Infection mechanism of *Edwardsiella piscicida* via glycoconjugates remodeling

(糖鎖リモデリングを介した *Edwardsiella piscicida* の
感染メカニズムの解明)

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Abstract

Edwardsiella piscicida, a gram-negative bacterium, belong to the family Enterobacteriaceae causing serious economic losses to aquaculture. *E. piscicida* produces NanA sialidase which cleaves sialic acids from α(2,3) sia-linked glycoprotein on host cell surface. Bacterial binding abilities to the host cell is dependent on the structure of glycoconjugates. Therefore, the cleavage of terminal sialic acids by bacterial sialidase is the most crucial step for the sequential degradation of host glycoconjugates and reveals binding sites for pathogens that seem plausible as a critical factor in bacterial infection. However, the significance of the removement sialic acid by NanA sialidase on this bacterial pathogenicity remains unclear. In addition, the roles of the resultant released sialic acid and its catabolism in *E. piscicida* pathogenicity remain to be elucidated. Firstly, we studied the relationship between sialidase activity and bacterial invasiveness using several *Edwardsiella* sp. strains. We found that the pathogenic strains showed higher sialidase activity as well as NanA expression level than non-pathogenic strain. The host cell invasion was significantly enhanced by NanA-overexpression *E. piscicida* and suppressed in the presence of the sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA). In addition, sialidase cleaved the terminal sialic acids on host glycoconjugates to unmask the glycoprotein containing *N*-acetylglucosamine and mannose that can be used as an adhesion anchorage. Secondly, we demonstrated that free sialic

acid significantly increased cellular infection toward goldfish scale fibroblast (GAKS) cells via sialic acids metabolic pathways. This study documented the presence of two metabolic pathways in sialic acid utilization, the degradation of sialic acid for use as a carbon source and the pathway of its conjugation on the glycoconjugates in *E. piscicida* as sialylation. Three enzymes involve in the metabolic pathway of sialic acid in *E. piscicida* have been identified including *N*-acetylneuraminate lyase (NAL), dihydrodipicolinate synthase (DHDPS) which removes a pyruvate group from sialic acid to yield *N*-acetylmannosamine, and one *N*-acetylneuraminate cytidyltransferase (CMP-Neu5Ac synthetase) which combined sialic acid with cytidine monophosphate (CMP) to form CMP-Neu5Ac. All these genes were up-regulated in sialic acid-treated *E. piscicida*. Among these enzymes, we focused on two enzymes belong to *N*-acetylneuraminate lyase family: NAL and DHDPS, which play a critical function in regulating of sialic acid catabolism in bacteria. We found that NAL significantly enhanced infection *in vitro* as well as the mortality of zebrafish larvae in bath-infection *in vivo,* whereas DHDPS did not. Moreover, NAL intensified the expression of *E. piscicida* phenotypes including biofilm formation and motility ability. Sialic acid-related genes, *N*-acetylmannosamine kinase (NanK), *N*-acetylmannosamine epimerase (NanE), and *N*-acetylglucosamine 1-phosphate uridyltransferase/ glucosamine-1-phosphate acetyltransferase

(GlmU) were up-regulated accompanied by an increase the level of total *N*-acetylglucosamine via NAL overexpression.

In summary, this study provides the evidence to elucidate the significance of desialylation by sialidase as well as free sialic acid and its catabolism regulated by *N*-acetylneuraminase lyase in the *E. piscicida* pathogenicity.

要　旨

Edwardsiella piscicida は、Enterobacteriaceae に属するグラム陰性細菌であり、水産業に深刻な経済的損失をもたらしている。一般に細菌の宿主細胞への結合能力は、複合糖質の構造に依存することから、シアリダーゼによる末端シアル酸の切断は、細菌感染に重要な糖鎖結合部位を露出させるために重要なステップである。*E. piscicida* は NanA シアリダーゼを産生することが知られているが、NanA によるシアル酸の切断が *E. piscicida* の病原性に与える影響についてはよくわかっていない。また、NanA により遊離したシアル酸の生理的意義についても不明である。

そこで本研究では、複数の菌株を用いて、シアリダーゼ活性と感染度の関係について調べた。その結果、病原性株は非病原性株に比べて高いシアリダーゼ活性と *nanA* 遺伝子発現レベルの亢進を示した。また、NanA を過剰発現させた *E. piscicida* では、宿主細胞への侵入が著しく促進され、それはシアリダーゼ阻害剤の存在下で抑制された。この NanA シアリダーゼは、宿主の糖タンパク質の糖鎖末端のシアル酸を切断し、*E. piscicida* が接着の足場として利用できる *N*-アセチルグルコサミンとマンノースを露出させていた。

また本研究では、遊離シアル酸が *E. piscicida* の培養細胞への感染を有意に増加させることを見出した。この *E. piscicida* には、シアル酸を分解して炭素源として利用する経路と、

複合糖質のシアリル化に利用する 2 つのシアル酸代謝経路が存在する。この経路には、シアル酸からピルビン酸基を除去して *N*-アセチルマンノサミンを生成する *N*-アセチルノイラミン酸リアーゼ（NAL）、およびジヒドロジピコリン酸合成酵素（DHDPS）、さらにシアル酸とシチジン一リン酸（CMP）を結合して CMP-Neu5Ac を生成する N-アセチルノイラミン酸シチジル転移酵素の 3 つが関与する。本研究ではこれらの酵素のうち、細菌のシアル酸異化作用の制御に重要な役割を果たしている *N*-acetylneuraminate lyase family に属する 2 つの酵素（NAL、DHDPS）に注目した。NAL 高発現株は培養細胞への感染、およびゼブラフィッシュ幼生への感染が有意に促進されたが、DHDPS 株では差が認められなかった。さらに、NAL はバイオフィルム形成や運動能などの病原性が著しく亢進していた。この NAL の過剰発現により、シアル酸代謝関連遺伝子である NanE 発現量が増加し、それに伴い遊離および結合型 N-アセチルグルコサミンの含有量が増加した。

以上より本研究では、*E. piscicida* の病原性におけるシアリダーゼによる脱シアル化、遊離シアル酸と N-アセチルノイラミナーゼによる異化の重要性を明らかにした。

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ABBREVIATIONS

bp	Base pairs
cfu	Colony forming unit
DHDPS	Dihydrodipicolinate synthase
GlcNAc	*N*-acetylglucosamine
KDN	3-deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid
LB	Luria Broth
Man	Mannose
ManNAc	*N*-acetylmannosamine
MOI	Multiplicity of infection
NAL	*N*-acetylneuraminate lyase
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-acetylneuraminic acid
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
TSA	Tryptic soy agar
TSB	Tryptic soy broth

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Chapter 1 General background

1.1 *Edwardsiella* genus and *Edwardsiella piscicida*

Edwardsiella was described as a new genus in 1965 and belongs to the family Enterobacteriaceae [1], which is a gram-negative, motile, rod-shaped and intracellular bacterium. This genus is distributed in diverse environments and causes severe infections in a broad range of hosts [2]. Until 2013, the genus comprised three species: *Edwardsiella hoshinae, Edwardsiella ictaluri,* and *Edwardsiella tarda.* Among these species, *E. tarda* have been well studied, show zoonotic potential with a wide range of hosts including freshwater fish, marine fish, birds, reptiles, amphibians and human [3]. *E. ictaluri* causes serious enteric septicemia in commercially raised fish such as tilapia and channel catfish in U.S. [4]. *E. hoshinae* was found in birds and reptiles [2]. Recently, based on genomic information and phylogenetic analysis, the species previously identified as *E. tarda* were reclassified into three different genetic groups: *E. tarda, E. piscicida* and *E. anguillarum. E. piscicida* is isolated from variety fish in freshwater and marine; and contains one type III and one type VI secretion system (T3SS and T6SS) [5]. *E. anguillarum* is a pathogen of eel and distinguishable from the other *Edwardsiella* species by the capacity of the acetoin production from glucose and the fermentation of arabinose [6]. *E. tarda* now describes in association with human or environmental isolates, which do not possess any T3SS and T6SS [5]. All *Edwardsiella* species can

grow in a temperature range from 25 to 37 °C; however, the optimum temperature for growth is 28-30 °C [7].

E. piscicida is a common pathogen in freshwater and marine fish including Japanese flounder, tilapia, carp, mullet, chinook salmon, and striped bass [5]. This bacterium causes severe infections in more than 20 fish host species to date, which induces dermal ulcerations, exophthalmia, hemorrhage of the skin and several internal organs, and erratic swimming [8]. *E. piscicida* is therefore a good model to study intracellular and systemic infection in fish. The disease called as Edwardsiellosis induces high mortality in fish, which bring severe economic losses to aquaculture. Although *E. piscicida* is known as a serious pathogen in fish, its pathogenicity mechanisms are still unclear and remain to be elucidated. Some virulence factors appear to be responsible for *E. piscicida* pathogenesis including T3SS and T6SS, hemolysins, flagellar structure, and chondroitinase [5]. Previous study suggested that the protein-protein interactions between outer membrane proteins (OMP) of *E. piscicida* and proteins in the gills are responsible for bacterial entry into the fish [9].

1.2 Sialic acids

Sialic acids are nine-carbon acidic sugars expressed as terminal monosaccharides of the glycans on the surfaces of various cell types. It has been reported the existence of over 50 different sialic acids [10]. The three main sialic acids found in nature are *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 3-deoxy-*D*-glycero-*D*-galacto-2-nonulopyranosonic acid (KDN). Neu5Ac presents widely among vertebrates, and in certain types of plants, fungi, bacteria, and viruses, whereas Neu5Gc is common in vertebrates but not humans due to a mutation in its synthase. Neu5Gc differs from Neu5Ac only by the presence of a hydroxyl group on the position five (C-5) of the respective carbon rings, while KDN is a deaminated form of Neu5Ac. Sialic acids are generally observed glycosidically linked to either galactose (Gal) as α(2,3)- and α(2,6)-linkage to Gal or as α(2,8)-linked homopolymers known as polysialic acid. Sialic acids contribute essential functions in a various array of physiological, pathological and immunological processes, including organ development, malignancy, immune regulation, microbial binding, and aspects of human evolution [11, 12]. In addition, sialic acids were decorated on cell surfaces that play important roles in a host-pathogen interaction.

Sialic acids can be used by pathogenic bacteria in at least two different ways: coating themselves in sialic acid to escape from the host immune system or using it as a nutrient source. In general, bacteria possess two major routes to acquire sialic acids: *de novo* biosynthesis or acquisition from the environment [13]. Therefore, sialic acids are involved in bacterial ability of adhesion, colonization, survival in host cell, resulting to distribute on bacterial pathogenicity.

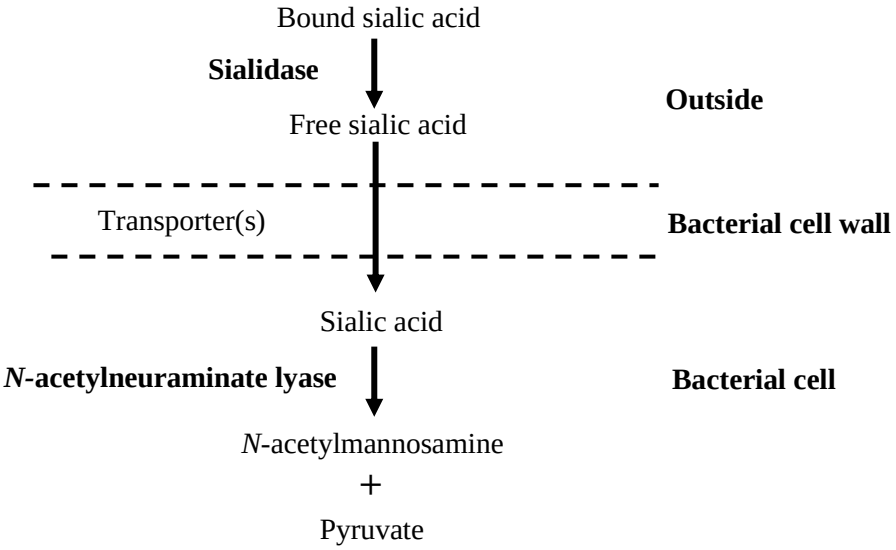

Figure 1-1. Schematic representation of the sialic acid usage pathway by bacteria.

Bacteria express extracellular sialidase allowing removal of sialic acid from bound sialoglycans, followed by importation of released-sialic acid into the cell via sialic acid transporters. Thereafter, sialic acid can be converted to the six-carbon sugar *N*-acetylmannosamine.

1.3 Sialidases

Sialidases, or neuraminidases (EC 3.2.1.18) are enzymes in a class of glycosyl hydrolases that removes terminal sialic acid residues, α-ketosidically linked to oligosaccharide chains of glycoconjugates including glycoproteins, glycolipids, and polysaccharides**.** The cleavage of sialic acids by sialidase is an initial step in the degradation of these glycoconjugates. Sialidases are presented in a wide range from mammals to micro-organisms including fungi, protozoa, bacteria, and viruses [14]. In mammals, sialidases are involved in many biological processes such as cell differentiation, cell growth, and malignant transformation [14]. In bacteria, sialidase can be used to scavenge sialic acids from sialylated glycoconjugates as nutrition for bacterial growth. Most sialidase are produced by pathogens or commensals such as *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Vibrio cholerae*, *Haemophilus influenza* and *Bacteroidetes* [15]. It has been reported that bacterial sialidase with hydrolysis activity contributes to promote biofilm formation and colonization [16]. In addition, sialidase assists with bacteria survival by modifying themselves surface and desialylaytion by sialidase also reveal the glycans receptors for bacterial adherence [17]. Hence, it has been suggested that sialidase play important roles in the interaction of host-pathogen or in the infection to specific tissue.

1.4 *N*-acetylneuraminate lyase

N-acetylneuraminate lyase (NAL, EC 4.1.3.3) is a class I aldolase which catalyzes the reversible cleavage of *N*-acetylneuraminic acid (Neu5Ac) from *N*-acetyl-D-mannosamine (ManNAc) and pyruvate. NAL is the archetype member of a sub-family of $(\beta/\alpha)_8$ barrel enzymes that share unifying mechanism in the reaction pathway by generating a Schiff base between a conserved lysine residue but catalyze reaction in different biological pathways [18, 19]. Other members in the family have been identified including dihydrodipicolinate synthase (DHDPS), D-5-keto-4-deoxyglucarate dehydratase (KDGDH), trans-*O*-hydroxybenzylidenepyruvate hydrolase-aldolase (HBPHA), trans-2′-carboxybenzalpyruvate hydratase-aldolase (CBPHA), and 2-keto-3-deoxygluconate aldolase (KDGA) [19]. Among these members, only NAL and DHDPS (EC 4.3.3.7) are well studied in molecular characterization and DHDPS shows the highest similarity of activity with NAL [20]. Furthermore, NAL activity is involve in the regulation of intracellular sialic acid concentration, whereas DHDPS is involved in the lysine biosynthesis [18]. It has been reported that the enzyme is popularly distributed in various mammals as well as in both pathogenic and non-pathogenic bacteria [21]. NALs have previously been cloned and purified from several bacteria, and the X-ray crystal structures have been determined for *Escherichia coli* (EcNAL), *Staphylococcus aureus* (SaNAL), *Haemophilus influenza* (HiNAL), *Pasteurella multocida* (PmNAL) and *Aliivibrio salmonicida*

(AsNAL) [22]. Since several pathogenic bacteria can utilize sialic acid as a carbon source, the primary function of NAL is to regulate bacterial sialic acid metabolism. Nowadays, an important application of NAL is that the utilization for *in vitro* chemoenzymatic synthesis of Neu5Ac and its derivatives [21]. However, to our knowledge, the physiological function of the enzyme in bacterial pathogenicity remains to be elucidated. In this study, we hypothesized that NAL could play a critical role in *E. piscicida* phenotypes and pathogenicity.

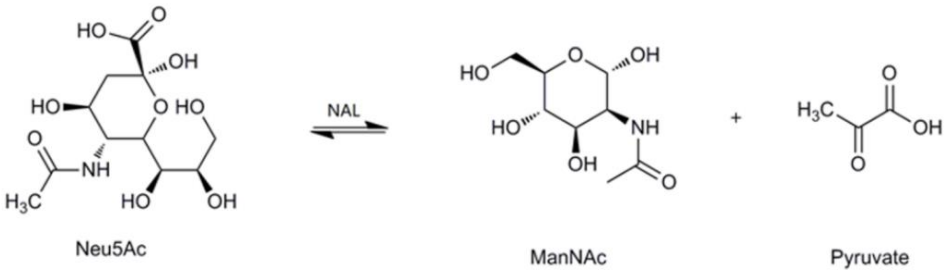

Figure 1-2. The reaction catalyzed by *N*-acetylneuraminate lyase (NAL) (Adopted from Wang et al., 2018).

1.5 Focus of this study

In fish aquaculture, infectious diseases caused by bacteria pose great threat to both freshwater and marine species. Among these bacterial diseases, Edwardsiellosis is a major disease affecting aquaculture, leading the severe economic loss. This disease is caused by *Edwardsiella piscicida* (reclassified from *E. tarda*), *E. ictaluri* and *E. anguilarum* [5]. The newly identified taxa, *E. piscicida*, is considered serious pathogen of cultured fish around the world, especially, in the USA, Asia, and Europe [5]. This emerging pathogen is a member of the Enterobacteriaceae and has been isolated from many fish host species such as channel catfish (*Ictalurus punctatus*), eel (*Anguilla japonica*), Japanese flounder (*Paralichthys olivaceus*), and red sea bream (*Pagrus major*) [24]. Several countries have used antibiotics to control Edwardsiellosis, although a few restrictions caused by development of the multi-antibiotics resistance strains and the policies in each country [8]. In addition, vaccination for the controlling of Edwardsiellosis in commercial aquaculture have been encouraged, but legal restrictions and the cost have to be considered [8]. Therefore, to solve that problem, it is necessary to establish an effective prevention and control strategy for *E. piscicida* regarding the molecular mechanism of its infection.

