Characterization of a novel N-acetylneuraminic acid lyase favoring N-acetylneuraminic acid synthesis

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N-Acetylneuraminic acid lyase (NAL, E.C. number 4.1.3.3) is a Class I aldolase that catalyzes the reversible aldol cleavage of N-acetylneuraminic acid (Neu5Ac) from pyruvate and N-acetyl-D-mannosamine (ManNAc). Due to the equilibrium favoring Neu5Ac cleavage, the enzyme catalyzes the rate-limiting step of two biocatalytic reactions producing Neu5Ac in industry. We report the biochemical characterization of a novel NAL from a “GRAS” (General recognized as safe) strain C. glutamicum ATCC 13032 (CgNal). Compared to all previously reported NALs, CgNal exhibited the lowest k_{cat}/K_{m} value for Neu5Ac and highest k_{cat}/K_{m} values for ManNAc and pyruvate, which makes CgNal favor Neu5Ac synthesis the most. The recombinant CgNal reached the highest expression level (480 mg/L culture), and the highest reported yield of Neu5Ac was achieved (194 g/L, 0.63 M). All these unique properties make CgNal a promising biocatalyst for industrial Neu5Ac biosynthesis. Additionally, although showing the best Neu5Ac synthesis activity among the NAL family, CgNal is more related to dihydrodipicolinate synthase (DHDPS) by phylogenetic analysis. The activities of CgNal towards both NAL’s and DHDPS’ substrates are fairly high, which indicates CgNal a bi-functional enzyme. The sequence analysis suggests that CgNal might have adopted a unique set of residues for substrates recognition.

Sialic acids are a family of 9-carbon amino sugars involved in the modulation of various biological processes. Among more than 50 structurally distinct sialic acids that have been found in nature, N-acetylneuraminic acid (Neu5Ac) is the most ubiquitous form. Neu5Ac has great potential in pharmaceutical and food industry: Neu5Ac is a potential raw material in the synthesis of anti-influenza drugs to prevent both influenza types A and B infections, and it is also an important additive in dairy products for its ability to strengthen the immunity of infants.

N-acetylneuraminic acid lyase (NAL, EC 4.1.3.3) has been employed in industrial production of Neu5Ac and it catalyzes the reversible aldol condensation of Neu5Ac from N-acetyl-D-mannosamine (ManNAc) and pyruvate (Fig. 1a). NAL is ubiquitously distributed in nature, such as bacteria and mammals (human, pig). Pathogenic bacteria colonized human respiratory tract and gut to utilize sialic acid as carbon and nitrogen source and disruption of the NAL gene could severely reduce the virulence of Vibrio vulnificus. In addition to pathogens, NAL was also reported from generally regarded as safe (GRAS) organisms, such as Lactobacillus plantarum WCFS1 and Taphylococcus carnosus TM300.

In order to understand its function and reaction mechanism, crystal structures of E. coli NAL (EcNal, PDB ID: 1NAL, 1FDY, 1FDZ and 1HL2), Haemophilus influenzae NAL (PDB ID: 1FSZ) and Pasteurella multocida NAL (PDB ID: 4ICM, 4IMD, 4IME and 4IMF) were solved and studied. NAL belongs to aldolase Class I superfamily, which has a classic (α/β)8 barrel profile, and activated as a homotetramer. The NAL family shares a unifying mechanism as showing in EcNal. The catalytic Lys165 of EcNal forms schiff base with α-keto moiety of pyruvate, and the highly conserved Tyr137 is associated with aldol cleavage/condensation step. Motif GXXGE (Gly46-Glu50 in EcNal) and the residues Asp191, Glu192 and Ser208 contribute to substrate recognition. Other members of the superfamily include dihydrodipicolinate synthase (DHDPS), D-5-keto-4-deoxyglucarate dehydratase (KDGDH), trans-o-hydroxybenzylidenepyruvate hydrolase-aldolase (HBPDA),...
trans-2'-carboxybenzalpyruvate hydratase-aldolase (CBPHA) and 2-keto-3-deoxygluconate aldolase (KDGA)\textsuperscript{18,22–24}. Among them, DHDPS (EC 4.3.3.7) is a member that shows the highest similarity with NAL. It catalyzes the first step in lysine biosynthesis and condensed L-aspartate-β-semialdehyde (ASA) with pyruvate to synthesize dihydrodipicolinate (Fig. 1b)\textsuperscript{15} The homology of Nal and DHDPS has been evidenced by a single mutation in \textit{E. coli} NAL (L142R) to shift NAL’s activity towards DHDPS\textsuperscript{17}.

As an important biocatalyst for \textit{in vitro} chemoenzymatic synthesis of Neu5Ac and its derivatives, recombinant NAL has been broadly applied with either \textit{E. coli}\textsuperscript{25} or plants\textsuperscript{26} as host. While naturally, NAL primarily functions to regulate intercellular sialic acid metabolism in mammalian cells; some microorganisms use NAL to catabolize sialic acid for a carbon and energy source\textsuperscript{19}. Through a long process of evolution, natural selection is more inclined to have a NAL with relatively high Neu5Ac cleavage ability. Therefore, all reported NAL to date suffered a common drawback: The equilibrium of the reversible reaction favors Neu5Ac cleavage rather than synthesis\textsuperscript{11,13,14,27–29}, which might lead to low yield and low efficiency for industrial production of Neu5Ac.

