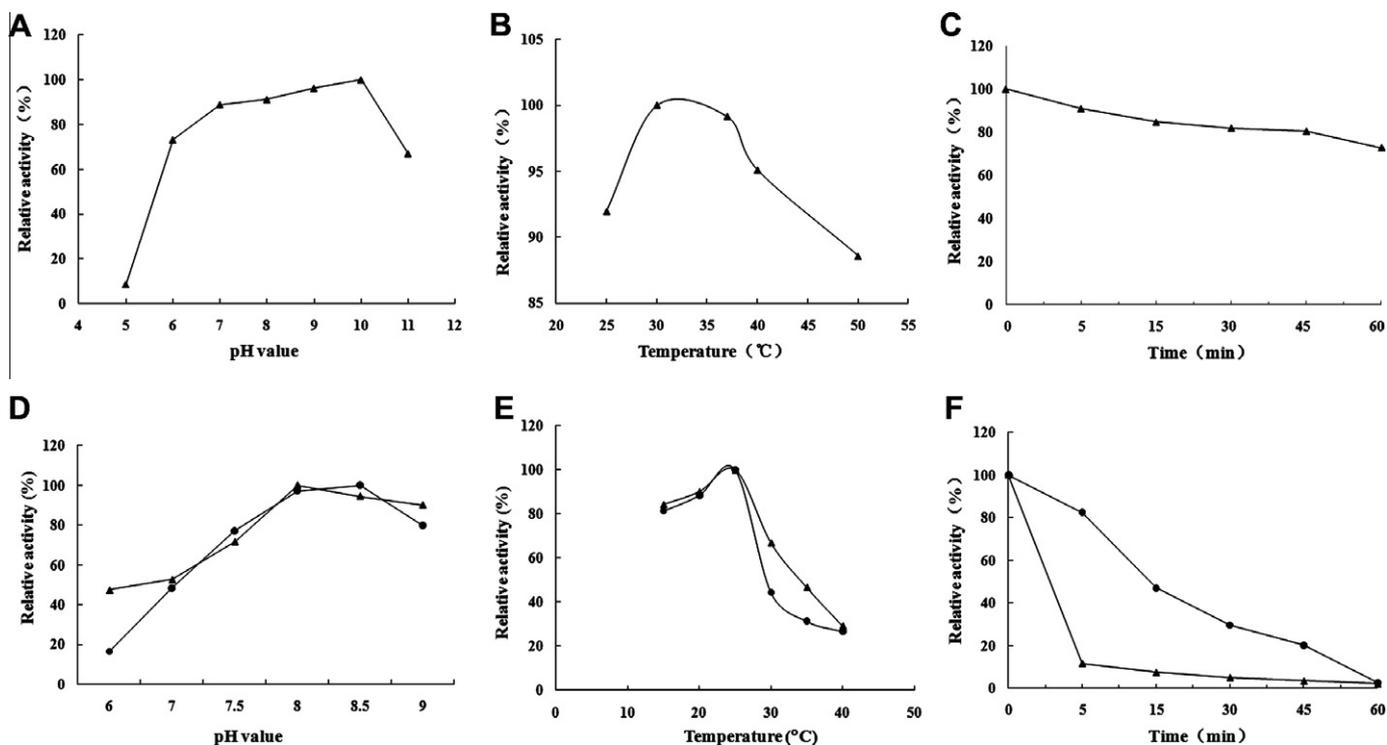

Fig. 1. Comparison of general properties of the SsNeuA and SsNeuA$_{234-410}$. (A) optimal pH of the SsNeuA synthetase activity; (B) optimal temperature of the SsNeuA synthetase activity; (C) thermostability of the SsNeuA synthetase activity; (D) optimal pH of the SsNeuA and SsNeuA$_{234-410}$ *O*-acetylesterase activity; (E) optimal temperature of the SsNeuA and SsNeuA$_{234-410}$ *O*-acetylesterase activity; (F) thermostability of the SsNeuA and SsNeuA$_{234-410}$ *O*-acetylesterase activity. ▲, SsNeuA; ●, SsNeuA$_{234-410}$.

it is interesting to note that the esterase domain in either EcNeuA or SsNeuA is resistant to proteolysis.

