

**Production and Characterization of Enterobactin-specific Antibodies Induced
by Novel Enterobactin Conjugate Vaccines**

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DEDICATION

This thesis is dedicated to my supportive parents and family. Thank you for the selfless support and constant care along the way I pursue my academic goal. Without your love, I would not be able to achieve this and move forward. Also, I would like to dedicate this to my future wife Shanshan Cao. Thank you for understanding and supporting me when I was working with 12 hours of time difference from you. You are the best girlfriend and wife I can ever ask for.

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ABSTRACT

Enterobactin (Ent)-mediated high affinity iron acquisition is critical for Gram-negative bacteria to survive in the host. Given the bacteriostatic effect of lipocalin resulting from its potent Ent-binding ability, immune intervention directly targeting Ent is promising for iron-dependent pathogen control. Moreover, passive immunization with Ent-specific egg yolk antibody (IgY) is a promising alternative to antibiotics to prevent and treat bacterial infections. In the first study, using a simple method, we successfully conjugated purified Ent to different carriers including keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), and CmeC, a vaccine candidate for *Campylobacter* control. Subcutaneous immunization of rabbits with the KLH-Ent conjugate triggered strong systemic IgG immune response with up to 16,384 fold increase in IgG titer directed against whole conjugate and up to 4,096 fold increase in the level of specific anti-Ent IgG. The Ent-specific IgG also displayed exceptional reactivity to ferric Ent, linear trimer of Ent, and different salmochelins. Growth assays further demonstrated the Ent-specific antibodies significantly inhibited Ent-dependent growth of *Campylobacter* spp. and *E. coli*. In the second study, using different vaccination regimens, we immunized laying hens with different Ent conjugate vaccines to produce Ent-specific egg yolk IgY. In the first trial, Barred Rock layers (n=3) were intramuscularly immunized with the KLH-Ent conjugate vaccine, which triggered significant immune response in layers, leading to moderately increased titers of Ent-specific IgY in serum (up to 8 folds) and yolk (up to 2 folds). In the second trial, Rhode Island Red layers were subcutaneously immunized with specific Ent conjugates (KLH-Ent, BSA-Ent, or CmeC-Ent; n=2 per conjugate). ELISA analysis indicated that anti-Ent IgY titers dramatically increased in serum (up to 2,048 fold) and yolk (up to 1,024 fold) of each individual layer upon vaccination with KLH-Ent or CmeC-Ent. However, the BSA-Ent conjugate failed to induce anti-Ent IgY.

The hyperimmune egg yolks were pooled and lyophilized for future evaluation of *in vivo* passive immune protection. Collectively, these studies have provided solid foundation for developing innovative and effective Ent-dependent immune interventions against Gram-negative bacterial infections in the future.

Key words: enterobactin, iron acquisition, conjugate vaccine, egg yolk IgY, Gram-negative pathogens

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CHAPTER I: Review of Literature

1.1 Siderophore-mediated iron acquisition in bacteria

Iron is a vital nutrient for nearly all prokaryotic and eukaryotic cells. Its role ranges from energy generation and DNA replication to oxygen transport and protection against oxidative stress (Skaar, 2010). Because of the insolubility of ferric hydroxide, the concentration of Fe(III) in water is very low (10^{-18} M). Mammalian iron-binding proteins further reduce the concentration of free Fe(III) to an estimated 10^{-24} M in human serum (Raymond et al., 2003). This scarcity of free iron presents a challenge for bacterial cells, which require a cytoplasmic iron concentration of $\sim 10^{-6}$ M for growth (Andrews et al., 2003). Bacteria have responded to this selective pressure by evolving the ability to acquire iron via numerous (and often redundant) mechanisms, primarily including the high-affinity iron acquisition mediated by utilization of small chelators with high affinity for Fe(III), known as siderophores, and the direct use of host iron-binding proteins (e.g., transferrin, lactoferrin, hemoglobin, and hemopexin) without utilization of siderophores (Fischbach et al., 2006; Skaar, 2010).

1.1.1 Direct use of host iron-binding proteins

As most iron in humans is contained within hemoglobin, many bacterial pathogens have developed mechanisms to liberate hemoglobin from erythrocytes and utilize heme as iron source. The requirement for heme may be satisfied through endogenous heme production, exogenous heme acquisition, or combination of both (Cassat & Skaar, 2013). There are two major classes of bacterial heme acquisition systems: hemophore-dependent system and direct heme uptake system (Anzaldi & Skaar, 2010). For hemophore-dependent system, bacterial pathogens can utilize hemophores to scavenge heme from host hemoproteins and deliver heme to bacterial cells through hemophore receptors. They are functionally analogous to siderophores but specifically target heme, while siderophores target iron atoms (Wandersman & Delepelaire, 2004). For direct

heme uptake system, it typically involves surface receptors that recognize either heme or heme-binding hemoproteins, such as hemoglobin or hemopexin. Subsequently, Heme is removed from hemoproteins and transported through envelope of bacteria into the cytoplasm. Once inside the cytoplasm, the iron is released from heme through the action of heme oxygenase or reverse ferrochelatase activity (Létoffé et al., 2009; Reniere et al., 2007; Wilks, 2002).