In this study, we report a novel NAL (CgNal) from a “GRAS” organism \textit{Corynebacterium glutamicum} ATCC 13032, which shows dual functions as NAL and DHDPS. More importantly, as NAL, CgNal shows a unique property of favoring Neu5Ac synthesis in the reversible reaction. In addition, the recombinant CgNal obtained a high expression level and achieved high production yield of Neu5Ac. These characteristics make CgNal a promising biocatalyst in industrial Neu5Ac biosynthesis processes.

**Results**

**Cloning, expression and purification of CgNal.** CgNal gene of \textit{C. glutamicum} ATCC 13032 (GenBank accession: NP_601846.1) encoding a 939 bp open reading frame, which corresponds to 312 amino acids, was cloned into pET-28a (+) vector with an N-terminal His6-tag (pET28a-CgNal). The \textit{E. coli} strain harboring pET28a-CgNal exhibited the highest CgNal activity when OD\textsubscript{600} reached 0.5 with 0.2 mM IPTG as inducer at 30°C (Fig. S1). Cultured under the optimal condition, CgNal was expressed mostly in soluble form. The purified CgNal showed a single band on SDS-PAGE image, corresponding to the molecular weight of CgNal (~33 KDa) (Fig. 2). When cultured and induced under the same conditions, CgNal showed higher expression level than EcNal as revealed by SDS-PAGE (Fig. 2). Up to 480 mg of purified CgNal could be obtained from 1 liter of \textit{E. coli} Rosetta (DE3) pLysS culture, which is 2.23 fold higher than the highest expression level reported for LpNal (215 mg/L cell culture)\textsuperscript{14} and 4.33 fold higher than EcNal which was cultivated and purified under the same conditions. This advantage of CgNal over EcNal would make CgNal industrially more competitive than EcNal, which is commonly used in the industry.

**Biochemical characterization of recombinant CgNal.** CgNal catalyzes a reversible aldo-cleavage/synthesis reaction. The effects of pH on CgNal were determined considering both Neu5Ac cleavage and synthesis activities. Interestingly, CgNal prefers more alkaline conditions (Fig. 3a) than most other NALs reported. In the Neu5Ac cleavage direction, the optimum pH of the CgNal was around pH 8.2 to 8.4, while it reached optimal Neu5Ac synthesis activity at pH 8.6. These values are obviously higher than the values described for other NALs, such as those from \textit{E. coli}\textsuperscript{28}, \textit{P. multocida}\textsuperscript{29}, \textit{C. perfringens}\textsuperscript{27}, \textit{S. carnosus} TM300\textsuperscript{14} and \textit{L. plantarum} WCF511\textsuperscript{13}. None of those NALs obtain the optimum pH above pH 8.0 in either Neu5Ac synthesis or cleavage direction (Table 1).

In contrast to other NALs, CgNal is much more pH sensitive. For both Neu5Ac synthesis and cleavage reactions, CgNal showed sudden enhancement in its activities when the pH increased from 8 to 8.5. And the Neu5Ac synthesis activity was even higher than Neu5Ac cleavage activity when pH reached pH 8.8. This is the first time to...
report a NAL that favors aldo-condensation rather than cleavage. Considering this sharp change of CgNal’s activity above pH 8, many CgNal characters were assayed at both pH 7.5 and pH 8.5.

The optimum temperature of CgNal was measured over a broad temperature range (30°C–65°C) at pH 7.5 and pH 8.5 for both Neu5Ac synthesis and cleavage directions (Fig. 3b). At pH 7.5 both directions adopted 40°C as the optimal reaction temperature, while at pH 8.5 the optimal temperature was 40°C and 45°C for Neu5Ac synthesis and cleavage direction, respectively. At both pHs, CgNal’s activities dramatically decreased on both directions when temperature was above 50°C. The optimal temperature of CgNal is much lower than 65°C of EcNal,65–70°C of C. perfringens NAL,65–70°C of S. carnosus TM300 NAL,14 and 70°C of L. plantarum WCFS1 NAL18 (Table 1), indicating that CgNal is not as thermo stable as other NALs.

The above results are in agreement with the following thermo stability studies (Fig. 3c). When CgNal was incubated at 40°C, pH 8.5 for 48 h, in Neu5Ac synthesis direction, CgNal kept 100% activity for the first 24 h but only remained 60% of its activity in 48 h. In the meanwhile, Neu5Ac cleavage activity decreased even faster than Neu5Ac synthesis activity. Only 75% and 20% of cleavage activity remained in 24 h and 48 h, respectively. Interestingly, although CgNal is less thermo stable than other NALs, for the reversible aldo-cleavage/synthesis reaction, Neu5Ac synthesis would be the main reaction for CgNal in long time catalysis.