Glycoconjugates based host-pathogen interactions is important for bacterial attachment and colonization toward the host cells [25]. The cleavage of terminal sialic acid from host cell

glycoconjugates by bacterial sialidase is concerned the most important step for the sequential degradation of host glycoconjugates, thus, its function is involved in bacterial pathogenicity. The previous study indicated that *E. piscicida* possesses NanA sialidase and its enzymatic profile was described [26]. In addition, free sialic acids are known to be utilized by pathogens and commensal bacteria [27]. The gene required for sialic acids associated with bacterial virulence such as NAL which is the first committed enzyme in the sialic acid degradation by bacteria [27, 28]. Hence, this study elucidated *E. piscicida* infection mechanism via glycoconjugates, especially the role of desialylation from host glycoconjugates by the bacterial sialidase in the pathogenicity as well as the roles of NAL in the *E. piscicida* phenotypes such as biofilm formation and motility ability, and pathogenicity.

1.6 Objectives

The current study clarified:

1.6.1 The role of sialic acid removement from host glycoconjugates by NanA sialidase in *Edwardsiella* sp. pathogenicity

1.6.2 The significance of free sialic acid in environment for *Edwardsiella* sp. pathogenicity via its metabolism

1.6.3 The involvement of sialic acid catabolism by its related genes in *Edwardsiella piscicida* phenotypes and pathogenicity

Chapter 2 Significance of sialic acids removement by NanA sialidase from host cell glycoconjugates in *Edwardsiella* sp. pathogenicity

2.1 Introduction

Sialidase is the enzyme which catalyzes the cleavage of sialic acids from the glycans such as glycoproteins, glycolipids, and polysaccharides. This enzyme has been found in many organisms from microorganisms to mammals. Several bacteria produce more than one sialidase as isoenzymes with different biochemical features; however, these biological functions have been still unclear. Bacterial sialidases can recognize sialic acids exposed on the surface of the host cell or scavenge sialic acids from various sialylated substrates [29]. They act toward various linkage such as the α(2,3)-, α(2,6)-, or α(2,8)-sialo-linkage, and diverse range of substrates [17].

Recently, NanA sialidase was identified in *Edwardsiella* sp. which shares low sequence similarities to other known bacterial sialidase [30]. NanA sialidase comprise a sialidase domain and an auto-transporter domain as a pre-mature 74 kDa form, and the auto-transporter domain is cleaved during generating the sialidase mature form. NanA is located on cell surface and modulates the content of glycoconjugates on host cells, that involved in the bacterial binding ability during infection. Our previous study revealed that NanA exhibited the activity toward α(2,3) linked sialic acids carrying sialoglycoconjugates and suggested that NanA-induced desialylation could be essential for exposing the internal mannose (Man) and *N*-acetylglucosamine (GlcNAc) [26].

In bacteria, endogenous sialidases have been known to enhance their virulence by promoting biofilm formation and adhesion to glycoconjugate epitopes on the plasma membrane of host cells [16, 31]. Recent research has shown that an endogenous sialidases is identified to involve in its infection and colonization ability toward Japanese flounder, although the actual mechanism still remains elusive [32]. In addition, sialidase is required to release sialic acids thereby exposing the binding epitopes on host cell surface that may be crucial for bacterial recognition and adhesion.

Herein, to better define the importance of sialic acids removal by NanA sialidase in *Edwardsiella* sp. infection, we utilized several *Edwardsiella* sp. strains expressing different pathogenic ability. Furthermore, we eluciated the relationship between NanA sialidase activity and the bacterial virulence, and the role of unmask-glycoprotein induced by sialic acids cleavage.

2.2 Materials and Methods

2.2.1 Bacterial strain and culture conditions

Several *Edwardsiella* sp. strains with different pathogenicity listed in Table 2-1, were anaerobically grown on tryptic soy broth (TSB) or on tryptic soy agar (TSA) and maintained at 28 °C. To assess bacterial growth, bacteria were adjusted to the same optical density at 630 nm (OD_{630} = 0.2) and cultured in glucose-depleted M9 minimal medium containing various concentrations of sialic acid: 0, 1, 5, and 10 mg/mL.

Table 2-1 Pathogenicity and origin of *Edwarsiella sp.* used in this study

Strain	Pathogenicity	Origin
FPC498	+	Japanese flounder
E381	+	Tilapia
NUF806	+	Japanese flounder
E22	+ (lost)	Eel
SU138	-	Eel

NanA-overexpressing strains was established as follows: NanA-inserted pSF-OXB20 plasmid was stably introduced into the FPC498 and E22 strain by electroporation, followed by selection by a kanamycin (20 μg/mL) supplemented-TSA plate. The mock was prepared using an empty pSF-

OXB20. The promoter in the plasmid is OXB20, a strong constitutive promoter, constructed based on the *E. coli* RecA gene.

2.2.2 Molecular cloning of *nanA* gene

Genomic DNA from *Edwardsiella* sp. strains were extracted by boiling the bacterial pellets for 3 min. The supernatant was obtained by centrifugation at 12000 *g* for 5 min and used as a template for PCR reaction. PCR was carried out using KOD-Plus-NEO (TOYOBO, Japan) with *nanA*-sequence specific primers as follows: *nanA*-5'HindIII (5'-GTAAGCTTCCACCATGCTGATTTTTGCCGA-3') (forward) and *nanA*-3'BamHI (5'-GTGGATCCCTAAAAGGTGTAGGTGAAGCTG-3') (reverse); under the following conditions: the initial denaturation at 94 °C for 2 min; followed by 30 cycles of two-step PCR including denaturation at 98 °C for 10 s; annealing and extension at 68 °C for 2 min. The amplified products were digested with appropriate restriction enzymes, sub-cloned into pBluescript SK (+) plasmid (TAKARA, Japan) and sequenced using the ABI 3130xl Genetic Analyzer (Applied Biosystems, CA).

2.2.3 Infection of cultured cells by bacteria

Goldfish scale fibroblast GAKS cells were obtained from RIKEN CELL BANK. The cells were cultured in Dulbecco modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum at 37 °C under a 5% CO_2 atmosphere.

To evaluate the cellular infection toward GAKS cells, *Edwardsiella* sp. were inoculated directly to the GAKS cell monolayers at the multiplicity of infection (MOI) of 100 in a 12-well plate for 1 h. Thereafter, the GAKS cells were washed twice with PBS and treated with RPMI medium containing 200 μg/mL gentamicin, 200 μg/mL streptomycin and 200 U/mL penicillin for 1 h to kill extracellular bacteria. The antibiotic treatment step was repeated using RPMI medium containing 100 μg/mL gentamicin, 100 μg/mL streptomycin and 100 U/mL penicillin for 1 h. Subsequently, the monolayers were washed thrice with PBS and lysed using 0.5% Triton X-100. The lysates were serially diluted in PBS, plated onto TSA plates, and incubated at 28 °C for 24h. The colonies which were formed on TSA plates counted and used for the evaluation of *Edwardsiella* sp. infection.

To clarify the involvement of desialylation of host cell glycoconjugates in *Edwardsiella* sp. infection, the GAKS cells were preincubated with 50 U/mL of recombinant NanA (rNanA, [26]) at 37 °C for 1 h before infecting by *Edwardsiella* sp. For endogenous NanA sialidase inhibition,

bacteria were preincubated with 2 mM *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA), a non-specific sialidase inhibitor, for 30 min and then inoculated to GAKS cells as described above.

To know the bacterial binding site on host cell surface, bacteria was preincubated with 0.1 mg/mL of asialo-glycopeptide (AGP) for 60 min, followed by infection toward GAKS cells.

2.2.4 Sialidase activity

Edwardsiella sp. strains were cultured in TSB until an optical density at 630 of 0.5 was obtained. The bacteria pellets were collected by 12000 *g* centrifugation and lysed in PBS containing 1% Triton X-100 and proteinase inhibitors (1 mM ethylenediaminetetraacetic acid, 0.5 mM phenylmethylsulfonylfluoride and 10 μg/mL leupeptin). The lysate obtained from 13000 *g* centrifugation were used as a crude enzyme. The protein concentration was determined using the CBB dye binding assay. A reaction mixture comprised 50 mM sodium acetate buffer (pH 5.5), 0.1% Triton X-100, 0.2 mM 4-methyllumbelliferyl-*N*-acetylneuraminic acid (MU-Neu5Ac), and the crude enzyme, and then incubated at 37 ℃ for 1 h. The released sialic acid level was estimated indirectly by quantifying the amount of released MU using a fluorescence spectrophotometer with excitation at 365 nm and emission at 448 nm.

The extracellular sialidase activity in *Edwardsiella* sp. strains was evaluated as followed: bacteria were cultured in M9 medium containing 0.15 M MU-Neu5Ac. The cleaved MU in the medium was continuously observed using a fluorescence spectrophotometer for 24 h.

2.2.5 Real-time PCR

Bacteria RNAs was extracted using Sepasol-RNA I Super G, followed by cDNA synthesis by the ReverTra Ace qPCR RT Master Mix with gDNA remover and used for PCR reactions as templates. Real-time PCR was conducted using SYBR Green KIT with the following conditions: the initial denaturation at 98 °C for 2 min; followed by 40 cycles of three-step PCR including denaturation at 98 °C for 10 s, annealing at 60 °C for 10 s and extension at 68 °C for 30 s; melt curve at 95 °C for 15 s, 60 °C for 60 s and 99 °C for 15 s. The primers used for estimation of the *nanA* gene expression level was designed based on *E. piscicida* genomic sequence of strain CK41 (accession No. CP047671) as follows: 5'-TCGATATCGCCACGAAGCTC-3' and 5'-GGGCACACGCTGACTATTCT-3'. The expression level of 16S rRNA was amplified with the primers 5'-TGCAAGTCGAGCGGTAGCAG-3' and 5'-AACGCTTGCACCCTCCGTAT-3' and used as the reference to determine the RNA quantity and quality among samples.

2.3 Results

In this study, five different *Edwardsiella* sp. strains were used including three pathogenic strains (FPC498, E381, NUF806), one pathogenicity-attenuated strain (E22), and one non-pathogenic strain (SU138). To assess the involvement of sialidase in *Edwardsiella* sp. pathogenicity, NanA sialidase activity was evaluated in each strain using MU-Neu5Ac as a substrate. Interestingly, the group of three pathogenic strains exhibited higher sialidase activity than the group of non-pathogenic strains. Among these pathogenic strains, the E381 strain had the highest sialidase activity (11.2 nmol/h/mg protein) followed by the FPC498 and NUF806 strain (2.1 and 1.1 nmol/h/mg protein, respectively, $p < 0.01$, Figure 2-1). Non-pathogenic strain SU138 possessed the lowest sialidase activity (0.2 nmol/h/mg protein), whereas the sialidase activity in the E22 strain was slightly higher (0.5 nmol/h/mg protein) compared to SU138 (Figure 2-1).

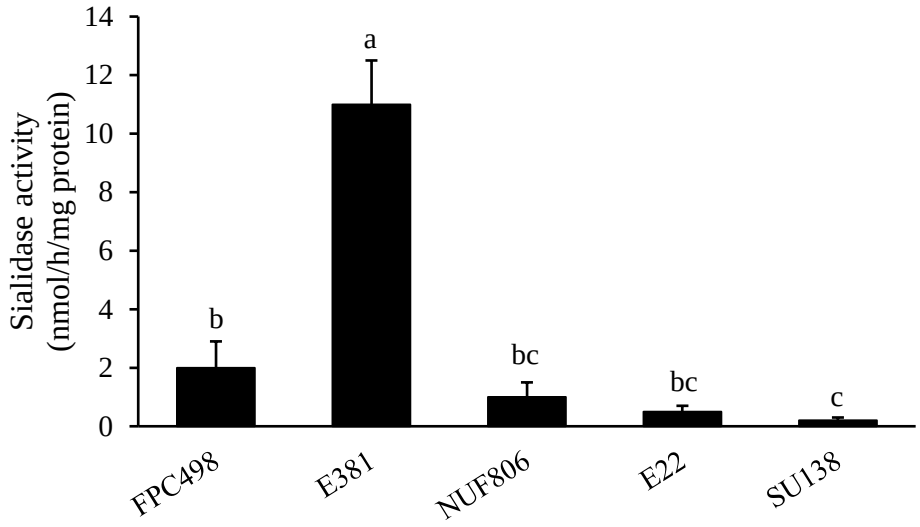

Figure 2-1. Sialidase activity in five different *Edwardsiella* sp. strains

Bacteria was cultured in TSB medium until OD_{630} of 0.5 was obtained and harvested by centrifugation. Bacterial lysates were incubated with MU-Neu5Ac, and the cleaved MU amount was evaluated using a fluorescence spectrophotometer. Significant differences are indicated using letters (Tukey-Kramer test, $p < 0.01$). Columns possess same letter were not statistically different, *vice versa*.

Next, to confirm whether high sialidase activity acts a role in pathogenicity, *in vitro* infection test toward goldfish GAKS cells by *Edwardsiella* sp. strains was conducted. As a result, the E381 strain indicated the greatest infection toward GAKS cells followed by the FPC498, NUF806, and E22 strain, whereas SU138 showed the lowest cellular infection rate (Figure 2-2A).

In addition, the significant correlation between the cellular infection in several *Edwardsiella* sp. strains and their sialidase activities was observed ($p < 0.01$, Figure 2-2B).

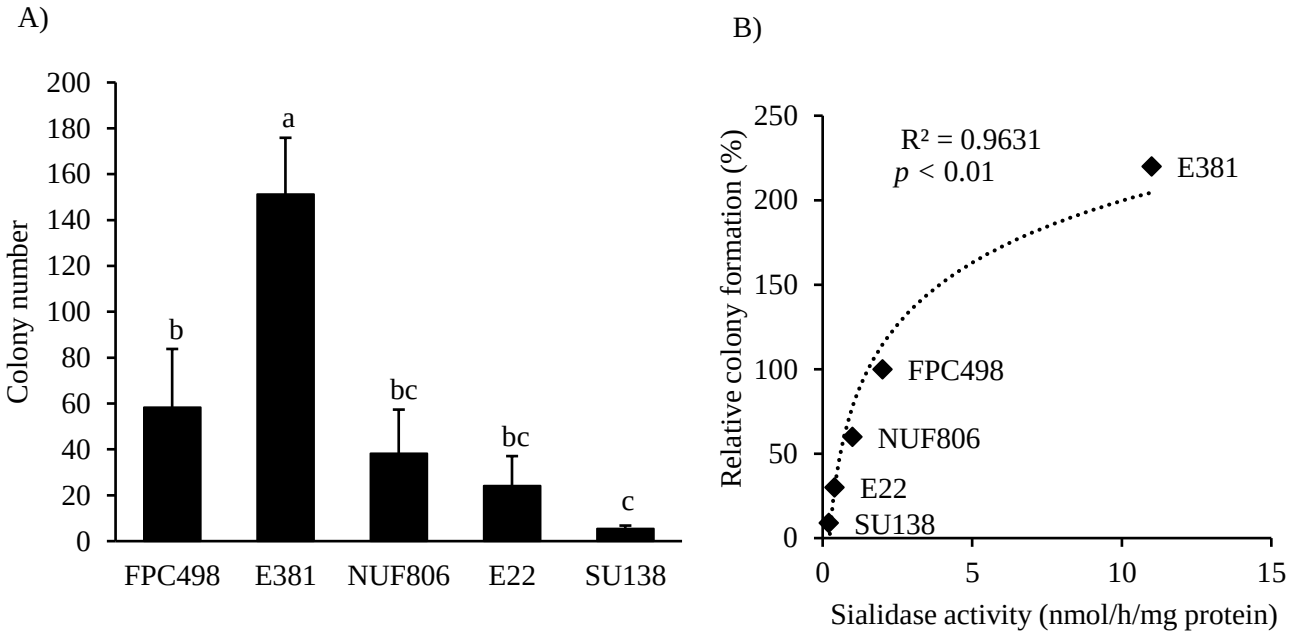

Figure 2-2. Involvement of bacterial sialidase and pathogenicity

A) Infection test using five *Edwardsiella* sp. strains toward GAKS cells. The GAKS cells were infected with the bacteria at MOI of 100, lysed with 0.5% Triton X-100/PBS and plated for colony count. The colony number was expressed as percentage with the FPC498 CFU designated as 100%. Significant differences are indicated using letters (Tukey-Kramer test, $p < 0.01$). Columns possess same letter were not statistically different, *vice versa*. B) Correlation between sialidase activity and infection rate from several strains.

To determine whether NanA sialidase distribute on bacterial infection, GAKS cells were preincubated with 50 U/mL of rNanA before infection test using the E22 strain. We found that the treatment by rNanA considerably enhanced E22 cellular infection compared to control ($p < 0.01$, Figure 2-3A). On the other hand, the infection caused by DANA- preincubated E381 strain toward GAKS cells was drastically suppressed compared to control ($p < 0.01$, Figure 2-3B).

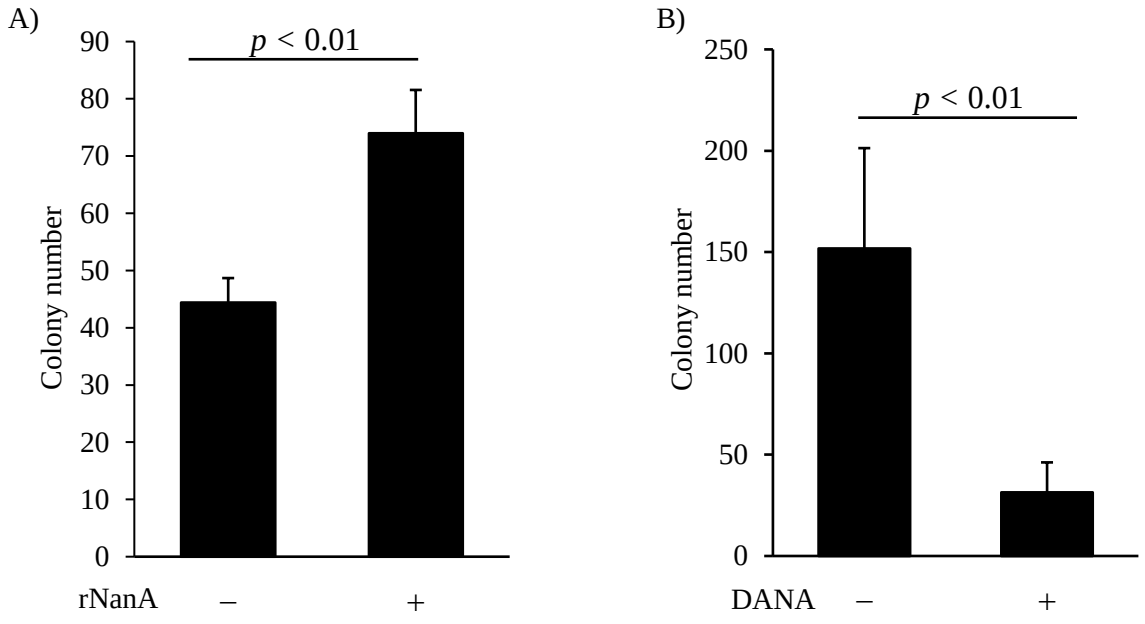

Figure 2-3. Promotion of *Edwardsiella* sp. infection by NanA sialidase

A) GAKS cell was pre-treated with recombinant NanA (rNanA) and then used for E22 infection. B) E381 strain was preincubated with 2 mM DANA, a sialidase inhibitor, and exposed to GAKS cells at MOI of 100. The data were compared using *t*-test.