3.3. De-O-acetylation catalyzed by the SsNeuA

Since the native substrate of the E. coli and GBS NeuA is monomeric O-acetyl-Neu5Ac [18,22]. We also tested the SsNeuA and SsNeuA$_{234-410}$ with monomeric O-acetyl-Neu5Ac. The SsNeuA did not show any detectable activity toward O-acetyl-Neu5Ac. When CTP and Mg^{2+} were added to the assay mixture, the SsNeuA was able to de-O-acetylate O-acetyl-Neu5Acs (Fig. 2A, left panel). In case of the SsNeuA$_{234-410}$, addition of CTP and Mg^{2+} did not trigger any de-O-acetylation, however, when the SsNeuA$_{S258A}$, a mutant enzyme devoid of O-acetylesterase activity, was added to the assay mixture containing CTP and Mg^{2+}, the activity toward O-acetyl-Neu5Acs could be detected (Fig. 2A, right panel). We also tested O-acetylesterase activity of the EcNeuA and EcNeuA$_{228-418}$. Both EcNeuA and EcNeuA$_{228-418}$ could directly hydrolyze O-acetyl-Neu5Acs (Fig. 2B). Activity of the EcNeuA toward O-acetyl-Neu5Acs was enhanced by the addition of CTP and Mg^{2+}, while no activity was detected with the EcNeuA$_{228-418}$, suggesting a higher activity of the EcNeuA toward CMP-O-acetyl-Neu5Ac.

Our results clearly demonstrate that SsNeuA esterase activity depends on its CS activity, suggesting that native substrate of the SsNeuA esterase is CMP-O-acetyl-Neu5Ac, instead of O-acetyl-Neu5Ac. While the EcNeuA preferentially acts on CMP-O-acetyl-Neu5Ac. Similar result has been reported in GBS [22]. It appears that S. suis adopts one enzymatic pathway to produce its capsular Neu5Ac by de-O-acetylation of the activated O-acetyl-Neu5Ac. Both EcNeuA and SaNeuA are thought to modulate their capsular O-acetylation via two pathways: de-O-acetylation of O-acetyl-Neu5Ac followed by CMP-activation of Neu5Ac, or activation of O-acetyl-Neu5Ac and then de-O-acetylation of CMP-O-acetyl-Neu5Ac [22]. In E. coli, the O-acetylesterase associated with the Ec-NeuA recycles most monomers to Neu5Ac before incorporation into polymer. The NeuD, an O-acetyltransferase responsible for O-acetylation of monomeric Nue5Ac in E. coli K1, and the NeuA esterase are proposed to be a minor pathway and play only a minor role in capsule modification [18]. However, the function of this proposed minor pathway is unknown.

3.4. The role of the NeuA in E. coli

In order to define the role of the NeuA O-acetylesterase in capsule synthesis, we analyzed de-O-acetylation catalyzed by the NeuA either in vitro or in vivo. As mentioned above, SsNeuA O-acetylesterase only utilized CMP-O-acetyl-Neu5Ac as its substrate in vitro, which is distinct from the EcNeuA or SaNeuA. To explore in vivo role of the NeuA esterase activity, we deleted the neuA gene in E. coli to obtain the neuA-KO strain and then complemented the mutant strain with genes encoding for the EcNeuA, SsNeuA, EcNeuA esterase domain or SsNeuA esterase domain, respectively. Deletion of the EcneuA gene led to a complete loss of both activities of CMP-Neu5Ac synthetase and O-acetylesterase in vivo. Either EcneuA or SsneuA could restore both CMP-Neu5Ac synthetase and O-acetylesterase activities in the complemented strains. However, the activities in the complemented strain were found to be different from those in the wild-type E. coli strain (Supplementary Table 3).