The effects of metal ions or detergents on the enzymatic activities were tested on both Neu5Ac synthesis and cleavage at pH 7.5 and pH 8.5. We used the CgNal’s Neu5Ac synthesis/cleavage activity at each pH without any metal ions’ or detergents’ addition as the standard to characterize the metal ions’ or detergents’ effects, respectively. For CgNal Neu5Ac synthesis activity, metal ions showed opposite effects at pH 7.5 and pH 8.5 (Fig. 4a). All metal ions, except Zn²⁺, activated the Neu5Ac synthesis at pH 7.5, while all metal ions inhibited the Neu5Ac synthesis at pH 8.5. There was no trend for the detergents’ effects. EDTA showed activation activity at pH 7.5. TritonX-100 activated the Neu5Ac synthesis by 49.5% at pH 8.5. CTAB and SDS inhibited the synthesis activity on both pH.

The effects of metal ions or detergents on the enzymatic activities were tested on both Neu5Ac synthesis and cleavage at pH 7.5 and pH 8.5 (Table 1). For Neu5Ac synthesis activity, both ManNAc and pyruvate were tested as substrates. Consistent with the results above, kinetic parameters of CgNal were significantly affected by pH. At pH 7.5, CgNal did not show significant differences in \( k_{cat} \) values, but the \( K_m \) values favored Neu5Ac synthesis. The CgNal \( K_m \) for Neu5Ac was found to be 33.5 mM, which is an order of magnitude higher than NALs from other organisms, meanwhile, the \( K_m \) for ManNAc and pyruvate was 53.3 mM and 14.7 mM, which is only 1/3–1/4 and half of the values from other organisms, respectively. This is the first time that the \( K_m \) values for Neu5Ac, ManNAc and pyruvate are in the same magnitude. This trend is more pronounced at pH 8.5. The \( K_m \) values for Neu5Ac, ManNAc and pyruvate were 87.7 mM, 92.1 mM and 72.4 mM, respectively. More interestingly, at pH 8.5 CgNal showed much higher activities for both Neu5Ac cleavage and synthesis. The \( k_{cat} \) values for Neu5Ac, ManNAc and pyruvate were 44.2 S⁻¹, 40.7 S⁻¹ and 42.6 S⁻¹, respectively. The \( K_m \) value for Neu5Ac cleavage was 4.4 fold of that from Lactobacillus plantarum19, which is the highest \( k_{cat} \) value reported. And the \( k_{cat} \) value for Neu5Ac synthesis was an order of magnitude higher than that from all other organisms. As a result, the \( k_{cat}/K_m \) values for CgNal for ManNAc and pyruvate were 0.44 s⁻¹mM⁻¹ and 0.59 s⁻¹mM⁻¹, respectively, while \( k_{cat}/K_m \) value for Neu5Ac is 0.50 s⁻¹mM⁻¹, on Neu5Ac cleavage. Compared to all previously reported NALs, CgNal exhibited the lowest \( k_{cat}/K_m \) value for Neu5Ac and highest \( k_{cat}/K_m \) value for ManNAc and pyruvate, which makes CgNal favor Neu5Ac synthesis the most.

**Sequence, structural and phylogenetic analysis.** In spite of its high NAL activity, the protein sequence of CgNal was annotated in GenBank (accession: NP_601846.1) as a putative DHDPs. Actually, CgNal showed low sequence identities with either EcNal (23.7%) (PDB ID: 1NAL, 1FDY, 1FDZ and 1HL2)15–17 or EcDHDPS (26.4%). It showed only 22.8% identity with Haemophilus influenzae NAL (PDB ID: 1F5Z)16, 26.4% identity with E. coli DHDPs (PDB ID: 1DFP)30 and 22.9% identity with C. glutamicum ATCC 13032 DHDPs (PDB ID: 3CPR).23 Sequence alignment showed that CgNal contained conserved catalytic sites (Tyr138, Lys166) and the GXXGE motif (Gly47, Gly50 and Gly51) of NAL family (Table 2, Fig. S2). However, significant differences occurred at specific residues for NALs’ substrate recognition. Asp191, Gly192, Ser208 of EcNal are responsible for Neu5Ac recognition and these three amino acids are conserved across all other NAL in the alignment (Fig. S2). Different residues were present at Neu5Ac recognition site of CgNal (Glu198, Thr199 and Val212). In the meanwhile, for DHDPs’ substrate recognition, CgNal (Ser143, Gly196 and Glu198) showed higher similarity with EcDHDPS (Arg138, Gly186 and Asp188) (Fig. S5, Table 2).