Mammals can also employ transferrin and lactoferrin to reduce the level of free extracellular iron. However, some bacteria are capable of direct recognition of transferrin and lactoferrin via surface-exposed receptors. Transferrin and lactoferrin receptors have been identified in various pathogenic bacteria, such as *Neisseria* species (Cornelissen & Sparling, 1994). These receptors are located in the outer membrane and induced by iron starvation. Iron is stripped from transferrin and lactoferrin at the bacterial cell surface and the iron-free proteins are released extracellularly rather than internalized and accumulated within bacterial cell (Andrews et al., 2003).

1.1.2 Production and utilization of siderophores for iron acquisition

Siderophores are of low molecular mass (<1,000 Da) and characterized by their high specificity and affinity towards Fe(III). When inside a host, these compounds capture iron from proteins such as hemoglobin, transferrin, or lactoferrin. Subsequently, iron-loaded siderophores are transported into the bacterial cell, where iron is released either by hydrolysis of the siderophores or by reduction of Fe(III) to Fe(II) (Faraldo-Gómez & Sansom, 2003). Siderophores are divided into three main classes based on the chemical nature of the moieties donating the oxygen ligands for Fe(III) coordination: catecholates, hydroxamates, and carboxylates. In addition, a panel of siderophores can have different classes of moieties within single molecule, resulting in “mixed-type” siderophores (Miethke & Marahiel, 2007).

The biosynthesis of siderophores in bacterial pathogens is induced by intracellular iron deficiency. There are two pathways involved in the synthesis of siderophores: one is dependent on nonribosomal peptide synthetases (NRPS) and the other is independent of NRPS (Saha et al., 2013). NRPS represents large multi-enzyme complexes that activate and assemble a broad array of amino, carboxy, and hydroxyl acids, leading to macrocyclic peptidic products with high structural variability (Grünewald & Marahiel, 2006). NRPS is mainly responsible for the synthesis of aryl-capped siderophores, such as pyochelin and pyoverdine in *Pseudomonas aeruginosa*, enterobactin in *Escherichia coli*, *Salmonella enterica*, and *Klebsiella* spp., and mycobactin in *Mycobacterium tuberculosis*. The NRPS-independent pathways are used for production of hydroxamate and carboxylate siderophores, such as petrobactin in *Bacillus anthracis*, alcaligin in *Bordetella pertussis*, and staphyloferrin A and B in *Staphylococcus aureus* (Saha et al., 2013).

In Gram-negative and low GC-content Gram-positive bacteria (e.g. *Bacillus* spp.), Fur (ferric uptake regulator) plays a critical role in regulation of siderophore production and iron homeostasis, whereas in high GC-content Gram-positive bacteria, such as *Streptomyces* and *Mycobacteria*, diphtheria toxin regulator (DtxR) controls siderophore-mediated iron acquisition (Saha et al., 2013). Fur protein (~17 kDa) acts as a repressor, using ferrous ion as a cofactor by binding to the consensus operator site, designated “iron box”, of iron acquisition genes (Skaar, 2010). With respect to DtxR, it displays very low or no sequence similarity to Fur but functions similarly to Fur by negatively regulating genes involved in iron acquisition and virulence factor (Andrews et al., 2003). In addition to these common repressors, there are several transcriptional regulators that control siderophore biosynthesis and utilization and act as activators by sensing

the iron-siderophore complex, either intracellularly or extracellularly (Miethke & Marahiel, 2007).

The export of siderophores out of bacterial cell is carried out by efflux pumps. Siderophore secretion requires the activity of at least one of the following efflux pump families: major facilitator superfamily (MFS), ATP-binding cassette (ABC), and resistance/nodulation/cell division (RND) that is specific for Gram-negative organisms (Wilson et al., 2016). The enterobactin secretion pathway has been well characterized in *E. coli*, which involves stepwise process using complex machinery systems. Briefly, enterobactin is transported from the cytoplasm to the periplasm via EntS (MFS-class efflux pump), followed by crossing outer membrane through the concerted action with aid of inner membrane RND-type efflux pump (AcrB, AcrD, or MdtABC) and the outer membrane protein channel TolC (Horiyama & Nishino, 2014).

Once Fe(III) is mobilized and captured by the secreted siderophore, the Fe(III)-siderophore complex is accessible for assimilation by bacteria (Miethke & Marahiel, 2007). In Gram-negative bacteria, the Fe(III)-siderophore complex is recognized by a specific outer membrane receptor (OMR) and subsequently transported through the receptor channel using TonB-ExbB-ExbD energy transduction system, in which TonB is an inner membrane-anchored, periplasm-spanning protein, while ExbB and ExbD proteins are embedded in inner membrane. The TonB-ExbB-ExbD system transduces the proton motive force energy to the OMR to allow translocation of Fe(III)-siderophore into cytoplasm via an inner membrane ABC transporter system (Schalk & Guillon, 2013). In contrast, Gram-positive bacteria have only a single membrane and therefore possess a simpler siderophore uptake system. In general, Gram-positive bacteria express siderophore-binding protein (SBP), associated with a permease. SBP binding to

an extracellular iron-siderophore causes a conformational change in the SBP-permease complex, allowing transport of the iron-siderophore across the membrane into the cytoplasm (Wilson et al., 2016).