Furthermore, DMB-HPLC analysis of both intracellular (free) and extracellular (capsular) Neu5Acs in the mutant strains showed that deletion of the neuA resulted in a significant accumulation of intracellular Neu5Ac, in which around 96% were O-acetyled (Fig. 3B), and a complete loss of extracellular Neu5Ac (Fig. 3A); while only 8% of extracellular Neu5Ac was detected as O-acetyl form and no intracellular O-acetyl Neu5Ac was detected in the wild type (Fig. 3B). The strain complemented with the SsneuA or EcneuA showed similar results as compared with the wild type, indicating that the wild type SsneuA or EcneuA was able to restore capsular Neu5Ac. On the other hand, the strain complemented with the genes encoding for various functional domains showed different intracellular levels of O-acetyl-Neu5Ac. As shown in Fig. 3B, the strain expressing the SsNeuA$_{234-410}$ or SsNeuA$_{167-410}$ exhibited the same O-acetyl-Neu5Ac level as the neuA-KO strain; while the strain expressing the EcNeuA$_{228-418}$ had lower Neu5Ac O-acetyl level (67%) than the neuA-KO strain (96%). These results demonstrated that S. suis O-acetylesterase domain alone was unable to act on intracellular O-acetyl-Neu5Ac in E. coli, whereas the E. coli O-acetylesterase domain was able to act on O-acetyl-Neu5Ac, which is consistent with our in vitro observations. These results also clearly demonstrate that SsNeuA strictly catalyzes de-O-acet-
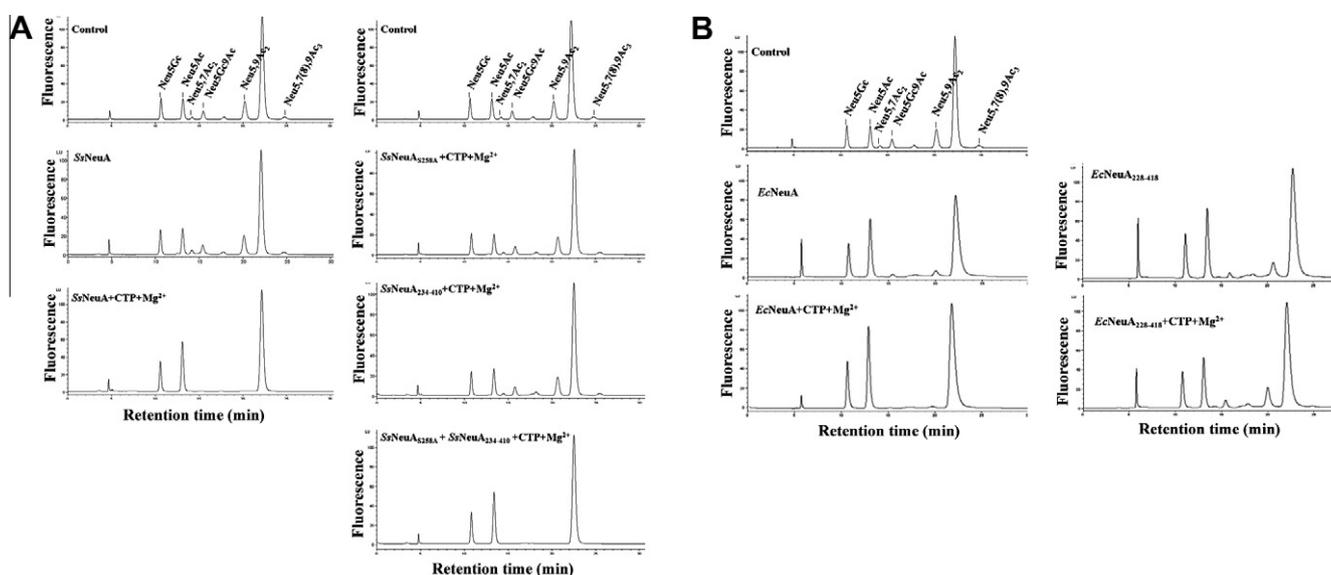