Although, the identity of primary structure between CgNal and other NALs was fairly low, the predicted CgNal secondary structure showed much higher similarity with that of EcNal (Fig. S2). CgNal shared the common (β/α)₂-barrel and the C-terminal extension of
| Table 1 | Enzyme characters and Kinetic parameters of NAL from different organisms |
|---------|----------------------------------------------------------------------------|
|         | Neu5Ac Cleavage | Neu5Ac Synthesis |
|         | Neu5Ac | ManNAc | Pyruvate | Neu5Ac | ManNAc | Pyruvate |
|         | Kₘ (mM) | Vₘₐₓ (U/mg) | kₗₓ/Kₘ (s⁻¹ M⁻¹) | Optimum pH | Optimum Temperature (°C) | Kₘ (mM) | Vₘₐₓ (U/mg) | kₗₓ/Kₘ (s⁻¹ M⁻¹) | Kₘ (mM) | Vₘₐₓ (U/mg) | Kₗₓ (s⁻¹ M⁻¹) | Optimum pH | Optimum Temperature (°C) | References |
| C. glutamicum ATCC 13032 (pH 7.5) | 33.50 | 16.74 | 9.30 | 0.28 | 8.4–8.8 | 40 | 53.30 | 10.20 | 5.67 | 0.11 | 14.70 | 10.98 | 6.10 | 0.41 | 8.2–8.4 | 40 | This study |
| C. glutamicum ATCC 13032 (pH 8.5) | 87.70 | 79.60 | 44.20 | 0.50 | 45 | 92.10 | 73.20 | 40.67 | 0.44 | 72.40 | 76.64 | 42.58 | 0.59 | 40 | This study |
| Escherichia coli (pH 7.5) | 3.50 | 151.10 | 83.11 | 23.75 | 287.1 | 35.28 | 19.4 | 0.07 | 206.1 | 40.42 | 22.23 | 0.11 | 40 | This study |
| Escherichia coli | 3.60 | 154.50 | 84.97 | 23.60 | 7.7 | 75 | - | - | 180 | 16.36 | 9 | 0.05 | 22 | 3.24 | 1.80 | 0.08 | - | - | [28] |
| Pasteurella multocida | 4.90 | 29 b | 16 | 3.27 | 7.5–8.0 | 220 | 20 b | 11 | 0.05 | 23 | 3.44 b | 1.90 | 0.08 | 7.5–8.0 | - | - | [29] |
| Clostridium perfringens TM300 | 3.20 | 27.50 | 16.04 | 5.01 | 7.6 | 65–70 | - | - | - | - | - | - | - | - | - | - | - | [27] |
| Lactobacillus plantarum WCFS1 | 1.80 | 18.27 | 10.08 | 5.60 | 7.5 | 70 | 160 | 8.72 | 4.80 | 0.03 | 19.90 | 3.96 | 2.19 | 0.11 | 7.50 | 60 | [13] |

Calculated according to Vₘₐₓ and MW = 33 KDa;
Calculated according to kₗₓ and MW = 33 KDa;
Calculated according to Vₘₐₓ and MW = 35 KDa;
Calculated according to kₗₓ and MW = 35 KDa.
several α-helices, which are usual in the NAL family, including NAL, DHDPS, KDGDH, HBPHA and other related enzymes. Obtaining 26.4% identity with EcDHDPS and 23.7% identity with EcNal, the phylogenetic analysis of CgNal would be very interesting. The phylogenetic tree of NAL and DHDPS was constructed by Mega 5.2 with NAL superfamily member KDGDH from *Pseudomonas putida* (GenBank accession: P42233.1) defined as the outgroup (Fig. 5). Not surprisingly, the phylogenetic tree could be divided into two groups, group 1 for NAL and group 2 for DHDPS (Fig. 5). But what do surprise us was that unlike previously reported NAL from *Clostridium perfringens*, *Escherichia coli*, *Pig*, *Pasteurella multocida*, *Staphylococcus carnosus* TM300, *Lactobacillus plantarum* WCFS1, *Trichomonas vaginalis*, *Haemophilus influenzae*, CgNal had higher similarity with DHDPS and it was classified into the DHDPS group. Sequence alignments of group 1 and group 2 were shown in Fig. S3 and the DHDPS group showed more conserved residues than the NAL group.

**Substrate specificity of CgNal.** Although CgNal showed very high NAL activity, it is more belonging to DHDPS according to sequence, structural and phylogenetic analysis. So we compared the enzyme activities of CgNal, EcNal and EcDHDPS with both substrates of NAL and DHDPS (Fig. 6a). Neu5Ac and ManNAc were used for characterizing NAL’s activities in Neu5Ac synthesis and cleavage directions, respectively. ASA was used for characterizing their DHDPS activities. CgNal, EcNal and EcDHDPS were active to both DHDPS’ and NAL’s substrates. Using ASA as substrate, CgNal showed only half the activity of EcDHDPS but twice the activity of EcNal. For NAL activity analysis, in Neu5Ac synthesis direction, CgNal showed higher activity than EcNal, while in the Neu5Ac cleavage direction, the activity of CgNal was much lower than EcNal. This special property made CgNal a potential enzyme for industrial synthesis of Neu5Ac.

**Discussion**

Enzymes from aldolase Class-I superfamily share a common structural framework of (α/β)₈ barrel, but catalyze different reactions on separate biochemical pathways. Among all the members of this superfamily, NAL and DHDPS are best-characterized. NAL takes in charge of the regulation of intracellular sialic acid by catalyzing the cleavage of Neu5Ac to produce ManNAc and pyruvate, while DHDPS catalyzes the aldol condensation of pyruvate and ASA, which is the first step in the biosynthesis of lysine via the diaminopimelate pathway. In this paper, we describe the cloning, expression and biochemical characterization of a novel NAL (CgNal) from the “GRAS” organism *C. glutamicum* ATCC 13032, which shows bi-function as NAL and DHDPS.

According to the primary sequence alignment, CgNal obtains 26.4% identity with EcDHDPS and 23.7% identity with EcNal. The phylogenetic analysis shows that CgNal is evolutionarily more related to DHDPS (Fig. 5). But the enzyme activity assay with different substrates of NAL (Neu5Ac synthesis/cleavage) and DHDPS illustrates that CgNal obtains the higher enzymatic activity as NAL in both cleavage and synthesis directions than that as DHDPS. More importantly, compare to all previously reported NALs, CgNal has higher conversion speed and conversion rate towards Neu5Ac synthesis, showing great potential in biotechnological application for industrial synthesis of sialic acid.