Moreover, we established the NanA-overexpressing FPC498 and E22 using for infection test toward GAKS cells. The bacterial transfectants showed higher sialidase activity compared to mock (223.1 and 60.0 nmol/h/mg, respectively, Figure 2-4A). Next, to infection test, the NanA transfectants of FPC498 and E22 notably enhanced cellular infection ($p < 0.01$, Figure 2-4B). Taken together, these results propose that NanA sialidase positively regulate *Edwardsiella* sp. infection.

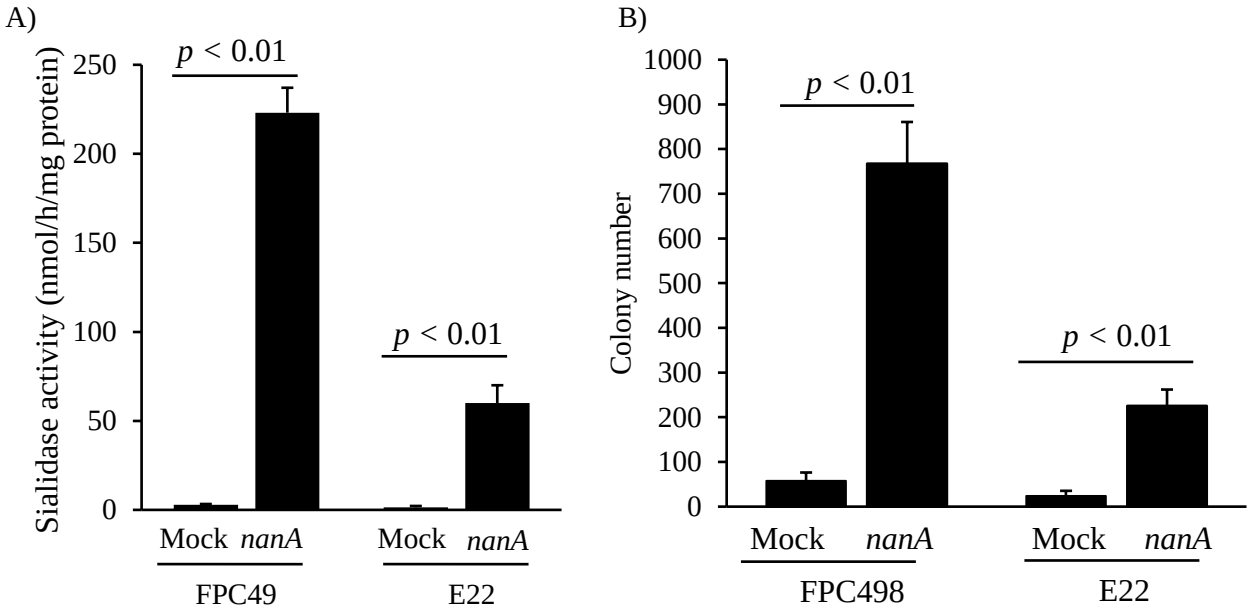

Figure 2-4. Infection toward GAKS cells using NanA-overexpression strains.

A) Sialidase activity of NanA overexpression FPC498 and E22 strains toward MU-Neu5Ac at pH 5.5. B) NanA-overexpression FPC498 and E22 were used for infection experiment to GAKS cells. The data were compared using *t*-test.

Our previous study suggested that the action of desialylation by NanA sialidase which located at the surface of the bacteria, occurs on the glycoprotein of the host cell during infection [26]. To confirm the location of NanA sialidase, the most potent sialidase activity strain, E381, and the weak sialidase activity strain, SU138 were cultured in MU-Neu5Ac-supplemented M9 medium for 24 h and we estimated the released MU amount by a spectrophotometer. We observed that the released MU amount was gradually and strongly increased in the E381 strain for 24 h, whereas the SU138 strain showed the slightly increased MU amount ($p < 0.01$, Figure 2-5).

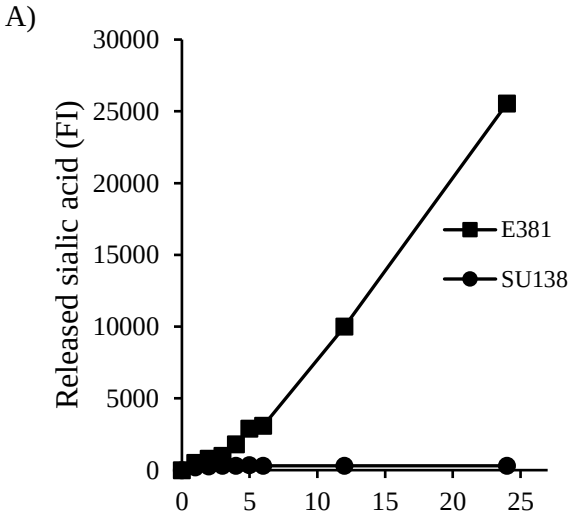

Figure 2-5. Action of NanA sialidase on the host cell surface.

E381 and SU138 strains were cultured in glucose-depleted M9 medium added 0.15 M MU-Neu5Ac. Extracellular sialidase activities of two strains were estimated by analysis of the released MU amount. The values were compared using *t*-test ($p < 0.01$).

To assess whether desialylation by NanA sialidase expose GlcNAc and Man in *N*-glycoprotein as a binding site for the bacteria, the FPC498 strain was preincubated with exogenous asialo-glycopeptide containing GlcNAc and Man (AGP) before infection toward GAKS cells. As expected, pre-incubation of the FPC498 strain with AGP, acting as a decoy, showed the suppression of GAKS cells infection ($p < 0.05$, Figure 2-6B). The result suggests that the binding of the bacteria to AGP reduced its attachment to host cell.

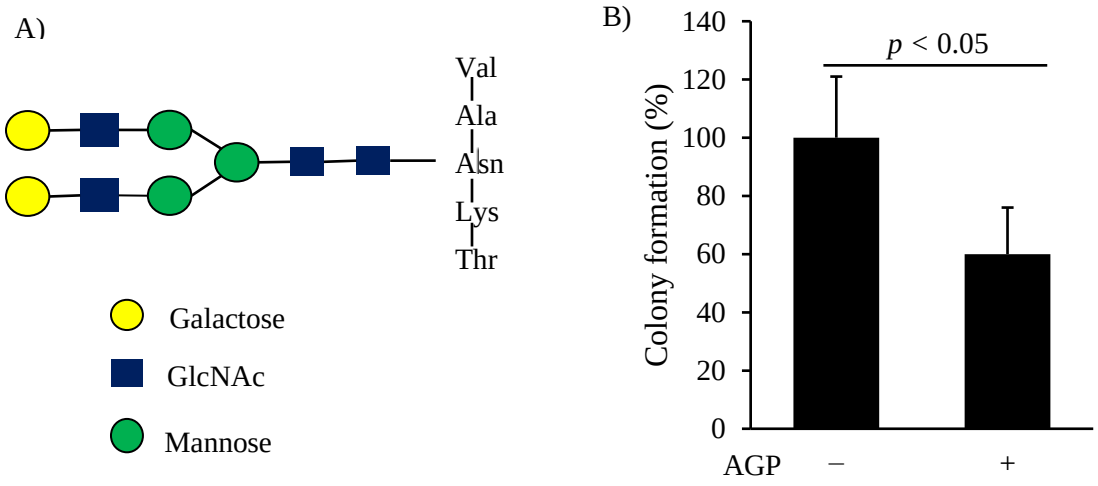

Figure 2-6. Identification of GlcNAc and mannose in *N*-glycoprotein as a binding site for the bacteria.

A) Structure of asialo-glycopeptide (AGP) used as a decoy in binding inhibition assay. B) FPC498 strain was preincubated in 0.1 mg/mL AGP-added medium before infection test.

To clarify whether the difference of NanA sialidase enzymatic activity in several *Edwardsiella* sp. strains involves in the synonymous mutations in each strain, NanA genes were cloned and identified the amino acid sequence. We determined that no mutation was observed in the FPC498, E22 and SU138 strains; however, some mutations were found including E588A in the E381 strain and 637E_670F deletion in the NUF806 strain. All these mutations were identified in the auto-transporter domains and no mutation was detected in the sialidase domains. As the auto-transporter domain is cleaved during generating the sialidase mature form, synonymous mutations in E381 and NUF806 may not be relative in the modulation of NanA sialidase activity.

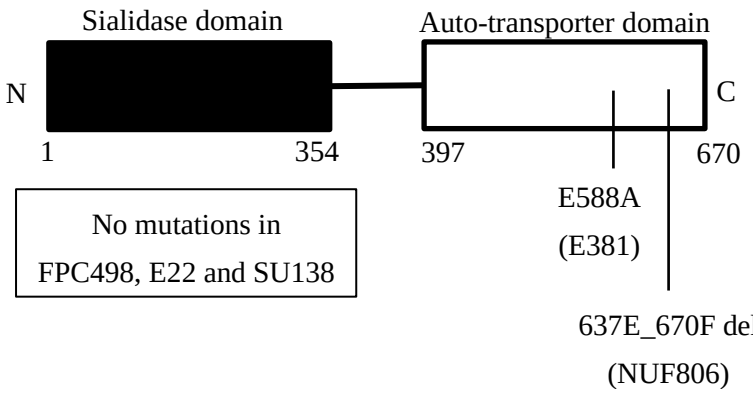

Figure 2-7. The illustration of amino acid mutations from the bacterial sialidase.

Next, NanA gene expression levels in the bacteria strains were analyzed using real-time PCR. The E381 strain exhibited the highest NanA expression level (3.6-fold increase compared to

that of FPC498, $p < 0.01$) among five tested strains, followed by the FPC498, NUF806 and E22 strains with moderate NanA expression level, whereas the small amount of NanA was detected in the SU138 strain (Figure 2-8). These results indicate that high NanA sialidase activity accompanied by high NanA gene expression found in pathogenic strains, whereas non-pathogenic strain did not. Therefore, we demonstrated the correlation between NanA gene expression level and sialidase activity and cellular infection to GAKS cells.

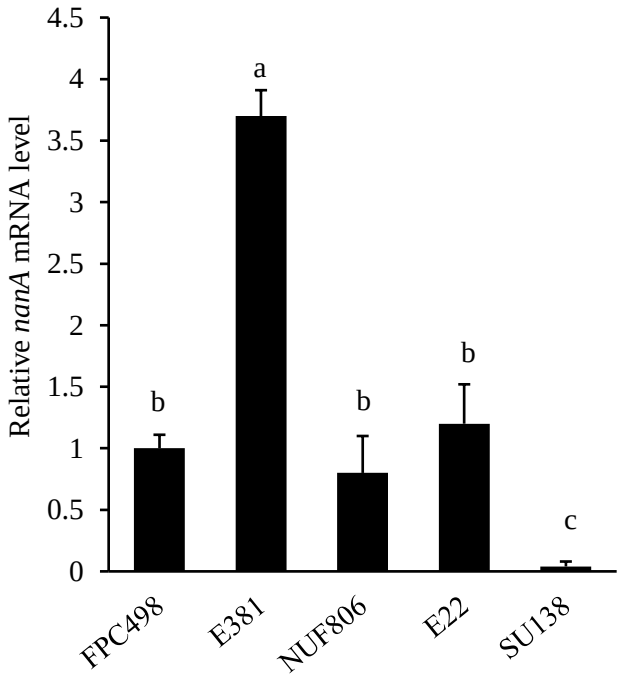

Figure 2-8. NanA gene expression level from five *Edwardsiella* sp. strains using real-time PCR. Significant differences are showed using letters (Tukey-Kramer test). Columns possess same letter were not statistically different, *vice versa*.

2.4 Discussion

Our previous study revealed that *Edwardsiella* sp. possess only NanA sialidase in its genomic sequence by NCBI database [26]; however, it has been reported several kinds of sialidase exist in other bacteria such as *Streptococcus pneumoniae*, *Gardnerella vaginalis*, and *Bifidobacterium longum* [17, 33, 34]. In this study, we revealed the relationship between NanA sialidase activity and bacterial invasiveness using five *Edwardsiella* sp. strains including three pathogenic strains: E381, FPC498, NUF806 and one pathogenicity-attenuated strain E22 and non-pathogenic strain SU138. Recently, bacterial sialidases have been considered virulence factors in several pathogenic organisms which colonize mucosal surfaces [15]. The sialidase function in *S. pneumoniae* has been well discussed that sialidase contributes to mucosal colonization, platelet clearance, and blood-brain barrier penetration [35]. Furthermore, the study revealed that AGP containing GlcNAc and Man served as a decoy for *Edwardsiella* sp. strains during infection. Our previous study proposes that GlcNAc and Man can be components of the binding site on host cell for the bacteria [26]. In general, β-galactosidase and β-*N*-acetylhexosaminidase are required for the exposure of GlcNAc and Man after desialylation by NanA sialidase. The putative sequences of these enzymes are found in *Edwardsiella* sp. genome, deposited in the GenBank database under the accession No. WP_024524403 for β-galactosidase and the accession No. WP_141112386 for β-*N*-

acetylhexosaminidase. This study has indicated that the cleavage of sialic acids by NanA sialidase from host cell glycoconjugates caused a series of events in positive regulation leading to bacterial invasion. In detail, *Edwardsiella* sp. sialidase cleaved the terminal sialic acids on host glycoconjugates to unmask the glycoprotein, after that the bacteria use it as an adhesion anchorage.

In conclusion, we suggest that desialylation by NanA sialidase is a crucial step for *Edwardsiella* sp. infection toward host cells. As *Edwardsiella* sp. are intracellular parasites and generate resistant strains, this bacterium is difficult to be eliminated by humoral immunity and antibiotic. NanA sialidase would be a novel target molecule to prevent *Edwardsiella* sp. infection; for instance, inhibition of sialidase activity can suppress bacterial infection, resulting to minimize the economic losses in aquaculture. Sialidase inhibitor decreased bacterial infection to host cells that studied in other bacteria such as *P. aeruginosa* and *S. pneumoniae* [36]. In addition, it has been reported that naringenin, a flavonoid originating from citrus, which acts as sialidase inhibitor, reduced *Edwardsiella* sp. infection [37].

Chapter 3 Significance of sialic acids in *Edwardsiella* sp. pathogenicity through its metabolism

3.1. Introduction

Sialic acid is a monosaccharide which is conjugated at the non-reducing terminal of glycans. Sialic acid has been known in the regulation of interaction between cell-cell, cell-protein, cell-hormones, and host-pathogen [13]. This monosaccharide is also a target molecular for lectins [38]. Moreover, free sialic acids can be utilized as a source of organic carbon for bacteria growth and/or biofilm formation that is determined in *Haemophilus influenza, Streptococcus preumoniae, Salmonella enterica,* and *Tannerella forsythia* [27, 39]. In addition, several bacteria can use the activated form of sialic acid, CMP-Neu5Ac (catalyzed by CMP-sialic acid synthase), to the lipopolysaccharide (LPS) sialylation pathway. As described before, LPS sialylation is an important virulence factors for some pathogens such as *H. influenza, E. coli* and *H. ducreyi* [13]. Besides, the incorporation of sialic acid into bacterial macromolecules has partly studied, for example, in *Neisseria meningitidis*, the synthesis of the polysialic acid capsules have been identified [13].

In previous study, NanA sialidase have been found in *Edwardsiella* sp. and its enzymatic properties was reported [30]. The NanA sialidase hydrolyzes the α2–3 sia linkages between sialic acids and its subterminal sugars in host glycoconjugates that reveals the potential binding site containing Man and GlcNAc on host cell for the bacteria [26]. However, the significance of released sialic acids by sialidase activity and its metabolism in *Edwardsiella* sp. growth and/or

infection remains unclear, for example, the effect of sialic acids on *Edwardsiella* sp. growth and infection. Furthermore, we hypothesized that two metabolic pathways involved in sialic acids utilization including degradation of sialic acids and incorporation to CMP for sialylated glycoconjugates on the bacteria regulate *Edwardsiella* sp. infection.

3.2. Materials and methods

3.2.1 Bacterial growth

Five *Edwardsiella* sp. strains (E381, NUF806, FPC498, E22 and SU138) with different pathogenicity used for the experiments in this chapter were listed in Table 2-1. To assess bacterial growth, bacteria were adjusted to the same optical density at 630 nm (OD_{630} = 0.2) and cultured in glucose-depleted M9 minimal medium containing various *N*-acetylneuraminic acid (Neu5Ac) concentrations of 0, 1, 5, and 10 mg/mL.

3.2.2 Infection of cultured GAKS cells

The infection test was carried out as described above. Briefly, bacteria were preincubated with 100 mM Neu5Ac for 30 min and exposed to GAKS cells at MOI of 100. Thereafter, the GAKS cells were treated with antibiotics, lysed, and plated on TSA plates. The colonies amount was used for evaluation of bacterial infection.