Fig. 2. De-O-acetylation of monomeric O-acetyl-Neu5Acs by the SsNeuA O-acetylesterase and the EcNeuA O-acetylesterase. A commercial available mixture of Neu5Gc, Neu5Ac, Neu5,7Ac$_2$, Neu5Gc9Ac, Neu5,9Ac$_2$, and Neu5,7(8),9Ac$_3$ were used as substrate in this assay (Glyko). For each assay, control was set by inactivating the enzyme in boiling water prior to addition to the reaction mixture. (A) His-tagged SsNeuA, SsNeuA$_{234-410}$, and SsNeuA$_{S258A}$ and (B) His-tagged EcNeuA and EcNeuA$_{228-418}$ were purified by Ni Sepharose™ 6 Fast Flow. Enzyme assays were performed in 100 mM Tris–Cl, pH 7.2, with 0.009 nmol of enzyme and 0.25 nmol of substrate and allowed to proceed for 90 min at 37 °C followed by DMB derivatization and HPLC analysis.
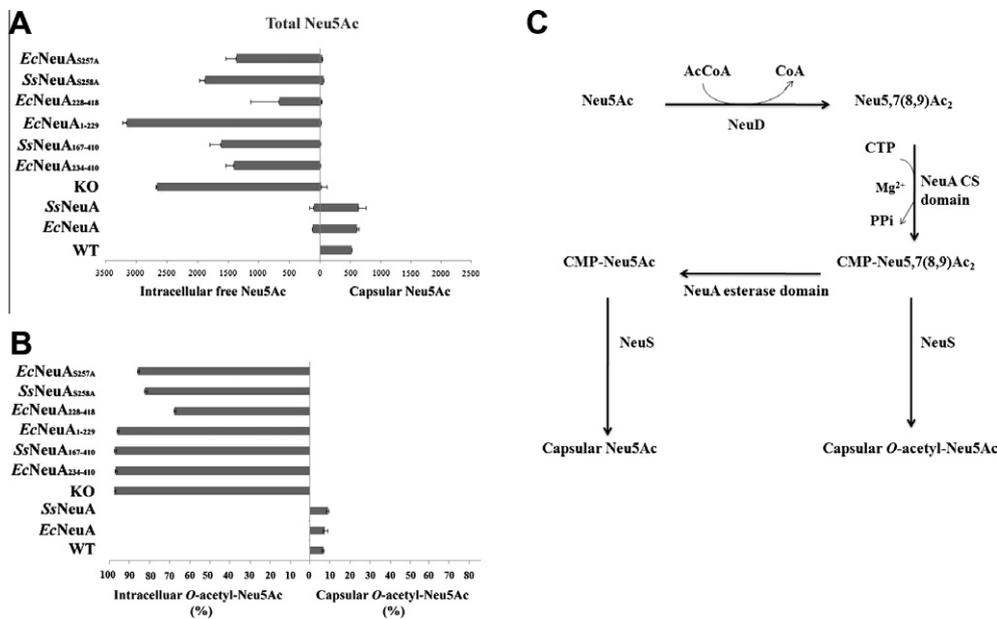
Fig. 3. Intracelluar and extracellular Neu5Ac (A) and O-acetyl-Neu5Ac (B) in *E. coli* strains and a model for O-acetylation of the capsular Neu5Ac in *E. coli* (C). Elimination of the NeuA (both CMP-Neu5Ac synthetase and esterase domains) in *E. coli* was accomplished as described under Section 2 to produce the *neuA*-KO strain. Mutant strain was complemented with various genes. Intracellular and extracellular Neu5Acs were isolated as described under Section 2. The amount of Neu5Ac and percentage of O-acetyl Neu5Ac were determined by DMB-HPLC analysis. Bars: S.D. of three independent experiments.

ylation of CMP-O-acetyl-Neu5Ac, while *Ec*NeuA preferentially catalyzes de-O-acetylation of CMP-O-acetyl-Neu5Ac.

The complemented strains only expressing the active CS domain, such as EcNeuA$_{1-229}$, EcNeuA$_{S257A}$, and SsNeuA$_{S258A}$, exhibited an accumulation of intracellular O-acetyl-Neu5Ac and were unable to produce either capsular O-acetyl-Neu5Ac (Fig. 3B) or capsular Neu5Ac (Fig. 3A). In contrast to the minor pathway proposed by Steenbergen et al. [18], our results imply that the O-acetylesterase is essential for the CPS biosynthesis. It appears that the strains only expressing the active CS domain was unable to directly use free Neu5Ac in *E. coli*. But this is not the case for *Sa*NeuA, O-acetylesterase activity is not required for capsular sialic acid synthesis in GBS.