Neu5Ac is an important 9-carbon amino sugar with huge market demand in pharmaceutical and food industry and NAL catalyzes the rate-limiting step for Neu5Ac synthesis. NALs catalyze the reversible aldol cleavage of Neu5Ac to form pyruvate and ManNAc. For the reversible reaction, all the NALs reported previously favored the Neu5Ac cleavage, resulting in the low yield for industrial Neu5Ac synthesis. CgNal illustrates the highest conversion speed and conversion rate towards Neu5Ac synthesis. At pH 7.5, the $k_{cat}$ values of CgNal for Neu5Ac and ManNAc are similar to other NALs, but $k_{cat}$ for pyruvate is about 3 times to others. At pH 8.5, the turnover rate increases dramatically for all substrates. The $k_{cat}$ values for Neu5Ac, ManNAc and pyruvate are 44.2 s⁻¹, 40.7 s⁻¹ and 42.6 s⁻¹, which are 3~20 times to those of other NALs. CgNal exhibits low affinity to Neu5Ac, which is the substrate for cleavage reaction. $K_m$ values of CgNal are 33.5 mM and 87.7 mM at pH 7.5 and pH 8.5, respectively. It is an order of magnitude higher than the $K_m$ values of NALs, which are 3.5 mM, 4.9 mM, 3.2 mM, 2 mM and 1.8 mM from *E. coli*, *P. multocida*, *C. perfringens*, *S. carnosus* TM300 and *L. plantarum*.

| enzyme | catalysis | DHDPS Sugar Recognition |
|--------|-----------|-------------------------|
| CgNal  | Tyr138    | Gly196, Gly198, Thr199  |
|        | lys166    | Val212                  |
| EcNal  | Tyr137    | Gly189, Asp191, Gly192  |
|        | lys165    | Ser208                  |
| EcDHDPS| Tyr133    | Gly186, Asp188          |
|        | lys161    | (Ala189)                |

**Table 2 | Comparison of key residues of CgNal, EcNal and EcDHDPS**
WCFS1 respectively (Table 1). Compared to other NALs, CgNal's affinities to ManNAc and pyruvate, the substrates for Neu5Ac synthesis, are relatively high. The $K_m$ values of CgNal are 53.3 mM and 92.1 mM to ManNAc at pH 7.5 and pH 8.5, respectively, which are only 1/4 to half of the $K_m$ values of NALs from other organisms. Therefore, at pH 8.5 CgNal shows the best enzymatic efficiency on Neu5Ac synthesis. The $k_{cat}/K_m$ values of CgNal for ManNAc and pyruvate are 0.44 s$^{-1}$mM$^{-1}$ and 0.59 s$^{-1}$mM$^{-1}$, respectively, on Neu5Ac synthesis, while $k_{cat}/K_m$ value for Neu5Ac is 0.50 s$^{-1}$mM$^{-1}$ on Neu5Ac cleavage. Compared to the previously reported NALs, whose $k_{cat}/K_m$ value for Neu5Ac cleavage is one or two orders of magnitude to those for Neu5Ac synthesis, CgNal favors Neu5Ac synthesis the most (Table 1). Therefore, CgNal shows extraordinary catalysis properties for Neu5Ac synthesis. 194 g/L Neu5Ac (0.63 M) was obtained from 2 M pyruvate and 0.8 M ManNAc with purified CgNal as catalyst (Fig. 6b), which was the highest among all reported Neu5Ac yield of 12.3 g/L$^{19}$, 19.1 g/L$^{25}$, 18.32 g/L$^{35}$, 59 g/L$^{36}$, 61.3 g/L$^{34}$, 122.3 g/L$^{37}$. Although CgNal illustrates the best properties for Neu5Ac synthesis, the phylogenetic analysis shows that CgNal is evolutionarily more related to DHDPs. As shown in Fig. 5, the phylogenetic tree of CgNal and other putative NAL and DHDPs, were clearly divided into two groups: the NAL group and DHDPs group. CgNal was grouped into the DHDPs group (Fig. 5 and Fig. S3). The alignment of the key residues from each group revealed different patterns in various motifs$^{13}$. The catalytic site in CgNal (Tyr 138 and Lys 166, residues in CgNal responsible for aldo-cleavage) showed homogeneity with both NAL and DHDPs. Slight difference was observed for “GXXGE” carboxylate binding motif. The $\alpha$-keto acid binding motif is highly conserved in all the NALs and the DHDPs subfamily of

**Figure 5** | Phylogenetic tree of CgNal and alignment of key residues. The phylogenetic tree was constructed and visualized with MEGA 5.2$^{29}$. BLAST identified DHDPs and NAL homologous to CgNal to construct the phylogenetic tree, *Pseudomonas putida* KDGDH (GenBank accession: P42233.1) was defined as the outgroup. The details of alignment outputs are shown in the middle of the figure. The schematic diagram showing modulation of residues within the active site of NAL and DHDPs is shown on the right side of the figure.