3.2.3 Real-time PCR

Neu5Ac-pretreated *Edwardsiella* sp. pellet was harvested in logarithmic growth phase and used for extracting RNAs by Sepasol-RNA I Super G followed by cDNA were prepared using the ReverTra Ace qPCR RT Master Mix with gDNA remover. The primers were used for real-time PCR reactions designed according to automated computational gene predictions in *E. piscicida* genomic sequence

of strain FL6-60 (CP002154) as follows: 5'-CTGGGTCGACGCCAG-3' and 5'-CGGATGATGGCGATCAC-3' for *N*-acetylneuraminate lyase 1, 5'-GTTTGCTTCAACGCCCCGAC-3' and 5'-GTGTACAACATTCCGGCGTTGA-3' for *N*-acetylneuraminate lyase 2, and 5'-TACAGATGCGGCGACGTAAA-3' and 5'-CTCCCTGACCCATTCAGCAG-3' for *N*-acetylneuraminate cytidylyltransferase. Real-time PCR was carried out using KOD SYBR qPCR Mix followed the program as 2 min denaturation step, 40 cycles of the step 98 °C for 10 s, 60 °C for 10 s and 68 °C for 30 s. The expression of 16S rRNA was used to compensate for RNA quantity and quality among samples.

3.2.4 Sialidase activity

Bacteria were preincubated with 100 mM Neu5Ac for 30 min and collected by centrifugation. The bacterial pellets were lysed by 1% Triton X-100 and the suspension was used as a crude enzyme. Sialidase activity were assessed using MU-Neu5Ac as a substrate. The cleaved MU amount was estimated by a fluorescence spectrophotometer. Protein concentration was analyzed using Bradford method by binding of Coomassie brilliant blue and its absorption was measured by a spectrophotometer at 595 nm.

3.2.5 Estimation of conjugate-sialic acid content in LPS, glycolipid and glycoprotein

Edwardsiella sp. pellet were harvested in logarithmic growth phase and dried by a vacuum freeze dryer. Crude LPS were prepared using the hot phenol-water method. Briefly, the dried bacterial pellet was added 90% aqueous phenol solution and stirred vigorously at 65 °C for 30 min followed by centrifugation at 7000 *g* for 20 min. Thereafter, an aqueous phase was collected, dialyzed (MWCO: 10000) for 24 h and lyophilized by a vacuum freeze dryer.

The lipid fraction was obtained using the Folch method [40]. Briefly, the dried bacterial pellet was added in sequence with chloroform/methanol 1:2, 1:1 and 2:1 (v/v) followed by solvents were removed by evaporation. The insoluble fraction was used as protein fraction. Lyophilized crude LPS, and lipid and protein fractions were hydrolyzed by 0.1 N H_2SO_4 for 1 h. The cleaved sialic acids were estimated by HPLC with derivation using 1,2-diamino-4,5-methylene-dioxybenzene (DMB). DMB-sialic acid was separated by reverse-phase column using a mobile phase containing acetonitrile:methanol:water (9:7:84, v/v) at an emission wavelength of 448 nm and an excitation wavelength of 373 nm.

3.2.6 Data analyses

All data were indicated as mean ± standard deviation (S.D.) and compared by Tukey-Kramer test or t-test. Each experiment was performed in triplicate.

3.3. Results

Previous chapter demonstrated the significance of the cleavage sialic acid from host cell by NanA sialidase in *Edwardsiella* sp. infection. However, the role of cleaved sialic acid in *Edwardsiella* sp. infection had not been identified. In this study, to elucidate the role of cleaved sialic acid specifically during *Edwardsiella* sp. infection, the E381 strain was pre-incubated with Neu5Ac for 30 min, then used for infection toward GAKS cells. As shown in Figure 3-1, free sialic acid significantly enhanced *Edwardsiella* sp. cellular invasion to GAKS cells.

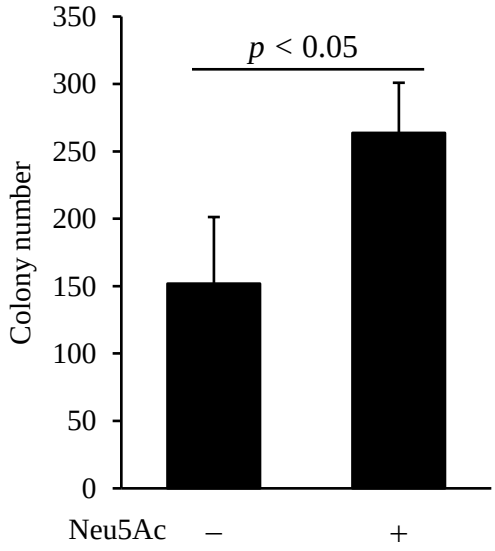

Figure 3-1. Regulation of *Edwardsiella* sp. infection by free sialic acids *in vitro*

E381 strain was pre-incubated with 100 mM Neu5Ac for 30 min and then inoculated into GAKS cells monolayer as an infection assay. The values were compared using *t*-test.

To determine the involvement of sialic acid and NanA sialidase during the infection, the alteration of NanA sialidase activity in the environment containing sialic acid was evaluated. We found that sialidase activity was significantly reduced by sialic acid treatment (11.3 mol/h/mg protein and 6.8 mol/h/mg protein, respectively, $p < 0.05$, Figure 3-2A), accompanied by a down-regulation of NanA expression level (10-fold decrease, $p < 0.05$, Figure 3-2B). These results suggested that enhancement of *Edwardsiella* sp. infection is regulated by free sialic acid, and it is independent on NanA sialidase. The results also suggest that sialic acid transporters may be present in *Edwardsiella* sp.

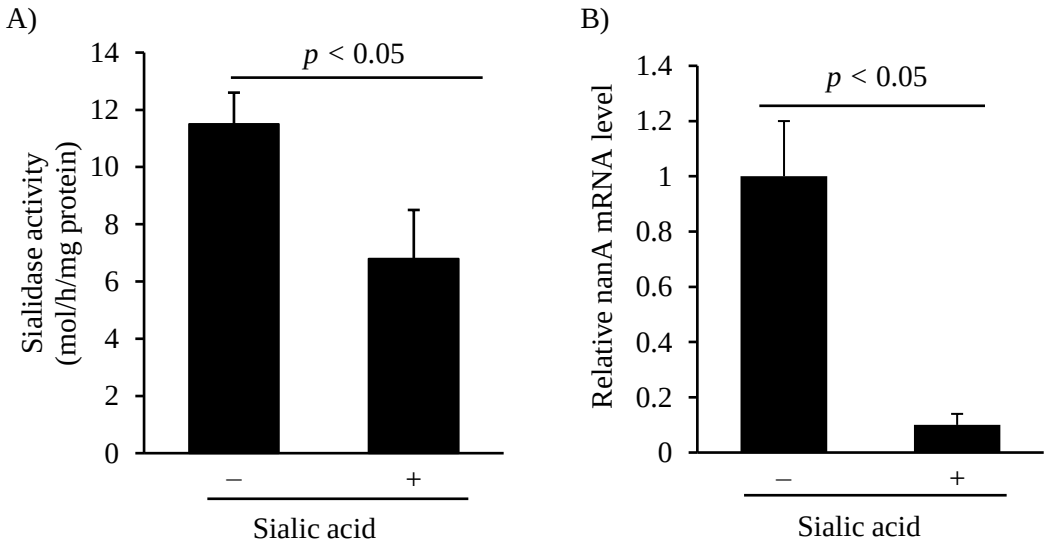

Figure 3-2. Sialidase activity and mRNA level of NanA gene by sialic acid-treatment.

A) E381 strain was pre-treated with Neu5Ac and harvested by centrifugation. The bacterial lysate was incubated with MU-Neu5Ac for estimation of sialidase activity. B) NanA gene expression level

in Neu5Ac-treated E381 strain was analyzed using real-time PCR. The values were compared using *t*-test.

Next, to understand the sialic acid metabolic pathway in *Edwardsiella* sp., based on the genomic sequence of *E. piscicida* FL6-60 strain (previously classified as *E. tarda,* accession No. CP002154) [41], we found three clusters associated with several putative sialic acids related genes in metabolism and putative sialic acid transporters as shown in Figure 3-3.

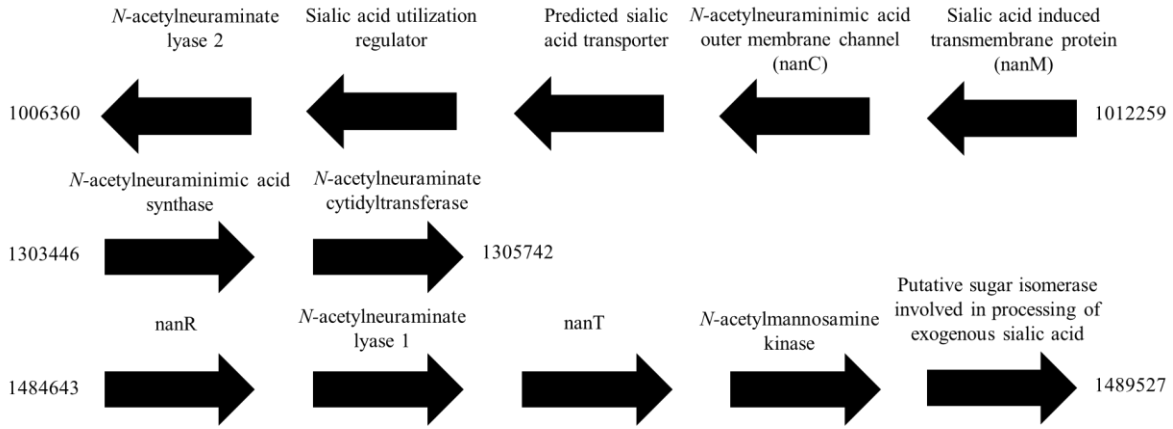

Figure 3-3. Putative sialic acid-related genes in clusters found on *E. piscicida* FL6-60 genome (accession No. CP002154).

The several putative sialic acid related genes are primarily located within three clusters. Three sialic acid metabolic enzyme are attentive including *N*-acetylneuraminate lyase 1 and 2, *N*-acetylneuraminate cytidylyltransferase.

Three sialic acid metabolic enzymes were concerned including two *N*-acetylneuraminate lyases which removes pyruvate group from sialic acid to yield *N*-acetylmannosamine, and one *N*-acetylneuraminate cytidylyltransferase (CMP-Neu5Ac synthetase) which combined sialic acid with cytidine monophosphate (CMP) to form CMP-Neu5Ac. Therefore, we hypothesized whether two metabolic pathways of sialic acid are involved in *Edwardsiella* sp. invasion. As two putative *N*-acetylneuraminate lyase genes were discovered in *E. piscicida* genome, they were temporarily referred as *N*-acetylneuraminate lyase 1 and 2, respectively. As shown in Figure 3-4A, B, C, the up-regulation of *N*-acetylneuraminate lyase 1 and 2 and CMP-Neu5Ac synthetase mRNA expression level was observed in sialic acid-treated *E. piscicida* using real-time PCR. The results indicates that NanA-induced sialic acid increases *E. piscicida* cellular infection via sialic acid metabolic pathway by the metabolism enzymes.

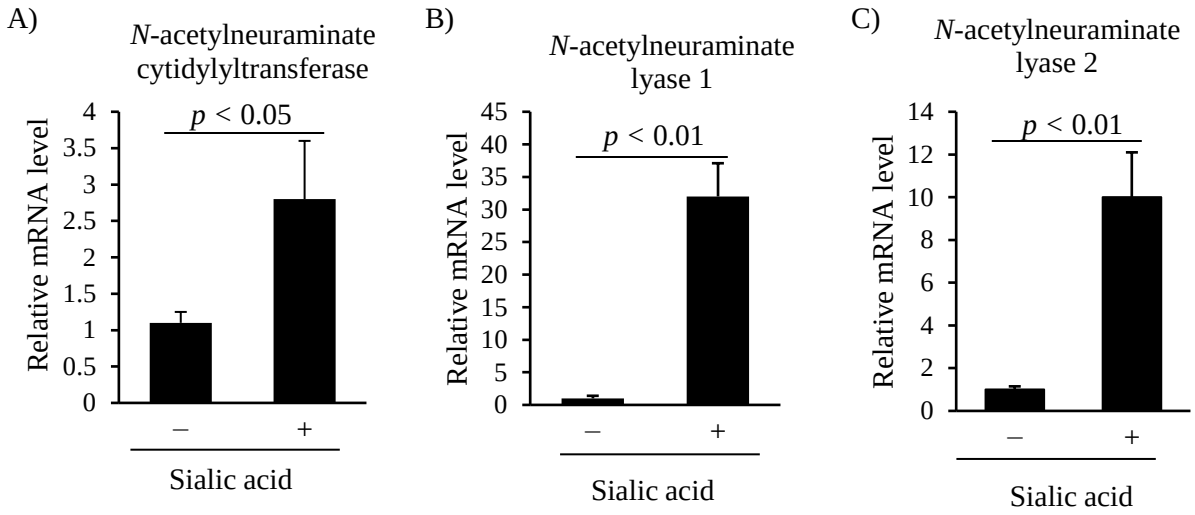

Figure 3-4. mRNA expression level in the Neu5Ac-treated E381 strain.

A) *N*-acetylneuraminate cytidylyltransferase, B) *N*-acetylneuraminate lyase 1, and C) *N*-acetylneuraminate lyase 2. The values were compared using *t*-test.

The mRNA levels of *N*-acetylneuraminate lyase 1 and 2 were estimated in several *Edwardsiella* sp. strains using real-time PCR. We found the expression level of *N*-acetylneuraminate lyase 1 did not significantly differ among five strains (Figure 3-5A), whereas the high *N*-acetylneuraminate lyase 2 mRNA level was observed in E22 and SU138 compared to other tested strains ($p < 0.05$, Figure 3-5B).

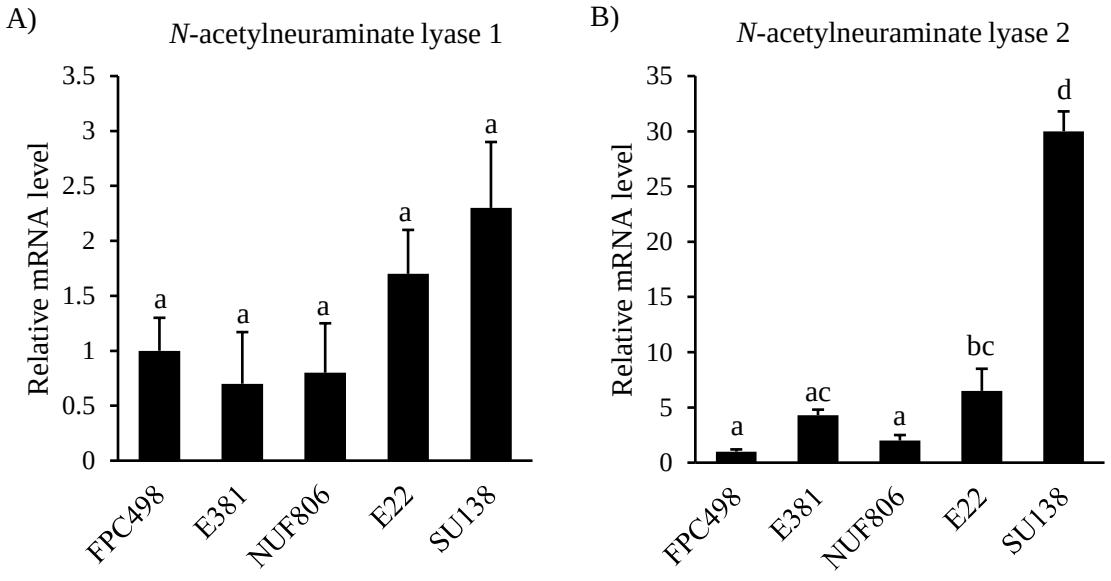

Figure 3-5. mRNA levels of *N*-acetylneuraminate lyase 1 and 2 gene evaluated in five *Edwardsiella* sp. strains (FPC498, E381, NUF806, E22 and SU138) using real-time PCR. Significant differences are showed using letters (Tukey-Kramer test). Columns possess same letter were not statistically different, *vice versa*.

In other bacteria, free sialic acid is meaningful for the bacterial growth. Hence, to assess the effects of sialic acid on bacterial growth, several *Edwardsiella* sp. strains were cultured in glucose-depleted M9 minimal medium adding Neu5Ac. As shown in Figure 3-6A, all five strains showed more notable growth rate in Neu5Ac-supplemented M9 medium than these growth in M9 medium without adding Neu5Ac. The better growth rate was observed in the higher concentration of

Neu5Ac in M9 medium. In addition, all tested strains displayed similarity growth under sialic acid treatment with the doubling time of 2.12, 1.98, 1.98, 1.88 and 2.00 in FPC498, E381, NUF806, E22 and SU138, respectively. This demonstrates that *N*-acetylneuraminate lyase 1 is preferred by *Edwardsiella* sp. for degradation of sialic acid rather than *N*-acetylneuraminate lyase 2.

In addition, the comparison to the utilization efficacy of between sialic acid and glucose by bacteria was evaluated. The efficacy of sialic acid utilization was 46.1% and 37.5 % of glucose utilization in E381 and SU138, respectively (Figure 3-6B). Taken together, these results indicate that *Edwardsiella* sp. can utilize sialic acid as a nutrient source for growth via *N*-acetylneuraminate lyase 1.