After the transport of Fe(III)-siderophore complex into bacterial cell, release of iron involves hydrolysis or modification of the siderophore scaffold and/or reduction of the coordinated Fe(III). It can occur either in cytoplasm or in periplasm, depending on specific siderophore pathway and organism (Schalk & Guillon, 2013). The reduction of Fe(III) to Fe(II) by reductases would greatly reduce its affinity to siderophore, consequently leading to release of iron. Most reductases have no significant specificity for their iron-siderophore substrates, suggesting that reductive iron release has not led to the development of highly specialized enzymes but led to the involvement of already established reductase activity (Miethke & Marahiel, 2007). In contrast, specialized enzymes are employed by iron-siderophore hydrolysis, leading to a dramatic loss of complex stability and hence facilitating the subsequent removal of the iron. This is a fairly limited strategy since it is feasible only if the siderophore structure provides a hydrolysable backbone that allows enzymatic degradation. It is also more cost-intensive to the bacteria than ferrisiderophore reduction because hydrolytic iron release results in destruction of siderophore scaffold (Miethke & Marahiel, 2007).

1.2 Enterobactin-mediated iron acquisition in Gram-negative bacteria

1.2.1 General features

Enterobactin, a catecholate siderophore that has the highest affinity for Fe(III) of all natural siderophore compounds, is an archetype for iron acquisition in Gram-negative bacteria (Raymond et al., 2003). Many species of Gram-negative enteric bacteria such as *E. coli*, *S.*

enterica, *Shigella dysenteriae*, and *K. pneumoniae* harbor a ~24-kb gene cluster encoding 13 proteins that collaborate to synthesize, transport, and process enterobactin. Enterobactin is a 2,3-dihydroxybenzoylserine (DHB-Ser) trilactone and assembled from 2,3-dihydroxybenzoic acid (DHBA) and serine by NRPS that comprises EntE, EntB, and EntF. The primary metabolite chorismate is diverted to DHB by successive action of EntC, EntB, and EntA, whereas EntD catalyzes the post-translational 4'-phosphopantetheinylation of EntB and EntF that is required for covalent tethering of assembly-line intermediates. Following synthesis in the cytoplasm, enterobactin is exported through the inner membrane by EntS, a major facilitator superfamily exporter, and through the outer membrane by a TolC-dependent mechanism (Fischbach et al., 2006).

Once secreted outside of bacterial cell, enterobactin can successfully compete for Fe(III) binding with all known protein and small-molecule ligands, given its remarkably strong affinity to Fe(III). The ferric enterobactin (FeEnt) complex is then imported through the outer-membrane enterobactin-specific OMR, such as FepA in *E. coli*, in a TonB-dependent process, escorted through the periplasm by FepB, and pumped through the two-protein inner-membrane channel FepDG into the cytoplasm by FepC-catalyzed ATP hydrolysis (Fischbach et al., 2006). Liberation of Fe(III) from FeEnt complex requires enzymatic degradation of its trilactone scaffold to three equivalents of DHB-Ser by the esterase Fes (Lin et al., 2005). The entire enterobactin system is under transcriptional control by the iron-dependent repressor Fur, which serves as a sensor for intracellular iron concentration. Thus, transcription of the enterobactin synthesis, export, and import genes is activated during low-iron conditions and repressed when iron is abundant (Fischbach et al., 2006).

1.2.2 FeEnt receptors

FeEnt receptors, as the outer membrane “gatekeepers”, can bind the FeEnt with high affinity for internalization in Gram-negative bacteria. Generally, the receptors are induced by iron starvation and are not present under iron-sufficient conditions. One important reason for this may be the targeting of OMRs by bacteriophages, microcins, and antibiotics as an entry point into bacterial cells (Andrews et al., 2003). There are often multiple FeEnt receptors within the same Gram-negative species, each possessing varied affinity to enterobactin and its derived products. For example, in *E. coli*, there are up to five FeEnt receptors (FepA, Cir, Fiu, IroN, and Iha) binding to enterobactin and its derivatives (Wang et al., 2019); in *Campylobacter*, CfrA and CfrB have been identified to bind FeEnt complex (Zeng & Lin, 2014). Such redundancy indubitably provides bacteria survival advantages via versatile FeEnt uptake. However, since such surface-exposed iron-regulated outer membrane proteins have been intensively targeted as vaccine candidates, the redundant FeEnt receptors within the same Gram-negative species may raise significant challenges for developing enterobactin receptors associated pathogen control (Wang et al., 2019).

The transport of FeEnt through OMRs also requires energy, which is provided by electrochemical charge gradient of the cytoplasm and is delivered by the energy-transducing TonB-ExbB-ExbD protein complex. ExbB and ExbD are integral cytoplasmic proteins whereas TonB is periplasmic and anchored to the cytoplasm by its hydrophobic N-terminal domain. It is believed that ExbB and ExbD use the membrane electro-chemical charge gradient to produce an “energized” form of TonB that subsequently mediates a conformational change in the liganded OMR. This in turn leads to translocation of the associated FeEnt to the periplasm and deenergisation of TonB (Andrews et al., 2003). There are diverse types of TonB-ExbB-ExbD

systems energizing FeEnt uptake in different Gram-negative bacteria. For instance, *Campylobacter* possesses up to three TonB systems while *E. coli* has only one. The presence of multiple TonB systems within a single organism implies that these TonB systems may be able to compensate each other for iron acquisition (Naikare et al., 2013).