**Figure 6** | (a) Comparison of enzyme activity with different substrate of CgNal, EcNal and EcDHDPs. (b) Synthesis of Neu5Ac by CgNal and EcNal. In a reaction mixture containing 0.8 M ManNAc, 2 M pyruvate and 0.5 M Tris-HCl (pH 7.5 and pH 8.5) purified EcNal or CgNal was added to initiate the synthesis.
incorporating from were designed according to Vazyme CloneExpress handbook with homology arms on

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Strains, plasmids and chemicals

C. glutamicum ATCC 13032 strain was obtained from ATCC (Manassas, VA). E. coli DH5a and E. coli Rosetta (DE3) pl except strains were from Promega (Shanghai, China). pET-28a(+) vector was from Novagen (Darmstadt, Germany). Restriction enzymes, DNA polymerase, genomic DNA extraction kit, DNA markers, protein markers were from Takara (Dalian, China). CloneExpress one-step cloning kit was from Vazyme (Shanghai, China). N-acetylamino and N-acetylneuraminic acid were from Sigma-Aldrich (Shanghai, China). All other chemicals were of analytical grade and commercially available.

Cloning of CgNal gene, EcDHDPS gene and EcDHDPR gene

Genomic DNA of C. glutamicum ATCC 13032 and E. coli Rosetta (DE3) pl except were isolated as PCR template, respectively, using Takara genomic DNA extraction kit. The CgNal gene sequence (GenBank accession: NP_601846.1), which was deposited in NCBI database as a putative DHDPS, was amplified from C. glutamicum genomic DNA with primers specific for its 5' and 3' ends. The E. coli dapA gene, encoding a dihydrosphosphate reductase (DHDPR) protein, were amplified from E. coli genomic DNA with primers specific for each 5' and 3' ends, incorporating EcoRl and Hindlll restriction sites, respectively. All primers (Table 1) were designed according to Vazyme CloneExpress handbook with homology arms on

β/γδ3 enzymes. But most enzymes of NAL subfamily adopt "GSTGE" motif in NAL group, while CgNal adopts the "GSSGE" motif shared by most of the DHDPS. In contrast, the sugar binding motif of the NAL group and the DHDPS group showed most distinguished differences due to different substrate specificity of these two groups. In NAL group, the Neu5Ac binding sites are relatively conservative. For example, in EcNal, Asp191, Ser208 and Glu192 interact with the hydroxyl groups O6–9. But CgNal equivalents Glu198, Thr199 and Val212 showed higher homogeneity with DHDPS group (Asp188, Ala189 and Val205 of EcDHDPS) (Fig. 5 and Table 2). In EcDHDPS, Asp188 is responsible for interacting with the ammonium group of ASA, while Ala189 and Val205, the equivalents of EcNal residues Glu192 and Ser208, showed no specific interaction with the pyruvate-ASA complex14. Arg138 in EcDHDPS was assigned to interact with the carboxylate group at the C4 position of the bound complex15. Absent in CgNal, Ser143 replaced Arg instead, while a conserved leucine shows in NAL group, which exhibits no interactions with the Neu5Ac (Fig. 5 and Table 2). This phenomenon elucidates that CgNal is phylogenetically more related to DHDPS, but during the evolution, CgNal might adopt a unique set of residues for either Neu5Ac or ASA recognition.

Although there is no three-dimensional structure for either CgNal or CgNal-substrate complex available to confirm our hypothesis, the comparison among enzyme activities with different substrates of NAL (Neu5Ac synthesis/cleavage) and DHDPS supports our assumption. CgNal activities towards the substrates of both NAL (ManNAc and Neu5Ac) and DHDPS (ASA) were assayed with EcNal and EcDHDPS as controls. CgNal, EcNal and EcDHDPS all showed detectable activities towards substrates of NAL and DHDPS (Fig. 6a), possibly because they belong to the same superfamily (with the same catalysis residues). But these aldolases showed different preferences towards the substrates. EcDHDPS demonstrated the highest DHDPS activity and EcNal showed the highest Neu5Ac cleavage activity. With the higher similarity to DHDPS, CgNal performed higher DHDPS activity than EcNal. But still CgNal illustrated high Neu5Ac cleavage activity and the highest Neu5Ac synthesis activity among all three. This result, combined with the analysis of the CgNal residues that are responsible for recognition of NAL's and DHDPS' substrate, indicated that CgNal might adopt a unique set of residues for substrates recognition, favoring Neu5Ac synthesis.

As a unique enzyme showing bi-function as NAL and DHDPS, CgNal illustrated a distinctive set of key residues for substrates recognition. Moreover, the outstanding Neu5Ac synthesis activity of CgNal made it an interesting enzyme for enzymatic research and a promizing catalyst in industrial synthesis of Neu5Ac. Due to the limited information we can extract from sequence alignment, further elucidation of the catalysis mechanism of CgNal can be achieved by resolving the crystal structure of CgNal itself and CgNal-substrate complex in our future study.

Methods

Strains, plasmids and chemicals. C. glutamicum ATCC 13032 strain was obtained from ATCC (Manassas, VA). E. coli DH5a and E. coli Rosetta (DE3) pl except strains were from Promega (Shanghai, China). pET-28a(+) vector was from Novagen (Darmstadt, Germany). Restriction enzymes, DNA polymerase, genomic DNA extraction kit, DNA markers, protein markers were from Takara (Dalian, China). CloneExpress one-step cloning kit was from Vazyme (Shanghai, China). N-acetylamino and N-acetylneuraminic acid were from Sigma-Aldrich (Shanghai, China). All other chemicals were of analytical grade and commercially available.