Based on these observations, we conclude that capsular Neu5Ac is synthesized from de-O-acetylation of CMP-O-acetyl-Neu5Ac, which is come from O-acetylation of free Neu5Ac followed by activation of O-acetyl-Neu5Ac, instead of direct activation of free Neu5Ac in *E. coli* (Fig. 3C). Therefore, the NeuA O-acetylesterase together with the NeuD should be the main pathway to produce capsular Neu5Ac, which recycles most CMP-O-acetyl-Neu5Ac to CMP-Neu5Ac before incorporation into polymer, only a small amount of CMP-O-acetyl-Neu5Ac is incorporated into polymer. It is likely that activation of Neu5Ac prior to O-acetyl removal is a fine-tuning mechanism to regulate capsular Neu5Ac in *E. coli*, and probably in GBS and *S. suis* as well. Several lines of evidences have been obtained to support this hypothesis: (i) all complemented strains devoid of O-acetylesterase activity lost their ability to produce capsular Neu5Ac, even non-O-acetylated one (Fig. 3A); (ii) the *S. suis* NeuA strictly de-O-acetylates CMP-O-acetyl-Neu5Ac; (iii) although the *E. coli* and GBS NeuA can de-O-acetylate both O-acetyl-Neu5Ac and CMP-O-acetyl-Neu5Ac, they preferentially de-O-acetylate CMP-O-acetyl-Neu5Ac; (iv) it has been shown that CMP-O-acetyl-Neu5Ac is not effective substrates for some sialyltransferases [26].

In conclusion, for the first time, we showed that the *Ss*NeuA strictly de-O-acetylated CMP-O-acetyl-Neu5Ac and the *Ec*NeuA preferentially de-O-acetylated CMP-O-acetyl-Neu5Ac. The O-acetylesterase associated with the NeuA plays a key role in capsule

synthesis by controlling the *in vivo* concentration of CMP-Neu5Ac. Therefore, the NeuA O-acetylesterase is essential for the CPS synthesis in *E. coli*. Our findings are key to understanding the biological functions of capsular Neu5Ac in *E. coli*, probably in *S. suis* and GBS as well, and may provide a new target for drug or vaccine development.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.092.

References

[1] F. Berthelot-Herault, M. Gottschalk, H. Morvan, M. Kobisch, Dilemma of virulence of *Streptococcus suis*: Canadian isolate 89–91 characterized as a virulent strain using a standardized experimental model in pigs, Can. J. Vet. Res. 69 (2005) 236–240.

[2] H. Yu, H. Jing, Z. Chen, H. Zheng, X. Zhu, H. Wang, S. Wang, L. Liu, R. Zu, L. Luo, N. Xiang, H. Liu, X. Liu, Y. Shu, S.S. Lee, S.K. Chuang, Y. Wang, J. Xu, W. Yang, Human *Streptococcus suis* outbreak, Sichuan, China, Emerg. Infect. Dis. 12 (2006) 914–920.

[3] K.S. Willenburg, D.E. Sentochnik, R.N. Zadoks, Human *Streptococcus suis* meningitis in the United States, N. Engl. J. Med. 354 (2006) 1325.

[4] H.F. Wertheim, H.D. Nghia, W. Taylor, C. Schultsz, *Streptococcus suis*: an emerging human pathogen, Clin. Infect. Dis. 48 (2009) 617–625.

[5] N. Charland, J. Harel, M. Kobisch, S. Lacasse, M. Gottschalk, *Streptococcus suis* serotype 2 mutants deficient in capsular expression, Microbiology 144 (Pt 2) (1998) 325–332.

[6] H.E. Smith, M. Damman, J. van der Velde, F. Wagenaar, H.J. Wisselink, N. Stockhofe-Zurwieden, M.A. Smits, Identification and characterization of the cps locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor, Infect. Immun. 67 (1999) 1750–1756.

[7] L. Benga, M. Fulde, C. Neis, R. Goethe, P. Valentin-Weigand, Polysaccharide capsule and suilysin contribute to extracellular survival of *Streptococcus suis*

co-cultivated with primary porcine phagocytes, Vet. Microbiol. 132 (2008) 211–219.

[8] M.R. Van Calsteren, F. Gagnon, S. Lacouture, N. Fittipaldi, M. Gottschalk, Structure determination of *Streptococcus suis* serotype 2 capsular polysaccharide, Biochem. Cell. Biol. 88 (2010) 513–525.

[9] E.J. McGuire, S.B. Binkley, The structure and chemistry of colominic acid, Biochemistry 3 (1964) 247–251.

[10] A.K. Bhattacharjee, H.J. Jennings, C.P. Kenny, A. Martin, I.C. Smith, Structural determination of the sialic acid polysaccharide antigens of *Neisseria meningitidis* serogroups B and C with carbon 13 nuclear magnetic resonance, J. Biol. Chem. 250 (1975) 1926–1932.