1.2.3 Enterobactin-dependent iron acquisition in *Campylobacter*

Campylobacter species, primarily including *C. jejuni* and *C. coli*, are the most common causative bacterial pathogen of human gastroenteritis in the United States and other industrialized countries. It establishes persistent and benign infection in chicken's gut (up to 10^{10} CFU per gram of feces) while causes significant inflammation and enteritis in humans (Zeng & Lin, 2014). To establish *in vivo* colonization, *Campylobacter* has evolved effective mechanisms, including siderophore-mediated iron acquisition, to counteract the iron-restricted intestinal conditions. Notably, *Campylobacter* does not inherently produce any siderophore, but it can utilize siderophores produced by other microorganisms. To date, enterobactin is the only known physiologically relevant siderophore utilized by *Campylobacter* for *in vivo* colonization, suggesting *Campylobacter* as an ideal model organism to examine key issues of enterobactin-mediated iron scavenging by Gram-negative bacteria (Zeng & Lin, 2014).

Two FeEnt receptors (CfrA and CfrB) on the outer membrane have been demonstrated to be induced under iron-restricted conditions and transport FeEnt from extracellular environment into periplasm. CfrA is prevalent in *Campylobacter* strains from diverse sources while CfrB is produced in the majority of *C. coli* and a small population of *C. jejuni*. Chicken studies indicate that both CfrA and CfrB play critical roles in *in vivo* colonization of *Campylobacter*, suggesting both as promising subunit vaccine candidates against *Campylobacter* (Xu et al., 2010; Zeng et al., 2009). Unlike many Gram-negative bacteria that have one type of TonB-ExbB-ExbD energy

transduction system to energize the transmembrane transport of FeEnt, *Campylobacter* possesses up to three TonB-ExbB-ExbD systems, whose redundancy may allow *Campylobacter* to utilize different iron sources (Zeng & Lin, 2014; Zeng et al., 2013b).

Upon arriving in the periplasm, FeEnt is hydrolyzed by the esterase Cee, which appears to be the sole enterobactin esterase in *Campylobacter*. Cee, located in the periplasm, is demonstrated to be not only essential for CfrB-dependent FeEnt acquisition but also able to utilize CfrA-transported FeEnt when the CeuBCDE ABC transporter on the inner membrane is not functional (Zeng et al., 2013a). The degraded products of FeEnt by Cee may follow two routes: one is the immediate release/transfer of Fe(III) from the low-affinity DHBS monomers and/or dimers; the other is the transport of linear Fe(III)-(DHBS)₃ into cytoplasm. In CfrA-dependent pathway, FeEnt is likely to be subject to uncharacterized reductive iron release mechanisms in the cytoplasm, which may confer an advantage of reutilizing intact enterobactin (Zeng & Lin, 2014).

1.3 Evolution of Ent-mediated iron acquisition: “tug of war” between host and bacterial pathogens

1.3.1 Enterobactin-binding protein lipocalin 2

The battle for iron between host and bacterial pathogens can be regarded as a scenario of coevolution (Miethke & Marahiel, 2007). The pathogen responds to iron scarcity within the host by developing siderophore-dependent high-affinity iron acquisition. Enterobactin has unmatched affinity for iron, however, its ability to acquire iron in the microenvironments of a mammalian host has been called into question (Fischbach et al., 2006). The quantity of FeEnt available for bacterial cell import is reduced by the hydrophobicity of its three catecholic acyl “arms”, which

cause Ent to partition into lipid bilayers (Luo et al., 2006). Also, mammalian protein serum albumin, present in serum microenvironments, has a reasonably high affinity for Ent (Konopka & Neilands, 1984), which also compromises the efficacy of Ent-mediated iron acquisition.

More importantly, Ent-mediated iron acquisition is efficiently impaired by the 24-kDa ligand-binding protein siderocalin (also named lipocalin 2 [Lcn2], neutrophil gelatinase-associated lipocalin [NGAL], or 24p3), which is a member of the lipocalin superfamily and is secreted as an acute-phase protein by neutrophils and epithelial cells during inflammation and rapid cell growth in mammals (Flo et al., 2004; Goetz et al., 2002). Lipocalin 2 is shown to have potent binding affinity for FeEnt complex and lower affinity for Fe(DHB)₃ complex. Thus, lipocalin 2 is able to successfully compete with the high-affinity FeEnt uptake system of enteric bacteria (Miethke & Marahiel, 2007). In addition, the crystal structure of the lipocalin 2:FeEnt ternary adduct also shows large sections of the lipocalin 2 calyx remain unfilled by FeEnt, therefore lipocalin 2 may be capable of binding larger catecholate siderophores (Raymond et al., 2003). It is also supposed that the employment of innate defense proteins for siderophore neutralization is likely due to the fact that siderophores are hardly recognized as antigens by the adaptive immune response if not presented in surface-immobilized forms (Miethke & Marahiel, 2007). Last but not least, L-norepinephrine (NE), a host neuroendocrine catecholamine, can support bacterial iron acquisition by mobilizing iron from a primary source such as *holo* transferrin to increase its bioavailability for bacterial uptake. However, it is shown that lipocalin 2 can also bind iron-complexed NE with high affinity and therefore inhibit the NE-mediated growth of *E. coli* strains that are either capable or incapable of producing the endogenous enterobactin. This indicates that NE directly serves as an iron source for bacterial uptake and

lipocalin 2 functions as an antagonist for this iron acquisition process complementary to enterobactin-mediated one (Miethke & Skerra, 2010).