Effect of induction conditions, including optical density (OD600) before induction, β-D-1-thiogalactopyranoside (IPTG) concentration and induction temperature on CgNal expression were examined. It was performed by changing OD600 between 0.1–1.5, IPTG concentrations between 0.1–0.6 mM and induction temperature between 20°C–40°C, respectively, while other conditions were fixed. Crude enzyme activities for Neu5Ac (OD95) and Neu5Ac (pH 7.5) after centrifugation and sonication were assayed to determine the effect.

Enzyme assay of CgNal and EcNal. Both enzyme activity of CgNal and EcNal was assayed by measuring its ability to condense ManNAc and pyruvate into Neu5Ac (Neu5Ac synthesis) as well as its ability to cleave Neu5Ac into ManNAc and pyruvate (Neu5Ac cleavage). The Neu5Ac synthesis reaction mixture consisted of 0.1 M pyruvate, 0.05 M ManNAc and 0.1 M Tris-HCl (pH 7.5 or 8.5) and the Neu5Ac cleavage reaction mixture contained 0.05 M Neu5Ac and 0.1 M Tris-HCl (pH 7.5 or 8.5). Protein concentration was determined by Bradford method24. CgNal or EcNal was added to 1 mL reaction mixture to final concentration of 130 μM. After incubation at 37°C for 15 min, reactions were terminated by boiling the mixture for 5 min, cooled down to 20°C and filtered at 12,000 × g for 10 min. The filtrate was centrifuged at 4°C for 30 min. The concentrations of the substrates and the products were analyzed by high performance liquid chromatography (HPLC). Samples were analyzed on an Agilent 1200 system equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) using a refractive index detector. The mobile phase consisted of 5 mM H2SO4 at 0.6 mL/min, 55°C. All tests were performed in triplicate and a unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol product per min.

Effects of pH and temperature on CgNal. The effect of pH on CgNal was determined using the following buffers: 0.1 M Tris-HCl (pH 7–8.5) and 0.1 M glycine-NaOH (pH 9). Optimum pH for CgNal was determined by measuring CgNal activities in reaction systems with different pH. Optimum temperature of CgNal was determined by measuring CgNal activities (at pH 8.5) under different reaction temperatures (30°C–65°C). CgNal stability was determined by measuring residual Neu5Ac activities after incubating the enzyme at pH 8.5, 40°C for 48 h.

Effects of salts and detergents on CgNal. Effects of salts and detergents on CgNal were determined by measuring Neu5Ac synthesis and cleavage activities of CgNal in 100 mM Tris-HCl buffer (at pH 7.5 and pH 8.5) in the presence of 5 mM salts and detergents, including CaCl2, NaCl, BaCl2, FeCl3, KCl, ZnCl2, CoCl2, MgCl2, NiCl2, Na2SO4, EDTA, Triton X-100, CTAB and SDS. Reaction mixture without salts and detergents was used as control.

Kinetic parameters of CgNal and EcNal

Kinetic parameters (Km, Vmax) for CgNal and EcNal were assayed by measuring their activities at the presence of various concentrations of substrates. For Neu5Ac synthesis reaction, ManNAc concentration was fixed at 50 mM with pyruvate concentrations varied (20 mM–450 mM) to determine kinetic parameters for pyruvate; and pyruvate concentration was fixed at 100 mM with ManNAc concentration varied (20 mM–450 mM) to determine kinetic parameters for ManNAc. As for Neu5Ac cleavage reaction, Neu5Ac concentration was varied from 1 mM to 200 mM to determine kinetic parameters for Neu5Ac. Km and Vmax were calculated with GraphPad Prism 5.0 (GraphPad, San Diego).
Sequence, structural and phylogenetic analysis. Basic Local Alignment Search Tool (BLAST) of NCBI was used to identify proteins homologous to CgNal, and MEGA 5.2 was used to construct the phylogenetic tree with bootstrap value calculated after 1000 generations. CgNal subunit structure was modeled by Phyre2 server at intensive mode. Sequences were aligned with ClustalW and ESPript.

Substrate specificity of CgNal. CgNal’s activity towards the substrates of both NAL (ManNAc and Neu5Ac) and DHDPS (ASA) were assayed with EcNal and EcDHDPS as controls. The activities of CgNal with ManNAc and Neu5Ac as substrate were assayed as mentioned in Enzyme assay of CgNal section. The activity of CgNal with ASA as substrate was measured using a coupled enzymatic assay as described by Yugari and Givarg. All enzymatic assays were replicated 3 times.

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Corrigendum: Characterization of a novel N-acetylneuraminic acid lyase favoring N-acetylneuraminic acid synthesis

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The original version of this article contained an error in testing the kinetic parameters of CgNal towards pyruvate, in which the concentration of ManNAc (50 mM) was not in excess. This error was corrected by re-running the assay in the presence of excessive ManNAc (180 mM). The corrected kinetic parameters of CgNal towards pyruvate were updated in Table 1 of the manuscript. These changes do not change the conclusions of the article.