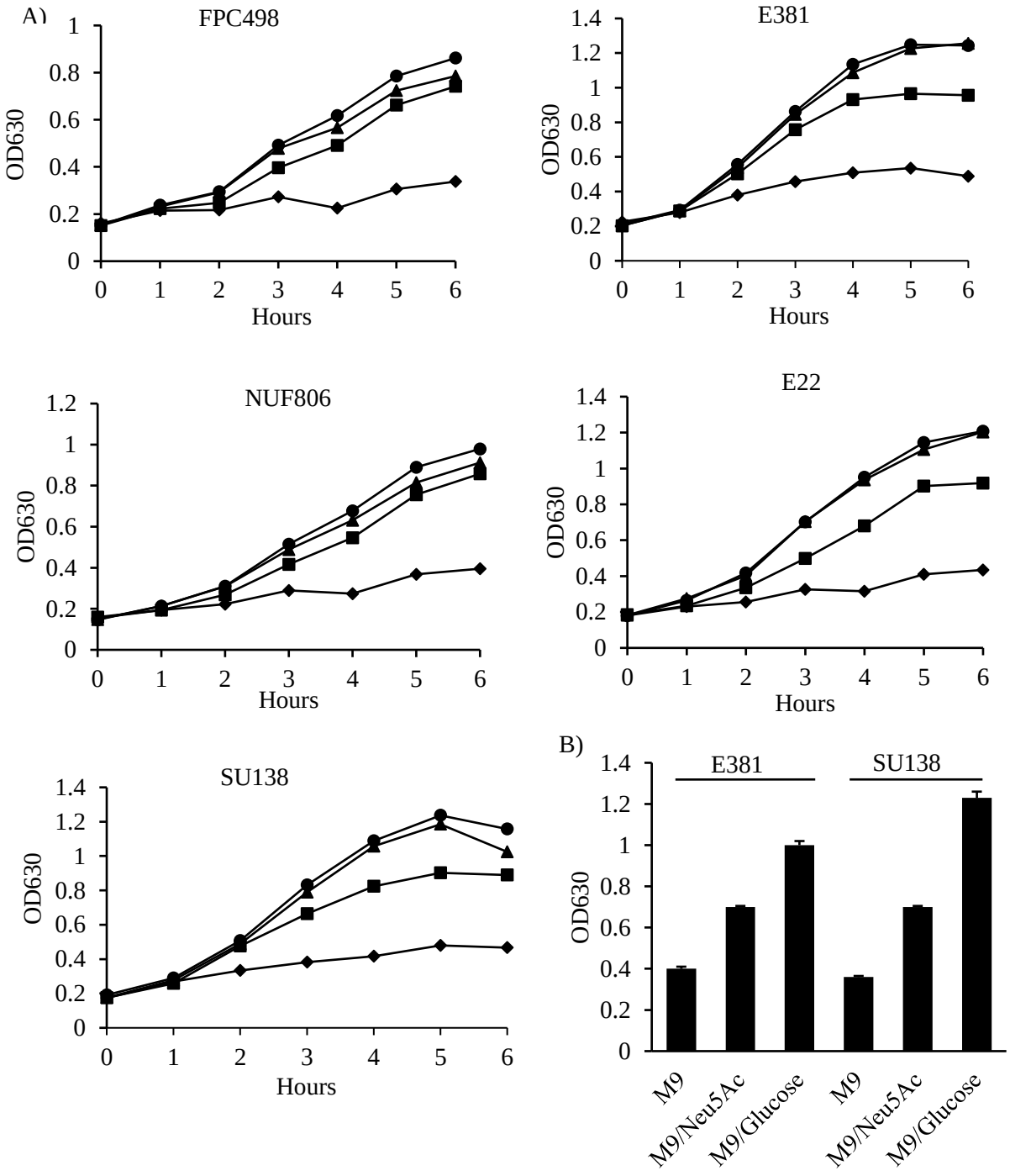

Figure 3-6. Utilization of sialic acids as a nutrient source for *Edwardsiella* sp. growth.

A) Five *Edwardsiella* sp. strains were cultured in a glucose-depleted M9 medium adding varying concentrations of Neu5Ac. Closed circle, triangle, square and diamond indicate Neu5Ac

concentration of 10, 5, 1 and 0 mg/mL, respectively. B) Comparison of sialic acid and glucose utilization efficacy in *Edwardsiella* sp. growth. Two strains (E381 and SU138) were cultured in either M9 medium, 1 mg/mL Neu5Ac or 1 mg/mL glucose for 3h. The bacterial growth in each medium was evaluated using the value of OD_{630}.

Recently, sialylation of LPS has been known to play a crucial role in bacterial pathogenicity. In other bacteria, sialic acid conjugates with CMP to form CMP-Neu5Ac and it were transferred to cell surface for the sialylation of glycans and LPS. In *E. piscicida,* since CMP-Neu5Ac synthase was explored that involved in the sialic acid metabolic pathway, its mRNA levels were estimated in several *Edwardsiella* sp. strains. As shown in Figure 3-7, two pathogenic strains, E381 and FPC498, showed comparatively high CMP-Neu5Ac synthase expression levels and NUF806 and E22 showed moderate its expression, whereas SU138 exhibited very small the expression level of CMP-Neu5Ac synthase. As CMP-Neu5Ac synthase is required for sialylation of glycoconjugates, the sialic acid contents in LPS, glycoproteins and glycolipid were estimated using HLPC analysis. Figure 3-8A illustrates that Neu5Ac was a main type of sialic acid found in *Edwardsiella* sp. and other sialic acid type such as *N*-glycolylneuraminic acid (Neu5Gc) and 3-deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid (KDN) were not detected. In *Edwardsiella sp,* LPS- and protein-

bound sialic acids were greater amount than lipid-bound sialic acids (Figure 3-8B). Moreover, E381, NUF806 and FPC498 strains possessed a large level of sialic acids conjugated with LPS and protein, whereas E22 and SU138 strains exhibited slight sialylation in LPS and protein. On the other hand, a small amount of lipid sialylation was found on all strains. These results demonstrated the involvement of CMP-Neu5Ac synthase dependent sialylation of glycoconjugates and *Edwardsiella* sp. infection.

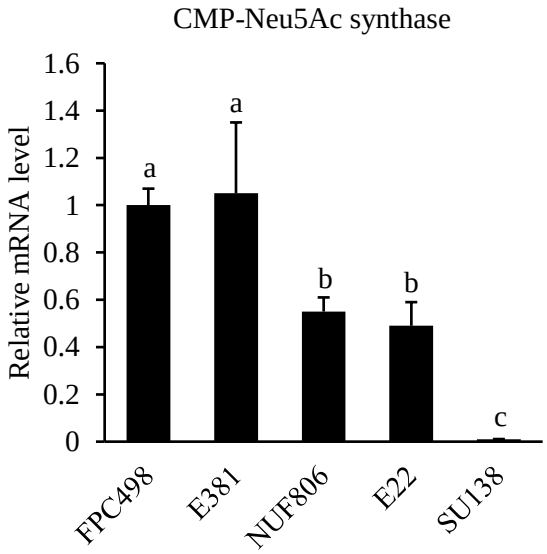

Figure 3-7. Expression level of CMP-Neu5Ac synthase gene in five *Edwardsiella* sp. strains (FPC498, E381, NUF806, E22 and SU138) using real-time PCR. Significant differences are shown using letters (Tukey-Kramer test). Columns possess same letter were not statistically different, *vice versa*.

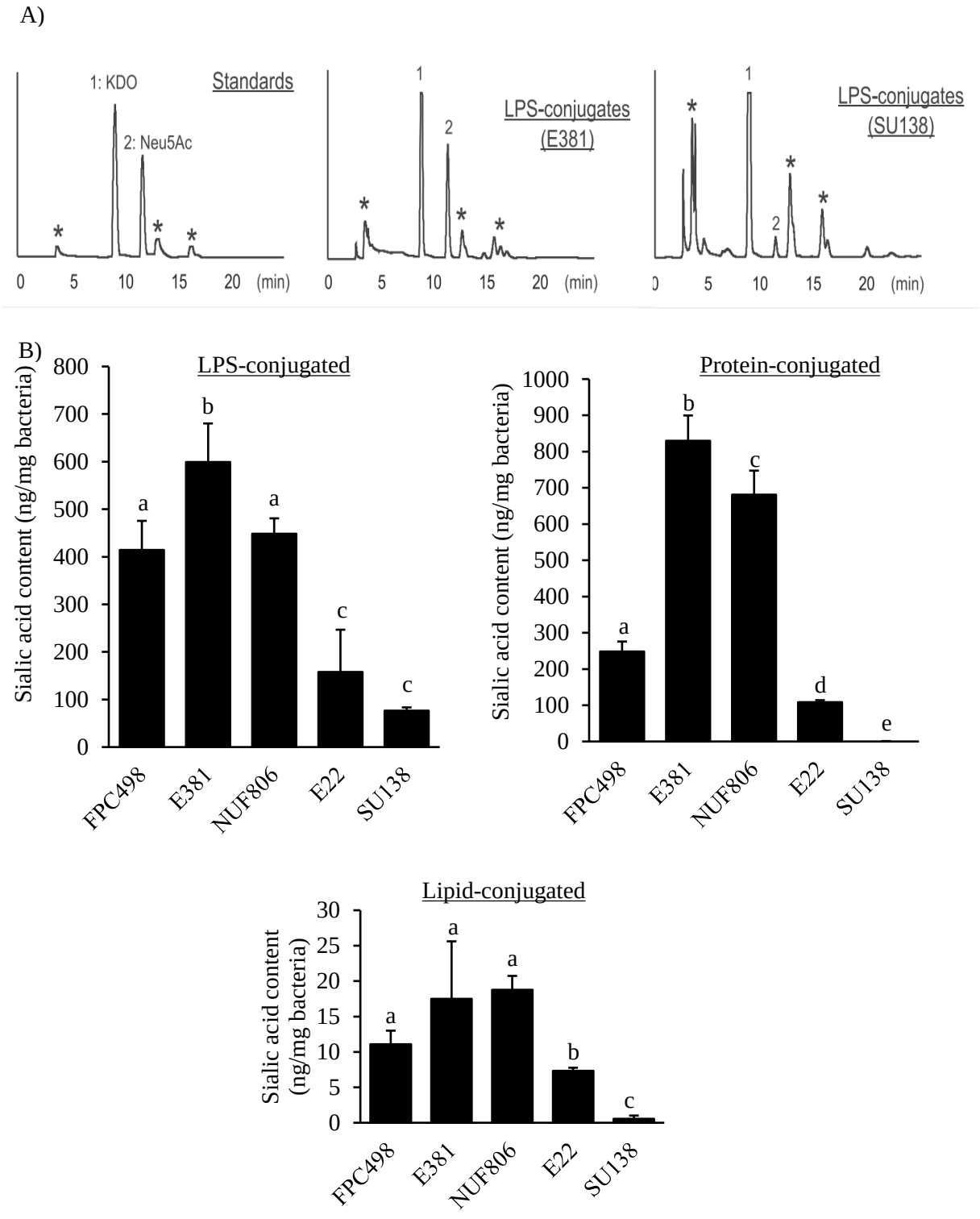

Figure 3-8. Sialic acid contents in glycoconjugates in the *Edwardsiella* sp. strains.

A) Reversed phase HPLC of DMB-Neu5Ac. Chromatogram of standards (derivatives of 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) and Neu5Ac), LPS-conjugates in E381 and SU138, from left to right, respectively. Asterisk indicates non-specific peaks. B) Sialic acid contents in LPS, glycoproteins and glycolipids fractions in five *Edwardsiella* sp. strains: FPC498, E381, NUF806, E22 and SU138. Significant differences are showed using letters (Tukey-Kramer test). Columns possess same letter were not statistically different, *vice versa*.

3.4. Discussion

This chapter details the significance of free sialic acids in *Edwardsiella* sp. pathogenicity via two its metabolic pathway including catabolism of sialic acid by either one of the two *N*-acetylneuraminate lyases and incorporation of sialic acid in the glycoconjugates by CMP-Neu5Ac synthase.

Our previous study shows that NanA sialidase produced by *Edwardsiella* sp. cleaves sialic acid from host cell glycoconjugates. Therefore, it suggests that the sialic acid source using by bacteria during infection can come from NanA-induced sialic acid. Indeed, utilization of sialic acids for bacterial growth have been reported in sialidase-possessed bacteria such as *S. pneumoniae*, *Tannerella forsythia* and *Bifidobacterium* sp. [33, 42, 43]. Our study showed that *Edwardsiella* sp. used sialic acid as a carbon source for the growth and the utilization efficacy of sialic acid was nearly half as much as the efficacy of glucose. In general, five bacterial enzymes related in sialic acid catabolism include *N*-acetylneuraminate lyase, ManNAc kinase, ManNAc-6-P epimerase, GlcNAc-6-P deacetylase, and glucosamine-6-P deaminase [13]. The final product of this pathway is fructose-6P, which is moved to the glycolytic pathway. Interestingly, we discovered the presence of two *N*-acetylneuraminate lyases in *E. piscicida* genome, named *N*-acetylneuraminate lyase 1 and 2, respectively These enzyme can be involved in *E. piscicida* infection.The degradation of sialic acid used for *Edwardsiella* sp. growth may proceed by *N*-acetylneuraminate lyase 1 which form a cluster

with several putative sialic acid-related genes: sialic acid transporter (*nanT*), ManNAc kinase and transcriptional regulators of both *nan* operons (*nanR*), which often found in other bacteria [28, 44], suggesting the successive lyase reaction is critical for *Edwardsiella* sp. growth. In general, most bacteria possess only one *N*-acetylneuraminate lyase. The SU138 strain, which possessed low sialidase activity, exhibited higher the expression level of *N*-acetylneuraminate lyase 2 compared to other *Edwardsiella* sp. strains. *N*-acetylneuraminate lyase 2 form a cluster with sialic acid utilization regulator, predicted sialic acid transporter, NanC (N-acetylneuramic acid outer membrane channel) and NanM (sialic acid-induced transmembrane protein). However, the function of *N*-acetylneuraminate lyase 2 have not understood.

The other pathway in the utilization of sialic acid by *Edwardsiella* sp. is a conjugation with CMP yielding CMP-Neu5Ac which used for the sialylation of glycoconjugates such as LPS, glycoproteins and glycolipids. Cell surface sialylation modulates its cell functions via the interaction of cell-cell and pathogen-host cell by sialic acid-binding immunoglobulin-like lectins (Siglec) in the host cell [38]. Meanwhile, it is no reports on the presence of sialo-glycoconjugates in *Edwardsiella* sp. Our study revealed LPS was one of the target molecules for the sialylation in *Edwardsiella* sp., that also reported in *E. coli*, in which sialic acids were detected in the O-antigen of LPS [13]. Recent studies reported that LPS sialylation is involved in the increment of the

bacterial escape ability from immune system. For example, in *H. influenza,* host derived sialic acid, which is transported into the its cell, incorporated into LPS, that is essential for resistance to host serum [27]. On the other hand, NUF806 strain, which possessed moderate CMP-Neu5Ac synthase, showed high sialylation in its glycoprotein and LPS, presumably due to unidentified sialic acid metabolic enzymes related to CMP-Neu5Ac, such as sialyltransferases.

In conclusion, this chapter has demonstrated the significance of free sialic acid in the positive regulation of *Edwardsiella* sp. infection and its utilization as a nutrient source for *Edwardsiella* sp. growth. However, further research on sialic acid metabolism in *Edwardsiella* sp., is necessary and important for the discovery of novel and effective preventive methods of Edwardsiellosis.

Chapter 4 The regulation of *Edwardsiella piscicida* biofilm formation, motility ability and infection by *N*-acetylneuraminate lyase

4.1 Introduction

Sialic acids and its catabolism have known to be involved in bacteria pathogenicity. In general, the sialic acid catabolic pathway relates to five steps: first NAL removes a pyruvate group from Neu5Ac generating ManNAc, and then *N*-acetylmannosamine kinase (NanK) adds a phosphate group to C6 position yielding ManNAc-6P; next *N*-acetylmannosamine-6-P epimerase (NanE) epimerizes the ManNAc-6P into *N*-acetylglucosamine-6-P (GlcNAc-6P) followed by the removement of the acetyl group from GlcNAc-6P by *N*-acetylglucosamine-6-P deacetylase (NagA) yielding glucosamine-6-P (GlcN-6P); lastly, glucosamine-6-P deaminase (NagB) removes the amino group from GlcN-6P generating fructose-6-P (Fru-6P) which enter the glycolysis pathway to producing energy for bacteria growth [13].

N-acetylneuraminate lyase (NAL) is responsible for the first step of sialic acid catabolism which convert Neu5Ac to *N*-acetyl-D-mannosamine (ManNAc) and pyruvate. Therefore, NAL plays an important function in regulating of sialic acid metabolism in bacteria. This enzyme has been found in various bacteria such as *Escherichia coli, Clostridium perfringens, Lactobacillus plantarum, and Aliivibrio salmonicida* [23, 45–47]. NAL enzymatic profile was greatly described in several bacteria such as *S. aureus*, Mycoplasma, and *A. salmonicida* [22, 23, 46].

Previous chapter has revealed the existence of two putative *nals* genes predicted based on the *Edwardsiella* sp. genome and their expression level showed different in the bacteria strains. In other bacteria, such as *E. coli*, NAL is recognized to be involved in biofilm formation [47]. In addition, the capability of sialic acids catabolic utilization has shown to be essential for bacterial pathogenesis. However, direct evidence of the involvement of sialic acid catabolism and bacterial pathogenicity is limited.

In this chapter, we hypothesized that NAL is involved in *E. piscicida* pathogenicity; thus, we investigated the effect of the overexpression of NAL genes on bacterial phenotypes such as growth, biofilm formation, motility, and infection.

4.2 Materials and methods

4.2.1 *Edwardsiella piscicida,* cultured cells, and zebrafish

Edwardsiella piscicida were grown in tryptic soy broth or on tryptic soy agar and maintained at 28 °C. Goldfish scale fibroblast GAKS cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) adding with 10% (v/v) fetal bovine serum at 37 °C under a 5% CO_2 atmosphere. Zebrafish RIKEN WT were obtained from the Centre for Brain Science, Institute of Physical and Chemical Research (Japan) and housed in a 3 L water tank under a 14/10 h light/dark cycle at 28 °C. For breeding, a pair (male and female) was transferred to a 1.3 L breeding tank.

4.2.2 Molecular cloning of putative *nal* genes and establishment of their overexpressing strains

Genomic DNA from *E. piscicida* was extracted by boiling the cell suspension for 3 min. Thereafter, the supernatant was harvest by centrifugation and used as template for PCR reaction. PCR amplification of putative *nal* genes was carried out using primers designed based on the *E. piscicida* genomic information (*E. piscicida* CK41 strain, accession No: CP047671) as listed in Table 4-1. The open reading frame (ORF) of the target genes were amplified using Tks Gflex DNA polymerase with the following conditions: the initial denaturation at 94 °C for 1 min followed by 30 cycles of three-step PCR denaturation at 98 °C for 10 s, annealing at 60 °C for 15 s, and extension

at 68 °C for 1 min 30 s. The PCR products were digested by restriction enzymes, cloned into pBluescript SK (+) vector (TAKARA), and sequenced using ABI 3130xl Genetic Analyzer (Applied Biosystem).

E. piscicida overexpressing putative *nal* genes were established using FPC498 strain by the liquid nitrogen transformation method [48]. Briefly, *E. piscicida* FPC498 was mixed with pSF-OXB20 plasmid containing *nals* genes, incubated on ice for 5 min, followed by submerge in liquid nitrogen for 5 min. Thereafter, the cells were recovered in Luria broth (LB) medium and incubated for 1 h at 28 °C. Transformed bacteria were selected on kanamycin plates (20 μg/mL). The mock was prepared using an empty pSF-OXB20. In this study, two *E. piscicida* transformant strains, referred as DHDPS and NAL strains, were successfully established and used for all experiments.