1.3.2 Salmochelins, glycosylated enterobactin derivatives, evade the binding of lipocalin 2

Rather than accepting a heavy loss to host defenses, enterobactin-producing Gram-negative bacteria, such as *E. coli*, *Salmonella*, *Shigella*, and *Klebsiella*, use enzymes encoded by a mobile cluster of genes to modify enterobactin so that the resulted iron-scavenging molecules, named salmochelins, can circumvent unfavorable partitioning and sequestration of lipocalin 2 and serum albumin (Fischbach et al., 2006). The five-gene *iroA* encodes two enzymes (IroB and IroE) that modify Ent into salmochelins before their secretion, two enzymes (IroC and IroN) that export salmochelins and import the iron-salmochelin complex, and one enzyme (IroD) that degrades the complex to release iron. This gene cluster was first discovered in *Salmonella* and is regulated by the iron-dependent repressor Fur (Fischbach et al., 2006).

IroB is a member of the glycosyltransferase family and catalyzes the C-glycosylation of enterobactin, adding a bulky glucose (Glc) to one or more of the catecholic “arms” of enterobactin. IroB is demonstrated to be necessary and sufficient for salmochelin production, suggesting a central role of this enzyme in the conversion of enterobactin to salmochelins (Bister et al., 2004). Purified IroB acts distributively to transfer one, two, or three Glc to C5 of the DHB moiety of enterobactin, creating mono- (MGE), di- (DGE), and triglucosyl-enterobactin (TGE) (Fischbach et al., 2005). The modification likely prevents enterobactin from being sequestered by lipocalin 2 but does not affect the ability of enterobactin to bind iron (Luo et al., 2006), as the binding pocket of lipocalin 2 does not appear to be large enough to accommodate a glucose appended to enterobactin (Fischbach et al., 2006).

IroE catalyzes single-time hydrolysis of enterobactin and DGE in the periplasm, yielding their respective ring-opened linear trimers before export. This modification increases the hydrophilicity of enterobactin and DGE, while decreasing their affinity for iron only modestly. Enterobactin has a high partition coefficient, so when enterobactin partitions into a lipid bilayer, its iron-binding rate decreases because it is sequestered from iron that resides in the aqueous phase. In contrast, DGE has a 5-fold lower partition coefficient, and linearized DGE has a 30-fold lower partition coefficient (Luo et al., 2006). As a result, both DEG and linearized DGE show higher rates of iron acquisition in the presence of phospholipid vesicles. Therefore, IroE-catalyzed hydrolysis of DGE prior to export will make linearized DGE a more efficient iron scavenger in membrane-rich microenvironments, such as those commonly found in animals (Fischbach et al., 2006). After the ferric complexes of enterobactin, DGE, and linearized DGE have been imported to the bacterial cytoplasm, IroD exhaustively hydrolyzes them to DHB-Ser and Glc-DHB-Ser monomers, which facilitates iron releases. It is worth noting that in enterobactin system, Fes can only efficiently hydrolyze enterobactin to release iron, but it is not able to hydrolyzes DGE, indicating its inability to liberate iron from DGE (Lin et al., 2005; Zhu et al., 2005).

IroN shows 52% amino acid sequence identity to FepA, the TonB-dependent outer-membrane receptor for enterobactin. It has been shown to mediate the import of FeEnt complex and salmochelins through the outer membrane (Bäumler et al., 1998; Hantke et al., 2003). In contrast, FepA and other catecholate-specific OMRs transport Fe(III)-bound Ent but not salmochelins, suggesting that IroN is necessary to import salmochelins arising from the action of IroB (Hantke et al., 2003). In addition, IroC has considerable similarity to eukaryotic ATP-driven MDR (multiple drug resistance) exporters (Müller et al., 2009). It is shown in *S. enterica*,

IroC exports both Ent and salmochelins, but it is not required for utilization of FeEnt or Fe-salmochelin complexes, while the ABC transporter protein FepD is required for utilization of both siderophores (Crouch et al., 2008).

1.4 Siderophore-mediated iron-dependent bacterial pathogen control

Siderophore-mediated iron acquisition is an important virulence factor in many bacterial pathogens. This process involves complex siderophore biosynthetic mechanisms, dedicated ferric siderophore export and import machineries, and various enzymes to release siderophore-bound iron following cellular uptake. Thus, the potential of utilizing siderophores or targeting siderophore biosynthetic and transport machineries for bacterial pathogen control has been attracting significant research interest (Zheng & Nolan, 2014). In general, three classes of strategies have been investigated to combat bacterial infection: 1) To use the “Trojan horse” concept by employing siderophore as a mediator to facilitate the cellular uptake of antibiotic compounds; 2) To create artificial iron starvation for pathogens by applying iron-competing metal ions or competitive siderophores that cannot be utilized by the pathogen; 3) To impair the availability of siderophore for pathogens by inhibiting corresponding siderophore synthesis; 4) To develop vaccine by targeting gatekeeper OMR to inhibit iron uptake (Miethke & Marahiel, 2007; Wilson et al., 2016).