[11] M.R. Wessels, C.E. Rubens, V.J. Benedi, D.L. Kasper, Definition of a bacterial virulence factor: sialylation of the group B *streptococcal* capsule, Proc. Natl. Acad. Sci. USA 86 (1989) 8983–8987.

[12] F. Orskov, I. Orskov, A. Sutton, R. Schneerson, W. Lin, W. Egan, G.E. Hoff, J.B. Robbins, Form variation in *Escherichia coli* K1: determined by O-acetylation of the capsular polysaccharide, J. Exp. Med. 149 (1979) 669–685.

[13] A.K. Bhattacharjee, H.J. Jennings, C.P. Kenny, A. Martin, I.C. Smith, Structural determination of the polysaccharide antigens of *Neisseria meningitidis* serogroups Y, W-135, and BO1, Can. J. Biochem. 54 (1976) 1–8.

[14] A.L. Lewis, V. Nizet, A. Varki, Discovery and characterization of sialic acid O-acetylation in group B *Streptococcus*, Proc. Natl. Acad. Sci. USA 101 (2004) 11123–11128.

[15] M.R. King, S.M. Steenbergen, E.R. Vimr, Going for baroque at the *Escherichia coli* K1 cell surface, Trends. Microbiol. (2007) 196–202.

[16] A.K. Bergfeld, H. Claus, U. Vogel, M. Muhlenhoff, Biochemical characterization of the polysialic acid-specific O-acetyltransferase NeuO of *Escherichia coli* K1, J. Biol. Chem. 282 (2007) 22217–22227.

[17] S. Weiman, S. Dahesh, A.F. Carlin, A. Varki, V. Nizet, A.L. Lewis, Genetic and biochemical modulation of sialic acid O-acetylation on group B *Streptococcus*: phenotypic and functional impact, Glycobiology 19 (2009) 1204–1213.

[18] S.M. Steenbergen, Y.C. Lee, W.F. Vann, J. Vionnet, L.F. Wright, E.R. Vimr, Separate pathways for O acetylation of polymeric and monomeric sialic acids and identification of sialyl O-acetyl esterase in *Escherichia coli* K1, J. Bacteriol. 188 (2006) 6195–6206.

[19] A.L. Lewis, M.E. Hensler, A. Varki, V. Nizet, The group B streptococcal sialic acid O-acetyltransferase is encoded by neuD, a conserved component of bacterial sialic acid biosynthetic gene clusters, J. Biol. Chem. 281 (2006) 11186–11192.

[20] G. Liu, C. Jin, CMP-N-acetylneuraminic acid synthetase from *Escherichia coli* K1 is a bifunctional enzyme: identification of minimal catalytic domain for synthetase activity and novel functional domain for platelet-activating factor acetylhydrolase activity, J. Biol. Chem. 279 (2004) 17738–17749.

[21] H. Yu, W. Ryan, X. Chen, Characterization of a bifunctional cytidine 5'-monophosphate N-acetylneuraminic acid synthetase cloned from *Streptococcus agalactiae*, Biotechnol. Lett. 28 (2006) 107–113.

[22] A.L. Lewis, H. Cao, S.K. Patel, S. Diaz, W. Ryan, A.F. Carlin, V. Thon, W.G. Lewis, A. Varki, X. Chen, V. Nizet, NeuA sialic acid O-acetylesterase activity modulates O-acetylation of capsular polysaccharide in group B *Streptococcus*, J. Biol. Chem. 282 (2007) 27562–27571.

[23] W.F. Vann, R.P. Silver, C. Abeijon, K. Chang, W. Aaronson, A. Sutton, C.W. Finn, W. Lindner, M. Kotsatos, Purification, properties, and genetic location of *Escherichia coli* cytidine 5'-monophosphate N-acetylneuraminic acid synthetase, J. Biol. Chem. 262 (1987) 17556–17562.

[24] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[25] K.A. Datsenko, B.L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products, Proc. Natl. Acad. Sci. USA 97 (2000) 6640–6645.

[26] H.H. Higa, J.C. Paulson, Sialylation of glycoprotein oligosaccharides with N-acetyl-, N-glycolyl-, and N-O-diacetylneuraminic acids, J. Biol. Chem. 260 (1985) 8838–8849.