4.2.3 Real time-PCR

In this study, we estimated the gene expression level in the mock, DHDPS and NAL strains pretreated with Neu5Ac including those of *nanK* (encoding *N*-acetylmannosamine kinase), *nanE* (encoding *N*-acetylmannosamine-6-phosphate epimerase), *nagA* (encoding *N*-acetylglucosamine-6-phosphate deacetylase), *nagB* (encoding Glucosamine-6-phosphate isomerase), *glmM* (encoding Phosphoglucosamine mutase), *glmU* (encoding *N*-acetylglucosamine 1-phosphate

uridyltransferase/glucosamine-1-phosphate acetyltransferase), *pgm3* (encoding Phosphoglucomutase) and *nagK* (encoding *N*-acetylglucosamine kinase). The primers were designed based on the *E. piscicida* genomic information (*E. piscicida* ETW41 strain, accession No: CP019440) summarized in Table 4-1. Total RNA was extracted from the bacteria using Sepasol-RNA I Super G solution (Nacalai Tesque, Japan), followed by cDNA synthesis using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Real-time PCR was carried out using KOD SYBR Green PCR Master Mix (TOYOBO). The expression level of the 16S rRNA gene was used as the reference to compensate for quantity and quality among samples.

Table 4-1. Primers sequences used for cloning and real-time PCR

	Gene name	Protein name	Forward primer (5'-3')	Reverse primer (5'-3')
Cloning	*dapA*	Dihydrodipicolinate synthase	GTGGTACCACCATGAAAAAGCTAACCG	GTGGTACCTTACAGGAACTCGGCGGC
	nal	*N*-acetylneuraminate lyase	GTGGTACCACCATGCCCGCATTGT	GTGGTACCTTAACGCGCCCGCTCG
Real-time PCR	*nanK*	*N*-acetylmannosamine kinase	ATCGCGGAATGGCTGATAGG	TTGAGAAGATGGCAGGGCAG
	nanE	*N*-acetylmannosamine-6-phosphate epimerase	ATCATCAAACGCGATCTGGA	ACGTCCATGGTGCTGTATTC
	nagA	*N*-acetylglucosamine-6-phosphate deacetylase	GGCGTGCAGTTTAACGACAG	CCCTTCAATATGCAGCCCCA
	nagB	Glucosamine-6-phosphate isomerase	GGTCTGGCAGAATCACACCC	CGGCGCGTTACCATTTAACA
	glmM	Phosphoglucosamine mutase	TGCTGGAGACGATGCAAGAG	GCGGTTCGGTACCGGATTTA
	glmU	*N*-acetylglucosamine-1-phosphate uridyltransferase/ glucosamine-1-phosphate acetyltransferase	TAAATATCGGCGCAGGGACC	ACTGAGCTGCACCTGTTTCA
	pgm3	Phosphoglucomutase	CAATGAGGCGCACATTCTGG	TCAGAATGGCGAGTGACACC
	nagK	*N*-acetylglucosamine kinase	CTTTAGCGAGCTGTTTGCCC	CGGGTGTCGCTCAGATGTAA

4.2.4 Estimation of cleavage and condensation activity

The mock, DHDPS and NAL strains were cultured in TSB at 28 °C under shaking until reaching the log phase, harvested, and resuspended in PBS containing 1% Triton X-100 and proteinase inhibitors (1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonylfluoride and 10 µg/mL leupeptin). After sonication for 10 s, the solubilized proteins were obtained by centrifugation at 13000 *g* for 15 min. The supernatant was used as the crude enzyme in this study.

NAL was assessed for both the cleavage and condensation activities. The cleavage activity determined by estimating pyruvate amount converted from sialic acid (Neu5Ac, Neu5Gc, or KDN). The reaction mixture contained 5 mM sialic acid, crude enzyme, and 50 mM phosphate buffer for pH 7.5. The mixture was incubated at 37 °C for 5 min or 16 h, depending on the enzyme, incubated with 0.2 mM NADH and 10 U/mL LDH at 37 °C for 5 min, and then measurement by a spectrophotometer at 340 nm.

The condensation activity was determined bases on the amount of sialic acid using the modified thiobarbituric acid (TBA) assay [22]. The reaction comprised 5 mM ManNAc or Man, 5 mM pyruvate and 1- 3 mg/mL of crude enzyme, and 50 mM phosphate buffer (pH 7.5). Thereafter, the reaction was stopped by adding 50 µL of 0.2 M metaperiodate solution, incubated at 25 °C for 20 min, and added 500 µL of 10% arsenite solution under vigorous shaking until the medium

became transparent, followed by the addition of 1.5 mL of 0.6 % TBA solution. The samples were incubated in boiling water for 15 min, immediately kept on ice water for 5 min. To extract the red chromophore, 1.5 mL of cyclohexanone was added into the mixture. The tubes were shaken for 10 s and centrifuged at 1500 *g* for 5 min at room temperature to divide into organic and inorganic phases. The red chromophore was present in the organic phase and its absorption was measured by a spectrophotometer at 549 and 532 nm.

4.2.5 *In vitro* infection test by *E. piscicida* transformants

The mock, DHDPS and NAL strains were pretreated with Neu5Ac (1 mg/mL) for 30 min and inoculated in the cell monolayers in a 12-well plate at MOI of 100 and incubated at 28 ℃ for 1 h. Thereafter, the monolayers were washed twice with PBS and incubated in fresh medium containing 200 μg/mL gentamicin, 200 μg/mL streptomycin and 200 U/mL penicillin for 1 h to kill the extracellular bacteria. The antibiotic treatment step was repeated with DMEM containing 100 μg/mL gentamicin, 100 μg/mL streptomycin and 100 U/mL penicillin for 1 h. Subsequently, the cells were washed thrice with PBS, lysed using 0.5% Triton X-100 in PBS and plated on TSA. The bacteria amount was quantified via formed colonies number on TSA plates. For the NAL inhibition assay, bromopyruvate (5 mM) were preincubated with the bacteria for 30 min, and then Neu5Ac treatment before the infection test.

4.2.6 *In vivo* infection test by *E. piscicida* transformants

Zebrafish larvae at 5 days post−fertilization (dpf) were used for bath immersion infection. They were distributed into three groups comprising 20 fish in the 6-well plate. The mock, DHDPS and NAL strains suspension were added to each well to attain a final concentration of 10^8 CFU mL^{-1} and exposed for 2 h. The plates were then washed and incubated at 28 °C throughout the experiment. The control groups were set without treatment with bacteria. The cumulative survival of zebrafish larvae was recorded for 7 days.

4.2.7 Estimation of bacterial growth

The mock, DHDPS and NAL strains suspension were adjusted to the same density (OD_{630} = 0.2) and cultured in sialic acid (1 mg/mL) supplemented M9 minimal medium at 28 °C under constant shaking. The OD_{630} was measured until the bacteria reached the stationary phase.

4.2.8 Estimation of bacterial biofilm formation

The mock, DHDPS and NAL strains were cultured in TSB medium at 28 °C until an OD_{630} of 0.5 was reached and pretreated with Neu5Ac (1 mg/mL) for 30 min before dilution (1:20) in fresh TSB, followed by inoculated in 96-well flat-bottomed microtiter plate with 200 µL per well. The plate was maintained at 28 °C for 72 h. Thereafter, the bacterial suspensions were removed, and the adherent bacteria were washed thrice with distilled water, followed by fixation with methanol for 20

min. The biofilm was stained with 150 μL of 0.1% crystal violet for 15 min and then washed thrice with water. After air-drying, 150 μL of 95% ethanol was added into the wells and the plate was shook for 15 min. The biofilm formation was evaluated by a spectrophotometer at 570 nm.

4.2.9 Estimation of bacterial motility

Swimming, swarming and twitching assays were performed on LB containing 0.3%, 0.5% and 1% (w/v) agar, respectively [49]. The mock, DHDPS and NAL strains were pretreated with Neu5Ac (1 mg/mL) for 30 min and then 1 μL of bacterial suspension was inoculated onto the LB plates. The plates were incubated for 72 h at 28 ℃. Thereafter, the swimming, swarming, and twitching motility was evaluated by measuring the visible growth zone every 12 h.

4.2.10 Estimation of the total GlcNAc amount in *E. piscicida* transformants

The mock, DHDPS and NAL strains were preincubated with Neu5Ac (1 mg/mL) for 30 min and harvested via centrifugation at 13000 *g* for 15 min. The bacterial pellets were hydrolyzed by 4 M Trifluoroacetic acid (TFA) at 100 °C for 3 h, followed by dried and soluble in water. The samples were estimated the GlcNAc amount using ABEE labeling kit (J-chemical, Japan) [50]. ABEE-derivatized monosaccharides were separated by HPLC using HonenPak C18 column (75 mm x 4.6 194 mm I.D.) with 0.01% TFA in 50% acetonitrile as mobile phase, and then detected by fluorescence detector (F-1150, HITACHI, Japan) with excitation at 305 nm and emission at 360nm.

4.2.11 Statistical analysis

All data were expressed as means ± standard deviations (SD) and compared using t-test and Tukey's test with $p < 0.05$ and $p < 0.01$ indicating statistical significance and distinct significance, respectively. Survival curves were compared using the Kaplan−Meier test. Each experiment was performed in triplicate.

4.3 Results

In the previous chapter, we mentioned that two putative NALs have been found in *E. piscicida* genome. Therefore, to confirm their existence in *E. piscicida*, these putative genes were cloned using *E. piscicida* NUF806 genomic DNA. The PCR product were checked by electrophoresis using 1% agarose gel. The results showed that a single band for each lyase gene similar to each nucleotide predicted sizes (Figure 4-1).

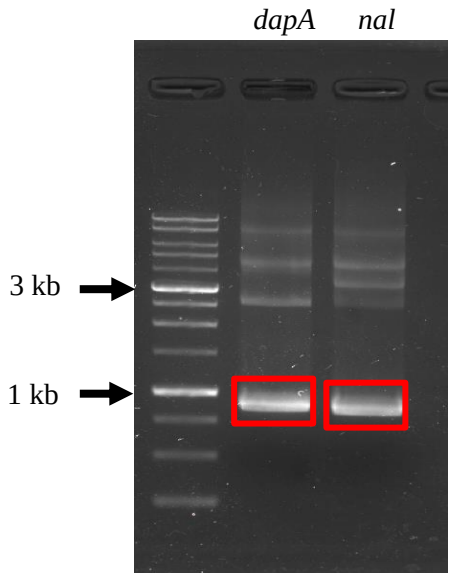

Figure 4-1. Two putative NAL genes in *E. piscicida* by electrophoresis.

The red box indicated amplicons of two target genes, *dapA* and *nal*, from left to right, respectively.

The PCR products were then purified and subcloned into pBluescript SK (+) vector using restriction enzyme treatment and ligation, followed by transformation. Subsequently, the samples collected from miniprep were confirmed the size of the insert and sequenced by ABI3130x1 Genetic Analyzer. After sequencing, the actual nucleotide sequences were obtained using Genetyx software. The deduced amino acid sequences revealed that the putative *N*-acetylneuraminate lyase 1 (previously mentioned in the chapter 3) was similar to *N*-acetylneuraminate lyase (NAL, EC 4.1.3.3) consisted of 873 bp and encoding 290 amino acids, and the putative *N*-acetylneuraminate lyase 2 (previously mentioned in the chapter 3) was similar to dihydrodipicolinate synthase (DHDPS, EC 4.3.3.7) consisted of 891 bp and encoding 296 amino acids. The similarity of amino acid sequences between DHDPS and NAL in *E. piscicida* was approximately 47%. In comparison with other bacteria, *E. piscicida* DHDPS was similar to DHDPS in some bacteria: 67.2% of *Aliivibrio salmonicida* (accession No: 5AFD_A), 19.9% of *Lactobacillus plantarum* (accession No: BBA83219), 19.6% of *Escherichia coli* (accession No: 1S5W_A), and 21.2% of *Haemophilus influenza* (accession No: ABQ99767); and, *E. piscicida* NAL was similar to other bacteria NALs: 35.9% of *Staphylococcus aureus* (accession No: 4AH7_A), 83.0% of *Escherichia coli* (accession No: 1HL2_A), 37.5% of *Clostridium perfringens* (accession No: AQW22573), and 37.5% of *Haemophilus influenzae* (accession No: 1F7B_A) (Figure 4-2). Furthermore, the converse

sequences were found in *E. piscicida* DHDPS and NAL, similar to other bacterial DHDPS and NAL, including the carboxylate binding site (sequence GxxGE), sugar-binding site (sequence GxDE), and aldol-cleavage zone (sequences KxT/Sx and xxG/ST) (Figure 4-2). This conserved motif suggested that *E. piscicida* NAL and DHDPS could be classified to group 2 and in subgroup 4.4 [45]. The nucleotide sequences of *E. piscicida dapA* (gene of DHDPS) and *nal* (gene of NAL) in this study were deposited in the GenBank database as the accession IDs LC602498 and LC602499, respectively.

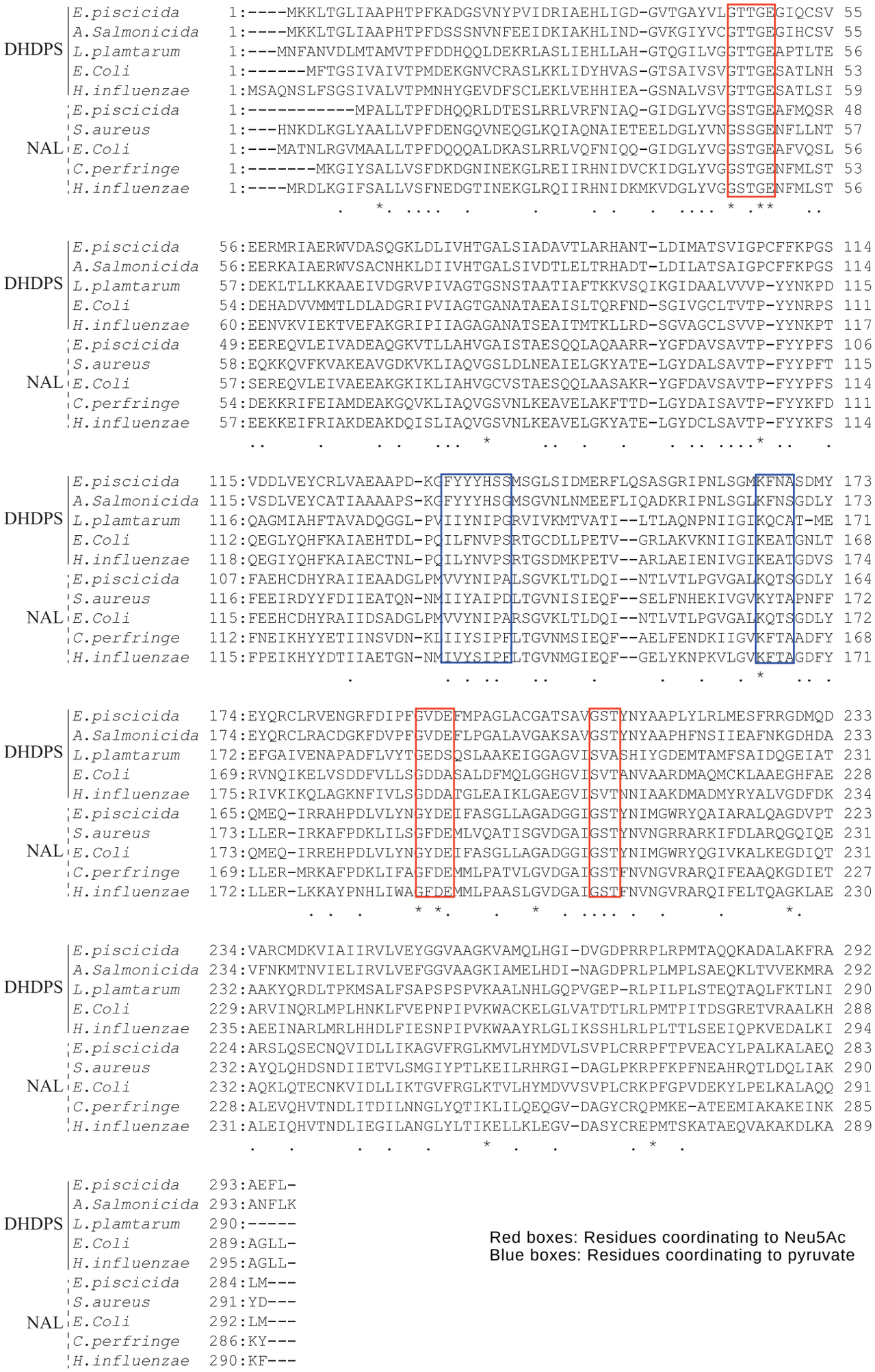

Figure 4-1. Sequence alignment of different bacterial DHDPSs and NALs.

In general, DHDPS and NAL are members of NAL sub-family which share a common catalytic step. In the chapter 3, *E. piscicida dapA* and *nal* mRNA level were up-regulated by Neu5Ac treatment, suggesting that the exogenous Neu5Ac may be metabolized by DHDPS and/or NAL. Besides, it remains to be clarified whether the other sialic acids type such as Neu5Gc and KDN could act as substrates for DHDPS and NAL. Hence, to evaluate the activity of DHDPS and NAL toward Neu5Ac, Neu5Gc and KDN for cleavage activity and ManNAc and Man for condensation activity, *E. piscicida* DHDPS or NAL strain lysates were prepared and used as crude enzymes. The results showed that the cleavage activity of DHDPS was obtained as 11.4, 7.4, and 1.0 μmol/min/mg protein for Neu5Ac, Neu5Gc, and KDN, respectively, whereas the mock exhibited negligible activities toward all tested sialic acids ($p < 0.01$, Table 4-2). In contrast, a longer reaction time was required for NAL enzyme activity compared to DHDPS, indicating that NAL activity was lower than the activity of DHDPS *in vitro.* NAL showed significant cleavage activity toward Neu5Ac, Neu5Gc, and KDN as 0.31, 0.16, and 0.08 μmol/h/mg protein, respectively, compared to mock (0.17, 0.12, and 0.01 μmol/h/mg protein, respectively, $p < 0.05$, Table 4-2).