1.4.1 Siderophore as “Trojan horse” for antimicrobial delivery

The “Trojan horse” strategy is inspired by natural antibiotic compounds called “sideromycins”, which are secondary metabolites comprised of a siderophore moiety and a toxic cargo. The siderophore part of these compounds is able to scavenge iron and is recognized by cellular iron-siderophore uptake systems, while the toxic cargo, bearing antibiotic activity, uses

the siderophore as a “Trojan horse” and exploits the iron-siderophore uptake system as a cellular entry gate (Miethke & Marahiel, 2007). Microcin E492, produced by a clinical isolate of *K. pneumoniae*, is a good example of natural siderophore-antibiotic conjugate. It is an 84-residue antibacterial peptide with a linearized MGE appended in ester linkage to its C-terminal carboxylic acid, exhibiting enhanced antibacterial activity against strains expressing siderophore receptor FepA, Cir, or Fiu (Thomas et al., 2004).

Although inhibition concentrations for some sideromycins are remarkably low, the main drawback for therapeutical use is the generally high frequency of resistant mutants that have gained mutations in the iron-siderophore-specific receptors or the TonB complex proteins. In this respect, synthetic development of sideromycins has proven to be an outgrowing field to generate a broad array of new antibiotic compounds (Miethke & Marahiel, 2007). The compounds can generally be produced by derivatizing siderophores and/or antibiotics isolated from cell cultures (semisynthesis) or by total synthesis. Semisynthesis may be advantageous if suitable functional groups are provided by the natural compound. However, total synthesis is often preferred since it allows the rational introduction of useful functional groups at appropriate positions, preferably in the periphery of the siderophore scaffold. In principle, each structural type of siderophore may be coupled with each type of antibiotic, yielding a broad spectrum of sideromycin variants. The siderophore part of the synthesized compounds comprises in most cases hydroxamate, catecholate, or mixed hydroxamate-catecholate tethered to various backbones. The use of mixed ligand siderophore that can be taken up via several pathways is seen as advantage since it reduces the frequency of resistant mutants and, if occurring, makes them more susceptible to iron starvation since the transport systems are found to be the main targets for resistance formation rather than the antibiotic targets themselves (Miethke & Marahiel, 2007).

It is reported that the β -lactam antibiotics ampicillin (Amp) and amoxicillin (Amx) were linked to a monofunctionalized enterobactin scaffold via a stable poly (ethylene glycol) linker. Under conditions of iron limitation, these siderophore-modified antibiotics provided enhanced anti-bacterial activity, compared to the parent β -lactams, against different uropathogenic, enterohemorrhagic, and enterotoxigenic *E. coli* strains. Moreover, Ent-Amp and Ent-Amx selectively killed *E. coli* co-cultured with other bacterial species such as *Staphylococcus aureus*, and Ent-Amp exhibited low cytotoxicity against human intestinal cells in both the *apo* and iron-bound forms (Zheng & Nolan, 2014). In another similar study, a ciprofloxacin (Cipro) cargo was attached to a synthetic enterobactin platform via an alkyl linker at one of the catechols. It exerted selective antibacterial activity, comparable to unmodified ciprofloxacin, against pathogenic *E. coli* strains. This selectivity was due to that intracellular activation of conjugated Cipro required the cytoplasmic enterobactin hydrolase IroD, which is expressed only by *E. coli* that harbor the *iroA* gene cluster and are predominantly pathogenic (Neumann et al., 2018).

1.4.2 Artificial iron starvation via competitive metal ions or iron chelators

The artificial iron starvation for bacterial pathogens can be created by iron-competing metal ions or competitive iron chelators that cannot be utilized by the pathogen. Gallium or Ga(III) is used in combating bacterial infections because it can compete with Fe(III) in binding to siderophores, while unable to participate in biologically necessary redox reactions, therefore arresting metabolic processes that depend on iron-mediated redox reactions (Wilson et al., 2016). Gallium maltolate, a gallium compound, has been evaluated as a potential therapeutic agent for methicillin-resistant *S. aureus* (Baldoni et al., 2010); Gallium nitrate, a commonly used drug for hypercalcemia, results in antimicrobial activity against multidrug-resistant *Acinetobacter baumannii* *in vivo* and *in vitro* (Antunes et al., 2012); This FDA-approved drug also inhibits *P.*

aeruginosa growth and biofilm formation and kills planktonic and biofilm bacteria *in vitro* by decreasing bacterial Fe(III) uptake and interfering with Fe(III) signaling (Kaneko et al., 2007). In addition, gallium-loaded siderophores present another approach for gallium-based therapeutics. Pre-loading pyochelin--a siderophore produced by *P. aeruginosa*--with gallium consistently leads to a stronger inhibitory effect on *P. aeruginosa* growth than gallium nitrate in *in vitro* bacterial cultures, suggesting treatment with gallium-siderophore complexes could be a more effective therapeutic option than using simple gallium salts (Frangipani et al., 2014).

Some iron chelators have been found to compete with siderophores for binding Fe(III) and therefore act as antimicrobial agents. Lactoferrin and other synthetic iron chelators such as conalbumin, ferric picolinate, ferric acetohydroxamate, 2,2'-dipyridyl (2DP), diethylenetriaminepentaacetic acid (DTPA), EDTA, deferoxamine mesylate (DM), and ethylenediamine-N,N'-diacetic acid (EDDA) have been shown to reduce growth and biofilm formation of *P. aeruginosa* (Saha et al., 2013). Moreover, it is demonstrated that iron chelators desferroxamine and desferasirox can improve the capability of antibiotic tobramycin to reduce bacterial biofilms caused by the antibiotic resistant *P. aeruginosa* in cystic fibrosis cells (Moreau-Marquis et al., 2009).