Apparent kinetic parameters (Km, Vmax) of CgNal towards pyruvate were re-assayed as follows. ManNAc concentration was fixed at 180 mM with pyruvate concentrations varied (20 mM–450 mM) to determine kinetic parameters for pyruvate. The reaction was run at 37 °C, at pH 7.5 and pH 8.5 and terminated by heat inactivation at 95 °C for 5 min. After centrifugation at 10000 rpm for 10 min at 4 °C, the supernatant was filtered using a 0.22 μm filter. Concentration of Neu5Ac was quantified by HPLC using a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) column with 5 mM sulfuric acid solution as mobile phase. Kinetic parameters were calculated with GraphPad Prism 5.0.

The original and revised kinetic parameters appear below as Table 1 and Table 2 respectively.

| C. glutamicum ATCC 13032 (pH 7.5) | Km (mM) | Vmax (U/mg) | kcat (s⁻¹) | kcat/Km (s⁻¹mM⁻¹) |
|---------------------------------|---------|-------------|-----------|-----------------|
| 14.70                           | 10.98   | 6.10        | 0.41      |
| C. glutamicum ATCC 13032 (pH 8.5) | 72.40   | 76.64       | 42.58     | 0.59            |

Table 1. Original kinetic parameters.

| C. glutamicum ATCC 13032 (pH 7.5) | Km (mM) | Vmax (U/mg) | kcat (s⁻¹) | kcat/Km (s⁻¹mM⁻¹) |
|---------------------------------|---------|-------------|-----------|-----------------|
| 18.85                           | 16.30   | 9.06        | 0.48      |
| C. glutamicum ATCC 13032 (pH 8.5) | 31.28   | 19.43       | 10.80     | 0.34            |

Table 2. Revised kinetic parameters.
In addition, the expression “favoring N-acetylenuraminic acid synthesis”, which was based on the enzymatic reaction under real industrial process conditions (non-equilibrium and non-standard conditions), caused some confusion about “shifting chemical equilibrium”. We have clarified the title and related expressions throughout the manuscript.

1. The title “Characterization of a novel N-acetylenuraminic acid lyase favoring N-acetylenuraminic acid synthesis” now reads “Characterization of a novel N-acetylenuraminic acid lyase favoring industrial N-acetylenuraminic acid synthesis process”.

2. Enzyme kinetic parameters “Km”, “Vmax”, “kcat” and “kcat/Km” now read “apparent Km”, “apparent Vmax”, “apparent kcat” and “apparent kcat/Km” in the main text.

3. In the abstract, “Due to the equilibrium favoring Neu5Ac cleavage...” now reads “Due to the high Neu5Ac cleavage activity in most isozyme forms...” and “...... which makes CgNal favor Neu5Ac synthesis the most” now reads “...... which makes CgNal favor industrial Neu5Ac synthesis process in a non-equilibrium condition”.

4. In the introduction section: “yield” is replaced with “space-time yield” to emphasize the non-equilibrium nature of industrial reactions; “...... of favoring Neu5Ac synthesis in the reversible reaction” now reads “...... of favoring industrial Neu5Ac synthesis in the reversible reaction under an optimized and non-equilibrium condition”.

5. In the “Biochemical characterization of recombinant CgNal” section: “...... a NAL that favors aldo-condensation rather than cleavage” now reads “...... a NAL that favors aldo-condensation rather than cleavage under an optimized condition”.

6. In the “Kinetic parameters of CgNal” section: “The kinetic parameters of CgNal were characterized in both Neu5Ac synthesis and cleavage directions at pH 7.5 and pH 8.5......” now reads “To evaluate the kinetic properties of CgNal, the second-order reaction was simplified as a pseudo first-order reaction by adding excessive amount of one substrate while varying the concentration of the other. To differentiate the values from theoretical kinetic parameters, we named them as apparent kinetic parameters to avoid confusion. The apparent kinetic parameters of CgNal were characterized in both Neu5Ac synthesis and cleavage directions at pH 7.5 and pH 8.5......”; “...... which makes CgNal favor Neu5Ac synthesis the most”. now reads “...... which makes CgNal favor industrial Neu5Ac synthesis process the most. It should be noted that unlike single-substrate reversible reactions, most multi-substrate reversible reactions could never attain equilibrium15. The shift in the apparent kinetic parameters does not imply a change in chemical equilibrium that is only associated with the free energy difference between the substrates and products”.

7. In the discussion section: "CgNal illustrates the highest conversion speed and conversion rate towards Neu5Ac synthesis”. now reads “......CgNal illustrates the highest conversion speed and conversion rate towards Neu5Ac synthesis under an optimized condition”; “yield” is replaced with “space-time yield” to emphasized that non-equilibrium nature of industrial reactions; “......CgNal favors Neu5Ac synthesis the most. Therefore, CgNal shows extraordinary catalysis properties for Neu5Ac synthesis”. now reads “......CgNal favors industrial Neu5Ac synthesis process the most”; “Although CgNal illustrates the best properties for Neu5Ac synthesis” now reads “Although CgNal illustrates the best properties for industrial Neu5Ac synthesis”; “favoring Neu5Ac synthesis” now reads “......favoring industrial Neu5Ac synthesis process”.

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