Table 4-2. Cleavage activity at 37°C for 5 mM substrate

Source	Substrate			Unit
	Neu5Ac	Neu5Gc	KDN	
Mock	0.027 ± 0.012	0.017 ± 0.013	0.012 ± 0.009	(μmol/min/mg)
epDHDPS	11.402 ± 0.166 **	7.433 ± 0.754 **	0.987 ± 0.056 **	
Mock	0.175 ± 0.014	0.118 ± 0.006	0.015 ± 0.004	(μmol/h/mg)
epNAL	0.310 ± 0.057 *	0.156 ± 0.010 *	0.081 ± 0.006 **	

*Significant differences are indicated using letters (t-test, * $p<0.05$, ** $p<0.01$) compared to mock.*

The condensation activity of DHDPS was drastically greater than that of mock for the substrates: ManNAc (77.9 and 0.28 μmol/min/mg protein, respectively, $p < 0.01$) and Man (2.7 and 0.14 μmol/min/mg protein, respectively, $p < 0.01$), whereas the condensation activity of NAL did not differ with that of mock (Table 4-3). Taken together, *E. piscicida* NAL and DHDPS showed different enzymatic properties toward the substrates of NAL, presumably due to their diverse physiological roles in the bacteria.

Table 4-3. Condensation activity at 37°C for 5 mM substrate

Source	Substrate		Unit
	ManNAc	Man	
Mock	0.275 ± 0.053	0.139 ± 0.006	(μmol/min/mg)
epDHDPS	77.903 ± 1.161 **	2.733 ± 0.213 **	
Mock	6.877 ± 0.598	2.640 ± 0.108	(μmol/h/mg)
epNAL	7.991 ± 2.320 n.s	2.244 ± 0.964 n.s	

*Significant differences are indicated using letters (t-test, * p<0.05, ** p<0.01) compared to mock.*

Recently, it has been reported that sialic acid and its related genes are involved in bacterial pathogenicity [51]. The previous study also showed that free sialic acid enhanced *E. piscicida* infection *in vitro*, presumably via sialic acid catabolism [52]. Therefore, we focused on the role of DHDPS and NAL in *E. piscicida* pathogenicity *in vitro* and *in vivo*.

The *in vitro* infection test was evaluated by using goldfish GAKS cells. The *E. piscicida* transformants strains were preincubated with Neu5Ac (1 mg/mL) for 30 min, and then inoculated in GAKS cell for 1 h. The result showed that the NAL strain significantly increased the cellular infection compared to the mock and DHDPS strains (64, 33 and 31 colonies/plate, respectively, $p <$

0.01, Figure 4-3A). To confirm the enhancement infection involved in NAL, the NAL strain was pretreated with bromopyruvate, a general NAL inhibitor, before infection test. The inhibitor pretreatment NAL strain drastically decreased the infection toward GAKS cells compared to vehicle (133 and 33 colonies/plate, respectively, $p < 0.01$, Figure 4-3B).

Next, the *in vivo* test was carried out using zebrafish larvae exposed to the DHDPS and NAL strains. Zebrafish larvae were used because of its high susceptibility [53]. The mock, DHDPS and NAL strains were immersed in wells with the 5 dpf zebrafish larvae for 2 h. The survival of zebrafish larvae was documented for 7 days. As shown in Figure 4-3C, the NAL strain induced lower fish survival compared to mock (55% and 75%, respectively, $p < 0.05$); however, the survival of zebrafish larvae infected by DHDPS was no changed compared to mock (71%, $p > 0.05$). These results suggested that NAL, not DHDPS, enhanced *E. piscicida* pathogenicity *in vitro* and *in vivo*, presumably via sialic acid catabolism.

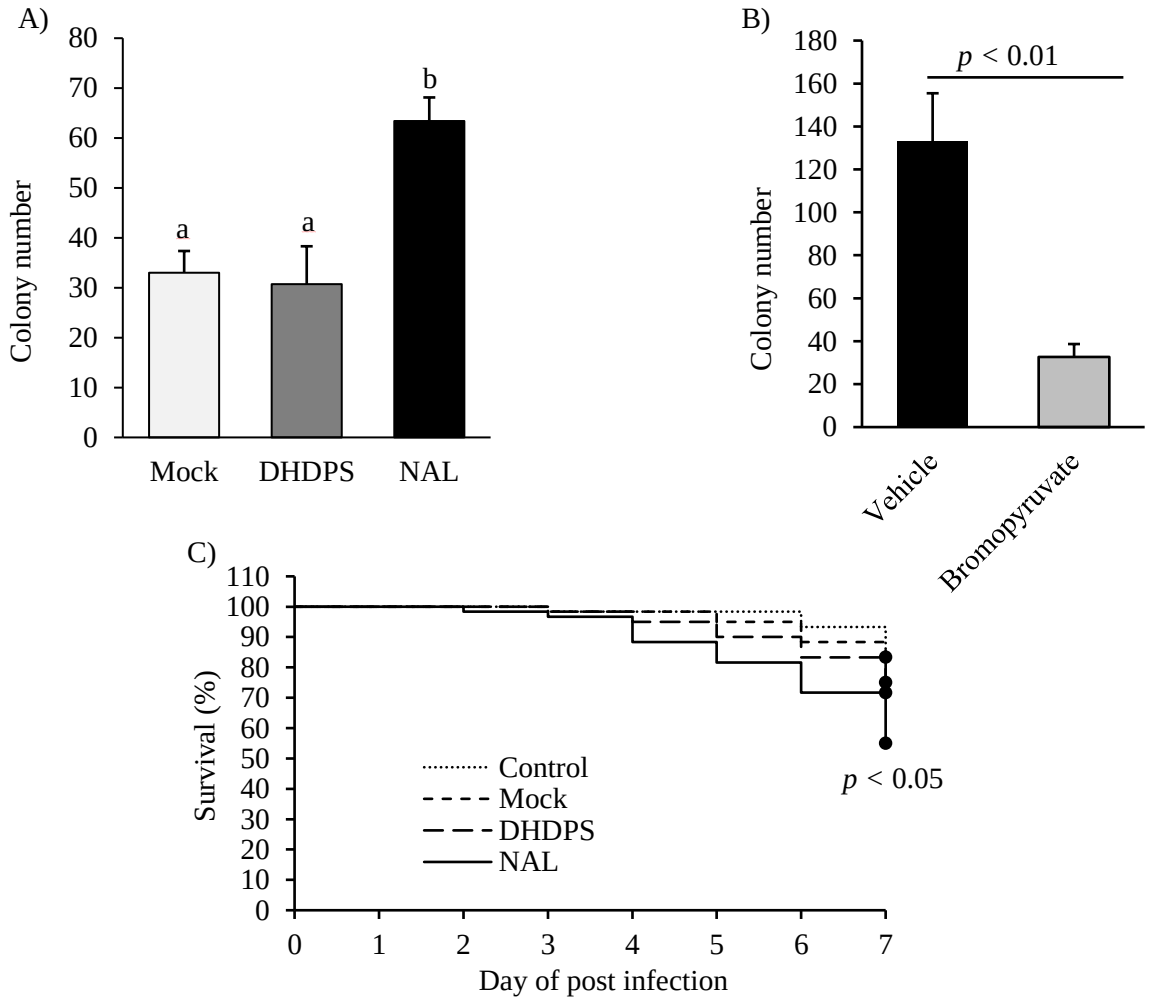

Figure 4-3. Involvement of DHDPS and NAL in *E. piscicida* pathogenicity.

A) Infection toward GAKS cell. The GAKS cells were inoculated with *E. piscicida* at MOI100 for 1 h at 28 °C. Formed colonies were enumerated after 24 h. The mock, DHDPS, and NAL strains are depicted with white, grey, and black bars, respectively. Significant differences are indicated using letters (Tukey-Kramer test, $p < 0.01$). B) The NAL strain was treated with 5 mM bromopyruvate, and the used for infection test. C) Bath-infection for zebrafish larvae. 10^8 CFU mL^{-1} of *E. piscicida*

were exposed to 5 dpf zebrafish larvae in wells for 2 h. The survival of zebrafish larvae was recorded for continuous 7 days. The control (fish only), mock, DHDPS, and NAL strains are depicted with dotted line, dashed line, long dashed line, and solid line, respectively.

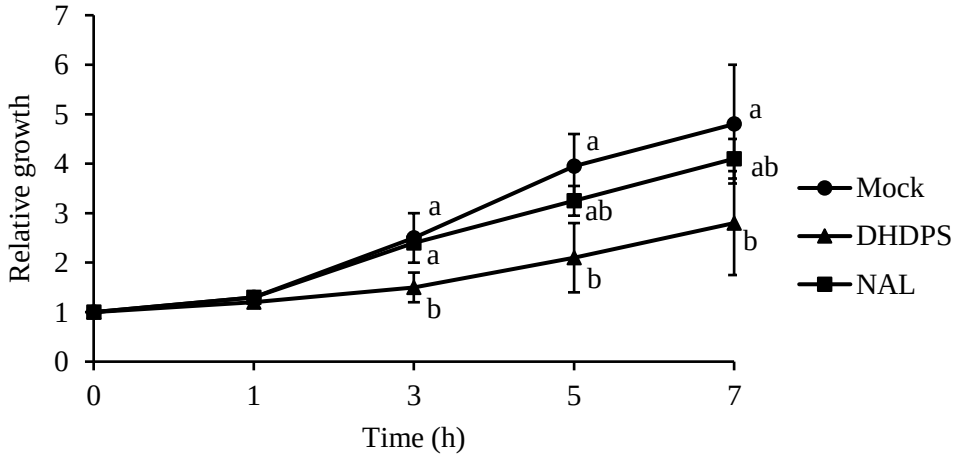

Figure 4-4. Involvement of DHDPS and NAL in bacterial growth.

The DHDPS and NAL strains were cultured in M9 medium containing Neu5Ac (1 mg/mL). Bacterial growth in each medium was assessed using the value of OD_{630}. The mock, DHDPS, and NAL strains are depicted with closed circle line, closed triangle line, and closed square line, respectively.

Recent studies reported that sialic acids can be used by pathogen via its catabolism. Hence, we hypothesized that DHDPS and/or NAL may involve in *E. piscicida* growth. The growth assay

was carried out as follows: the *E. piscicida* transfectant strains were cultured on M9 minimal medium adding Neu5Ac (1 mg/mL) and recorded OD_{630} every hour. As a result, the DHDPS strain exhibited slow growth in the sialic acid supplemented medium compared to mock; however, no change was observed in the NAL strain (Figure 4-4).

In general, biofilm formation plays an important role in the enhancement of the bacterial survival under adverse conditions. In this chapter, we assessed the effect of DHDPS and NAL on biofilm formation. The mock, DHDPS and NAL strains were inoculated in 96-well flat-bottomed microtiter plates. After cultivation for 72 h, the biofilm of the DHDPS and NAL strains were evenly distributed on the bottom of the 96-well plate; however, the NAL strain showed thicker biofilm with darker violet color (stained by crystal violet dye), whereas the mock cells were not (Figure 4-5A). The difference of these strains' biofilms may be due to the disparity of the biofilm amount. Indeed, the biofilm amount of the NAL strain was significantly increased compared to the mock strain (202% of mock, $p < 0.05$), and the biofilm formation in the DHDPS strain was slightly enhanced (126% of mock, $p > 0.05$, Fig. 4-5B).

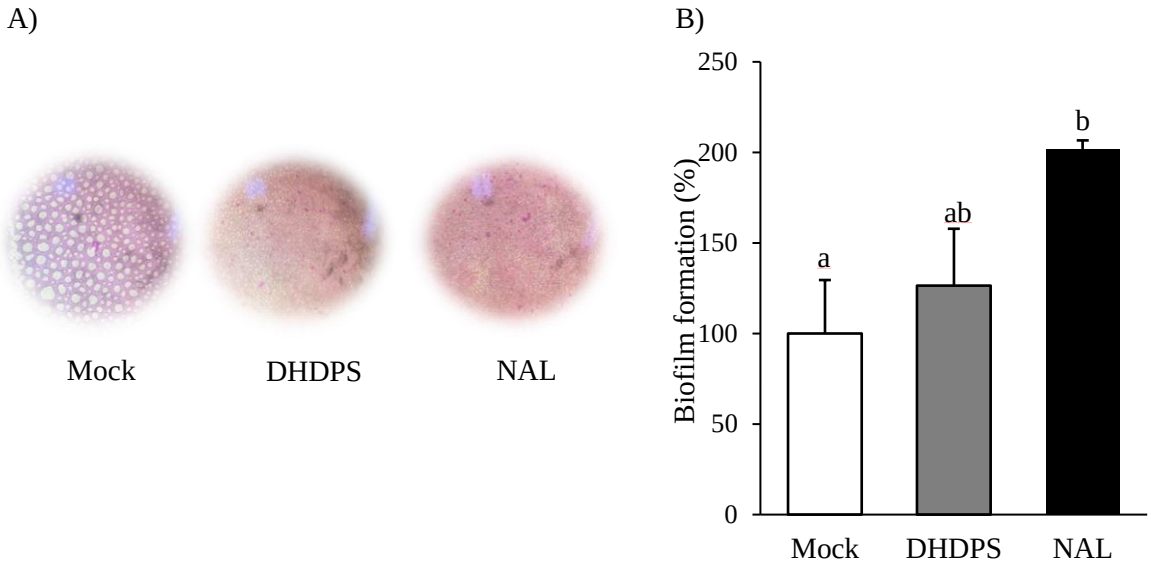

Figure 4-5. Effect of DHDPS and NAL on biofilm formation.

The mock, DHDPS and NAL strains were inoculated in a 96-well-plate for 72 h.(A) The adherent bacterial was stained with 150 µL of 0.1% crystal violet for 15 min, washed with water, and observed under microscope.. (B) After air-drying, 150 µL of 95% ethanol was added and evaluated by a spectrophotometer at 570 nm. Mock, epDHDPS, and epNAL are depicted with white, grey, and black bars, respectively. Results from three independent experiments are presented as mean ± SD. Significant differences are indicated using letters (Tukey-Kramer test, $p < 0.01$).

In natural, motility is defined the movement of bacteria, as the initial phase in biofilm formation. Bacterial motility comprises three types: swim, swarm, and twitch. To evaluate whether

the sialic acid catabolism drives *E. piscicida* motility via NAL and/or DHDPS, the swimming, swarming, and twitching test were conducted using 0.3%, 0.5% and 1% LB agar plates, respectively. The results showed that the swimming zone of the NAL strain was wider than that of the mock (74 and 25.6 mm, respectively, $p < 0.01$), whereas that of the DHDPS strain did not differ (16.7 mm, $p > 0.05$, Figure 4-6A) after 36 h of cultivation. Furthermore, the swarming of the NAL strain was greater active than that of the mock and DHDPS strains (9.7, 5.3, and 5.2 mm respectively, $p < 0.05$, Figure 4-6B). However, none of the tested strains exhibited twitching (Figure 4-6C, $p > 0.05$). Collectively, NAL, not DHDPS, may act a crucial role in bacterial movement and biofilm development.

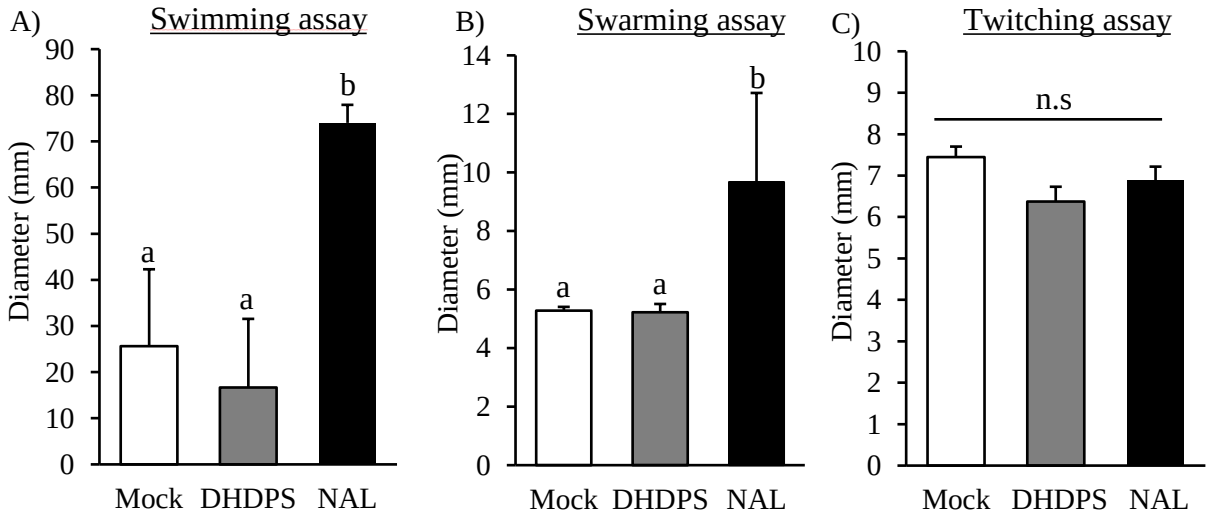

Figure 4-6 Effect of DHDPS and NAL on motility.

The DHDPS and NAL strains were inoculated on 0.3%, 0.5%, and 1% LB agar to test for (A) swimming, (B) swarming, and (C) twitching, respectively, and then incubated at 28 °C. The motility zones of swimming, swarming, and twitching were measured at 36, 48, and 72 h, respectively. Significant differences are indicated using letters (Tukey-Kramer test, $p < 0.01$).