1.4.3 Inhibition on siderophore synthesis pathways

The strategy of target-specific siderophore biosynthesis inhibition is proved to be an attractive research field in recent years due to the availability of crystal structures that allow the rational design of catalytic site inhibitors. (Miethke & Marahiel, 2007). Most of the studies have targeted the NRPS which is involved in the synthesis of all aryl-capped siderophores and requires salicylate (SAL) or DBH as prerequisite precursors. The aryl acid A domain of NPRS catalyzes the initial step of siderophore formation by activating the aryl acid substrates (SAL or

DHB) via adenylation. Therefore some inhibitors have been designed to inhibit the catalytic activity of aryl acid A domain, thus preventing the growth of corresponding pathogens in iron-limited conditions (Saha et al., 2013). Examples of such inhibitors include *p*-aminosalicylate (PAS) in *M. smegmatis* and *M. bovis* (Brown & Ratledge, 1975) and aryl acid A domain analogue SAL-AMS (salicyl sulfamoyl adenosine) in *M. tuberculosis* and *Yersinia pestis* (Ferrerias et al., 2005). Moreover, siderophore modification processes like tailoring can also be inhibited. It is shown that brominated enterobactin derivatives are potent inhibitors of IroB, a bacterial enzyme catalyzing *iroA*-dependent C-glycosylation of enterobactin in order to evade the host innate immunity mediated by lipocalin 2. Those IroB inhibitors can potentially be used as antibiotics against *iroA*-harboring bacterial pathogens (Lin et al., 2006).

1.4.4 Vaccine development by targeting OMR

In Gram-negative bacteria, OMRs are the “gatekeepers” for cellular uptake of iron-siderophore complex. They display high ligand affinity and specificity (Miethke & Marahiel, 2007). There are considerable studies demonstrating that the OMRs are induced under iron-restricted conditions *in vivo* and bacterial virulence are attenuated after the genetic knockout of corresponding genes (Lin et al., 2002). Together, these evidences suggest OMRs as promising targets for iron starvation and pathogen control. Monoclonal antibody specific for blocking the FeEnt-binding site of the FeEnt receptor FepA could inhibit the growth of *E. coli* derived from a bovine intramammary infection *in vitro* (Lin et al., 1998). OMRs IreA, Hma, and IutA involved in iron acquisition of uropathogenic *E. coli* were utilized as vaccine candidates. Intranasal immunization of these receptor proteins in mice elicited systemic and mucosal immune response and provided significant protection from experimental infection of this uropathogenic *E. coli* (Alteri et al., 2009). In *Campylobacter*, CfrA and CfrB, two types of ferric enterobactin receptors

on outer membrane, are demonstrated to be induced and critical for *Campylobacter* growth under iron-limited condition. Antisera directed against CfrA or CfrB could diminish the FeEnt-mediated *in vitro* growth promotion under iron-restricted condition (Xu et al., 2010; Zeng et al., 2009).

Though extensive efforts have been placed on development of vaccines targeting surface-exposed iron-regulated OMRs, these attempts have had only partial success, primarily due to complexity of iron acquisition system and redundancy of receptors for utilization of the ferric-siderophore complex in various Gram-negative pathogens. For example, there are up to five ferric Ent receptors in *E. coli* (FepA, Cir, Fiu, IroN, and Iha) that show different affinities to Ent and its derived products. Clearly, such redundancy, together with other issues (e.g., antigenic variation), raises significant challenges for developing iron-dependent pathogen control by targeting OMRs (Wang et al., 2019).

1.4.5 Emerging novel siderophore-based intervention

Rather than focusing on iron acquisition-related large cellular targets (e.g., iron-regulated outer membrane proteins), an innovative ‘out-of-the-box’ strategy focusing on extracellular Ent siderophore has been inspired by a unique antibacterial function of lipocalins as reviewed above, and has been supported by a recent study (Sassone-Corsi et al., 2016). It is widely accepted that huge repertoire of different antibodies can be generated in individual human beings or animals. In addition, specific high-affinity antibodies could be produced against small molecule (called ‘hapten’) if hapten is conjugated with a carrier protein. Therefore, specific high-affinity antibodies directed against Ent, a hapten with low molecular mass of 669 Da, could be theoretically obtained and such Ent-specific antibodies may display a bacteriostatic effect similar to that observed for lipocalins.

Recently, intranasal immunization of mice with an enterobactin conjugate vaccine triggered anti-enterobactin antibodies in the gut mucosa (Sassone-Corsi et al., 2016). When mice were infected with the enteric pathogen *Salmonella*, they exhibited reduced intestinal colonization and reduced systemic dissemination of the pathogen (Sassone-Corsi et al., 2016). Nevertheless, there are still some weaknesses in this study. The preparation of the enterobactin conjugate was lengthy and complicated, which required the addition of a linker to enterobactin for its conjugation with carrier protein; the enterobactin-specific immune response was weak upon immunization of mice (i.e., <4-fold increase in enterobactin-specific antibodies); it also failed to provide any direct evidence demonstrating that the induced anti-enterobactin antibodies could function similarly as lipocalin 2 to interfere with enterobactin-dependent growth promotion on enteric bacteria under iron-restricted conditions, a critical evidence required for validating the concept of the enterobactin conjugate vaccine. In another study, yersiniabactin and aerobactin, the most prevalent siderophores among pathogenic *E. coli* for iron acquisition, were utilized as protective vaccine antigens. Intranasal immunization of these siderophore conjugates elicited an adaptive immune response against the stealth siderophores and reduced bacterial burdens and inflammation in the urine and kidneys caused by the uropathogenic *E. coli* (Mike et al., 2016).