Next, we assessed the alteration of the sialic acid related genes in the *E. piscicida* transformant strains to understand the mechanism of NAL-induced pathogenicity. Figure 4-7 illustrates the sialic acid catabolic pathway which involved in NAL, NanK, NanE, NagA, NagB, NagK, Pgm3, GlmM, and GlmU. Thus, we analyzed the mRNA levels of these genes in the Neu5Ac-treated *E. piscicida* transformant strains by real-time PCR. As shows in Figure 4-8A and 4-8B, the expression levels of *nanK* and *nanE* were significantly elevated in the NAL strain than that of the mock (1.8-and 3-fold increase than mock, respectively, $p < 0.01$), whereas that of the DHDPS strain did not change ($p > 0.05$). In contrast, the expression levels of *nagK*, *nagA*, *pgm3*, and *glmM* did not differ between the mock, DHDPS and NAL strains ($p > 0.05$, Figure 4-8C, 4-8E, 4-8F and 4-8G). On the other hand, *nagB* gene was significantly decreased in the DHDPS strain and up-regulated in the NAL strain ($p < 0.05$, Figure 4-8D). The mRNA level of *glmU* was also significantly higher in the NAL strain (1.9-fold increase, $p < 0.05$, Figure 4-8H). These results

suggested that NanK, NanE and GlmU may be critical in regulating the enhancement pathogenicity in the NAL strain, presumably accompanied by the synthesis of UDP-GlcNAc.

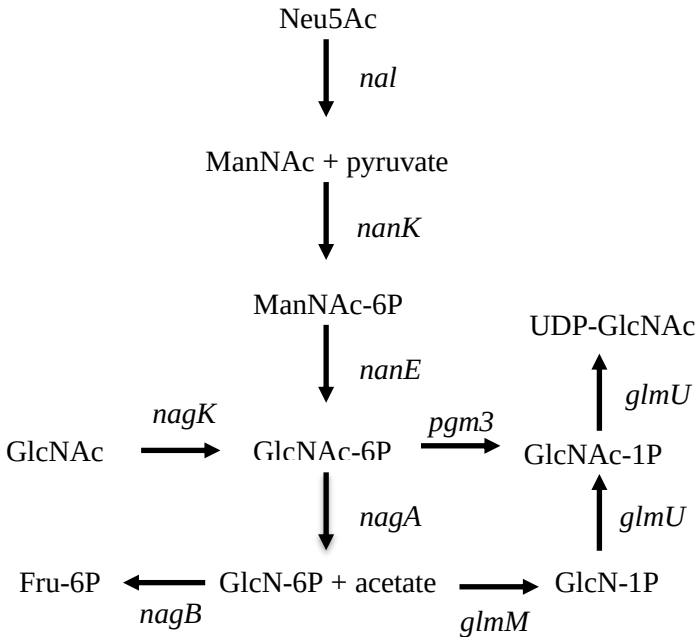

Figure 4-7. Schematic view of sialic acids catabolic pathway.

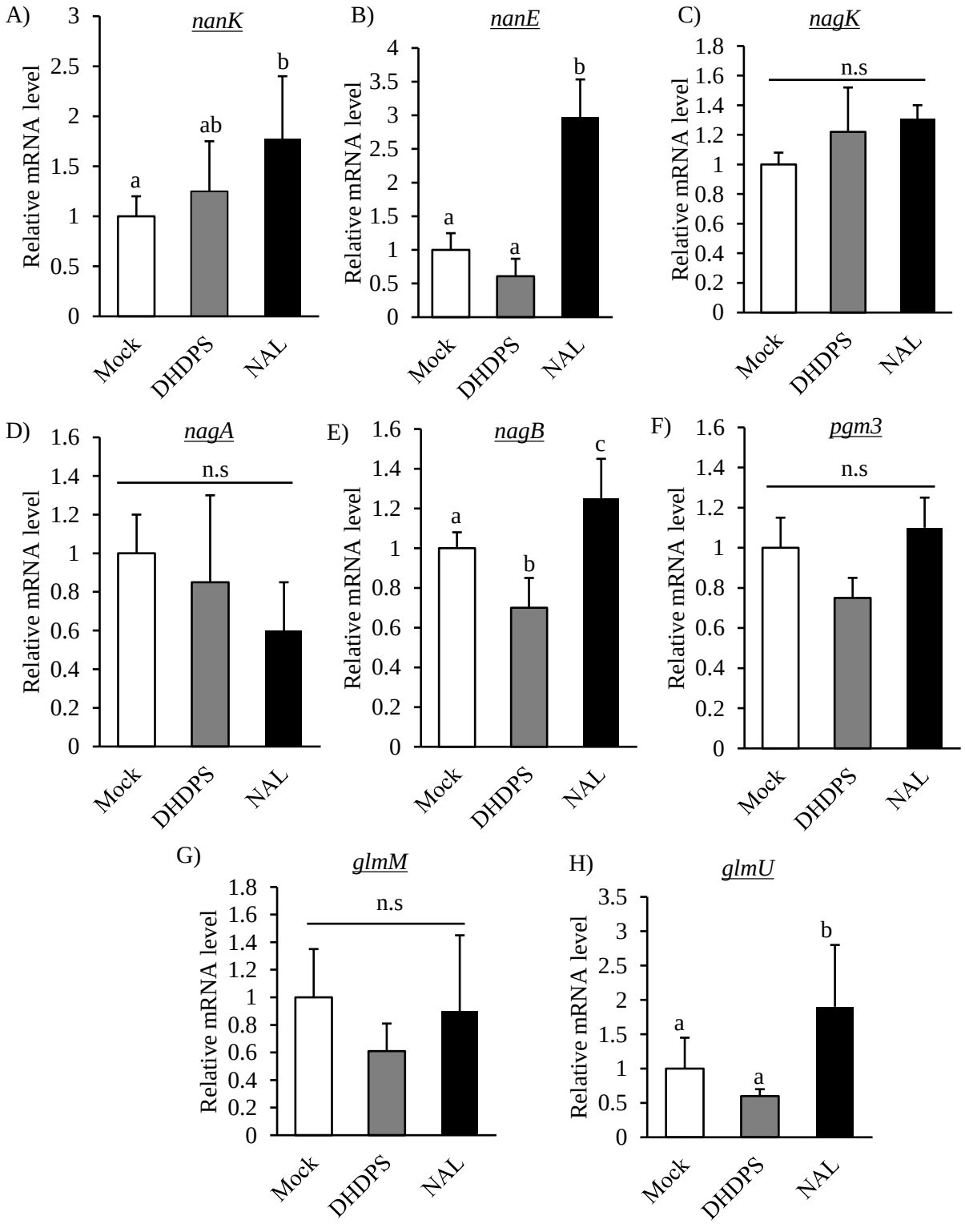

Figure 4-8. Expression level of sialic acid catabolism related genes.

As GlcNAc can intensify cell motility by regulating the curli expression in *Escherichia coli* [54], we estimated the total GlcNAc amount in the NAL and DHDPS strains. The result indicated that the NAL strain significantly elevated the amount of total GlcNAc compared to that of the mock (1.40 and 1.14 nmol/mg bacteria, respectively, $p < 0.05$), whereas the total GlcNAc content in the DHDPS remained unchanged ($p > 0.05$, Figure 4-9). These results suggested that the upregulation of NanK, NanE and GlmU in the NAL strain may increase the total GlcNAc content, thereby increasing the pathogenicity.

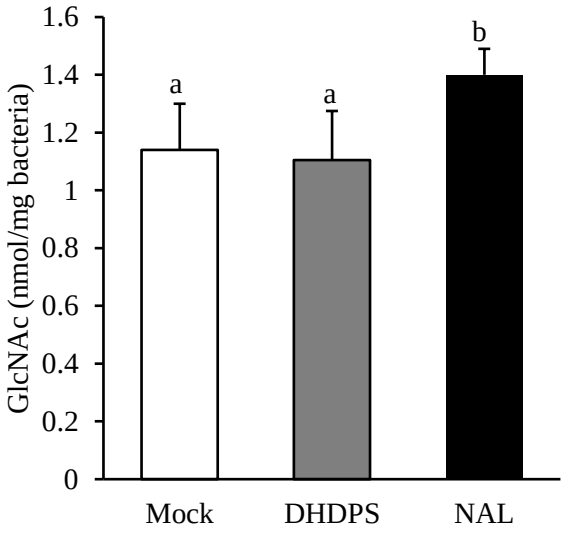

Figure 4-9. Total GlcNAc amount in the *E. piscicida* transformant strains.

The pellets of Neu5Ac-pretreated bacteria were hydrolyzed by trifluoroacetic acid. The GlcNAc amount were analyzed using HPLC with ABEE derivatization. Significant differences are indicated using letters (Tukey-Kramer test).

4.4 Discussion

In the previous chapter, we suggested that free sialic acid can enhance *E. piscicida* infection, presumably via sialic acid catabolism. Interestingly, two putative NAL genes were discovered based on the *E. piscicida* genome and their expression patterns differed in *E. piscicida* strains. Therefore, we cloned these genes and then clarified the role of that in *E. piscicida* pathogenicity.

After cloning, the actual NALs genes were revealed and identified as dihydrodipicolinate synthase (DHDPS) and *N*-acetylneuraminate lyase (NAL). Generally, NAL and DHDPS have been known to be belong to the NAL subfamily of $(\beta/\alpha)_8$-barrels which shared involved in catalyzing the cleavage/condensation of sialic acids [18, 55]. In nature, bacteria can be classified into three groups according to NAL: 1) only DHDPS, 2) only NAL 3) both DHDPS and NAL found in genome; however, there are no reports to compare the physiological functions of DHDPS and NAL.

In this study, DHDPS was detected to show potent enzymatic activity with substrates of NAL *in vitro* compared to NAL. In general, DHDPS which catalyzes the aldol condensation of pyruvate and L-aspartate-β-semialdehyde (ASA) reported that is responsible for the first step in the biosynthetic pathway for the production of l-lysine in bacteria and also associate with the production of essential metabolites for cell wall and protein synthesis [56, 57]. Nevertheless, a few studies reported that DHDPS can act to both DHDPS and NAL substrates [20]. On the other hand,

the enzymatic activity of NAL was evaluated under a long incubation time, from several hours to days in other bacteria [22, 45]. Hence, the *E. piscicida* NAL activity may be equivalent to that for other bacterial NAL. The study revealed DHDPS impaired *E. piscicida* growth in the Neu5Ac-supplemented M9 medium, whereas the NAL strain exhibited similar growth to the mock. This study also found that the expression level of *nagB* was lower in the DHDPS strain than that in the mock and NAL strain, presumably to be a reason for the slow growth in the DHDPS strain.

The study demonstrated that NAL involved in the enhancement of *E. piscicida* pathogenicity, whereas DHDPS did not. The increased expression levels of *nanK* and *nanE* were found only in the NAL strain. In general, NanK and NanE are the key enzymes for the utilization of sialic acids and appears to be a conserved protein among gram-positive and gram-negative bacteria [44, 58]. GlcNAc-6P yielded by NanE can be convert to GlcNAc [59].; however, responsible enzyme has not been identified in *E. piscicida.* The product of NagA, GlcN-6P, can be convert to UDP-GlcNAc by GlmM and GlmU; indeed, the expression levels of *nagA* and *glmM* did not alter, whereas the mRNA level of *glmU* was upregulated in the NAL strain. Besides, the increment of total GlcNAc amount was detected only in the NAL strain. Collectively, the synthesis of UDP-GlcNAc may be intensify through GlmU in the NAL strain, leading the alteration of GlcNAc in

glycoconjugates. Furthermore, NAL enhanced the expression of biofilm formation and motility in *E. piscicida*. In general, GlcNAc is the major component for the biosynthesis of cell envelope such as capsular polysaccharide (CPS) in bacteria [60], lipid A, peptidoglycan, and extracellular polymeric substances (EPS) including curli and fimbriae [54]. The involvement of NAL in the bacterial morphotype has been reported in several bacteria. For example, *Vibrio vulnificus* possesses NAL and the knockout NAL strain induces the alteration of the bacterial morphotype such as CPS, resulting the impairment adhesion, colonization, and survival during infection [61]. In addition, EPS, especially curli, is a major component of the matrix in the biofilm for gram-negative bacteria [62]. Recent study reported that curli fibers are involved in biofilm formation, swarming and swimming motility [63]. In *E. coli*, the depletion of NanE and the low level of GlcNAc induced the decrease of curli expression [54]. The functions of *E. piscicida* in biofilm formation, motility and infection are similar to other bacteria [47, 61]. Collectively, this chapter revealed the relationship between NAL and *E. piscicida* pathogenicity via the regulation of biofilm formation, motility, and infection by the upregulation of NanK, NanE and GlmU level and the elevation of GlcNAc content.

Chapter 5 Conclusion

The current study clarified the molecular mechanism of *E. piscicida* infection via sialidase or sialic acids and its catabolism. Firstly, our study described the relationship between sialidase activity and *Edwardsiella* sp. pathogenicity using several strains including pathogenic strains (E381, FPC498, NUF806), one pathogenicity-attenuated strain (E22), and one non-pathogenic strain (SU138). Generally, sialidase has been identified in many pathogens such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Salmonella typhimurium*, and *Clostridium perfringens* [17]. The role of sialidase in bacterial virulence has been reported, for instance, *Streptococcus pneumoniae* sialidase is identified to play an important role in the promotion of *S. pneumoniae* adhesion and invasion toward human brain microvascular endothelial cells [64]. *S. pneumoniae* sialidase deletion mutants are defective for host colonization and the ability to cause sepsis *in vivo* [27]. Furthermore, this study demonstrated that the cleavage of terminal sialic acid from glycoconjugates by NanA sialidase is a crucial step in *E. piscicida* infection. The cleavage of sialic acids from host glycoconjugates unmask Man and GlcNAc which can act as the potential binding site on host cells for *E. piscicida*. Our study showed that a glycoprotein containing Man and GlcNAc acts as a decoy for the bacteria during infection, leading diminished *E. piscicida* infection. Other report revealed that *E. piscicida* was invasive from microdomains through endocytosis, which may occur after the bacterial attachment to host cell [65].

Secondly, the study also details the function of free sialic acid for the *Edwardsiella* sp. growth as well as *E. piscicida* infection via two metabolic pathways in sialic acid utilization: the degradation of sialic acid for use as a nutrient source and the conjugation of sialic acid with CMP for the sialylation of glycoconjugates in *E. piscicida.* Several bacteria can utilize sialic acids as a carbon source for their growth, for example, *Bacteroides fragilis* showed the growth ability in glucose-depleted medium containing sialic acids [29]. NanA-induced sialic acids may be transfer to *E. piscicida* by the sialic acid transporter and immediately metabolized by *N*-acetylneuraminate lyases or CMP-Neu5Ac synthase. The metabolites from the catalyzation by CMP-Neu5Ac synthase are likely used for sialylation of glycoconjugates, such as LPS, glycoproteins, and glycolipids, within *E. piscicida* itself. In other bacteria, such as *H. influenza*, they have defined the capability to scavenge host sialic acid for both catabolism and sialylation of its LPS [27].

E. piscicida genome revealed the presence of putative sialic acid utilization-related genes: sialic acid transporter, *N*-acetylneuraminate lyase, ManNAc kinase and potentially ManNAc-6-P epimerase. Among these genes, we focused on *N*-acetylneuraminate lyase family which found with two putative genes as same name, that acts a pivotal role in the regulation of sialic acid catabolism. The two putative genes were cloned and sequenced, that referred as dihydrodipicolinate synthase (DHDPS) and *N*-acetylneuraminate lyase (NAL). In general, DHDPS is shown the main catalysis

toward L-aspartate-β-semialdehyde (ASA) and pyruvate, nevertheless, this enzyme also acts to NAL substrates. Indeed, DHDPS impaired *E. piscicida* growth by suppressing NagB expression level. However, the detail mechanism of DHDPS reaction pathway have been unknown. On the other hand, the catabolites from the degradation by NAL are not only used for *E. piscicida* growth, because the NAL strain did not growth faster than mock.

This study revealed that NAL plays a crucial role in promoting biofilm formation and motility in *E. piscicida.* In addition, the study also demonstrated that NAL, not DHDPS, enhanced *E. piscicida* infection *in vitro* and *in vivo*. In other bacteria, for example *Vibrio vulnificus*, the *nal* mutant showed a low level of cytotoxicity toward epithelial cells and reduced virulence in a mouse model [61]. Down-regulation of NAL level in *E. coli* induced the impairment of biofilm formation and invasion toward eukaryotic cells [47]. Moreover, the current study has shown that the overexpression of NAL induced the up-regulation of NanK, NanE, and GlmU levels accompanied by the increment of total GlcNAc contents. In *E. coli*, the involvement of NanE and GlcNAc level in the curli expression, resulting the expression ability of motility and biofilm have been reported [54]. Besides, sialic acid and its metabolites, GlcNAc and GlcNAc-6P, have shown to involve in the regulatory mechanisms affecting type I fimbriae and curli expression [66, 67].

Since *E. piscicida* is intracellular parasites, its elimination by humoral immunity is difficult [68]. Besides, *E. piscicida* is also a multidrug resistant pathogen, thus it makes the medical challenges to control Edwardsiellosis disease. The present study has indicated that the removal of sialic acids by *E. piscicida* NanA sialidase from host glycans induces a series of events in positive regulations resulting its invasion. Therefore, *E. piscicida* sialidase would be a novel target molecule in the treatment of Edwardsiellosis. Recent report showed that naringenin, a flavonoid originating from citrus, suppressed *E. piscicida* infection by interrupting sialidase activity [37]. Other natural compound, curcumin analogues, originating from *Turmeric,* is also shown as an inhibitor of *S. pneumoniae* sialidase, that could be a potent agent for treating bacterial infection [36]. In other bacteria, such as *Pseudomonas aeruginosa*, sialidase inhibitor also impaired the adhesion to host cell [69]. Besides, a putative NANA-synthase gene is found in cluster with CMP-NANA synthase on *E. piscicida* genome, suggesting that sialic acids metabolism is critical on the development of *E. piscicida* phenotypes. In addition, our study has demonstrated NAL plays a crucial role in *E. piscicida* infection. Sialic acid and its catabolism have been demonstrated to be important in promoting *E. piscicida* phenotypes and infection. Thus, NAL could be a viable target molecule for control of *E. piscicida*. Previous study indicated that NAL is recognized as a capable antibiotic drug target [46]. Furthermore, *nal* deletion strains of *V. cholerae* showed the decrease of colonization

and survival ability to the mouse intestine [61]. However, natural compounds which act as NAL inhibitor has no report. Collectively, this study provides the insights of *E. piscicida* infection mechanism via sialidase and sialic acid catabolism; and further research on inhibition of sialidase and NAL activity is useful for the discovery of novel and effective approach to prevent Edwardsiellosis in fish, that can diminish the burden of antimicrobial pollution.

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