1.5 Egg yolk IgY: a promising alternative to antibiotics for pathogen control

1.5.1 Overview of egg yolk IgY

Hens' eggs have long been recognized as a rich source of nutrients. It also contains large quantities of egg yolk antibodies. Immunoglobulin Y (IgY) is the functional equivalent of IgG in mammals and is selectively transferred from serum to yolk via specific receptor on the surface of

yolk membrane during egg formation. It therefore provides passive immune protection for the developing chick. Since the discovery of egg yolk IgY, this process has been harnessed to produce antigen-specific egg yolk IgY for numerous applications in the medical and production fields (Kovacs-Nolan & Mine, 2012).

Although the exact mechanisms of action of orally administered IgY remain unclear, it is well known that they largely depend upon efficient binding of IgY to specific antigens of the pathogens, thus impairing their growth or biological functions. In the case of bacteria, IgY binds to structures such as flagella, fimbriae (pili), outer membrane proteins, and lipopolysaccharide (LPS), and therefore inhibits the bacterial growth, colonization, and/or invasion of host cells (Gadde et al., 2015). Other mechanisms of IgY include: bacterial agglutination, leading to death by immobilization and expulsion from the gut; toxin neutralization; inhibition of enzyme activity; interference of bacterial cellular signaling cascades, reducing bacterial pathogenicity (Gadde et al., 2015).

There are a number of advantages to using hens for the production of antibodies. First, when considering antibody production for passive immunotherapy, hens present a much more economical source of large quantities of specific antibodies. It is shown that an immunized hen can produce approximately 1500 mg of egg yolk IgY in one month, 2-10% of which is antigen-specific, whereas an immunized rabbit produces 18 times less IgG from serum in the same time period (Gadde et al., 2015). Second, IgY production is less invasive, only requiring the daily collection of eggs, compared to blood collection in mammals (Karlsson et al., 2004). Third, in contrast to mammalian serum, egg yolk contains only a single class of antibody (IgY), which can easily be isolated from the yolk by precipitation techniques. Meanwhile, IgY does not activate mammalian complement or interact with mammalian Fc receptors so that it will not mediate

inflammatory responses when applied to passive immunotherapy in mammals (Kovacs-Nolan & Mine, 2012). Last but not least, the vaccination of hens and automated collection and processing of eggs have already been carried out on an industrial scale, making the large-scale production of IgY technically feasible (Kovacs-Nolan & Mine, 2012).

1.5.2 Stability of egg yolk IgY

The stability of IgY under various processing and physiological conditions is a critical consideration for passive immunotherapy applications. IgY is fairly stable during storage and processing. Its stability is not affected by freezing and freeze-drying unless repeated several times. The activity of IgY is shown to decrease with increasing temperature and time. Minimal loss of activity was observed following heating between 60 °C and 65 °C but the activity was decreased markedly by heating for 15 min at 70 °C or higher. However, the addition of carbohydrates was found to improve its heat resistance. Likewise, it is shown that the addition of disaccharides or complex carbohydrates to IgY prior to lyophilization could improve the stability during storage (Kovacs-Nolan & Mine, 2012). IgY is relatively stable between pH 4 and pH 11 but displays a rapid reduction in activity above pH 12 or below pH 3. However, pH stability may be improved by the addition of stabilizers, such as sugars, complex carbohydrates, and polyols (Kovacs-Nolan & Mine, 2012). As with any protein, antibodies are susceptible to proteolytic digestion. IgY is relatively resistant to trypsin and chymotrypsin but quite sensitive to pepsin digestion, especially at low pH (Gadde et al., 2015).

A major concern for oral immunotherapy application of IgY is the inactivation of IgY during digestive processes, which depends on gastric pH, enzyme levels, and gastric transit times. In order to prevent inactivation of IgY following oral administration, a number of encapsulation techniques have been examined (Kovacs-Nolan & Mine, 2012). The

macroencapsulation of IgY using enteric-coated gelatin capsules was found to significantly improve antibody stability (Akita & Nakai, 2000). The use of a pH-sensitive methacrylic acid copolymer, chitosan-alginate microcapsules, and whey protein-based microcapsules have also been shown to increase IgY stability to gastric conditions, both *in vitro* and *in vivo* (Kovacs-Nolan & Mine, 2012). The use of cytoprotective agent sucralfate is described to be able to improve IgY resistance to low pH and pepsin treatment, as well as enhance storage stability, in a dose-dependent manner (Xun et al., 2010). Finding an effective method to protect IgY from degradation will not only ensure the delivery of viable IgY but could also allow the effective dose to be lowered, thereby reducing cost.

1.5.3 Applications of hyperimmune egg yolk IgY

The use of hyperimmune egg yolk IgY to prevent and treat human and animal infections has gained popularity in recent years as an alternative to antibiotic use. Because the absorption of orally administered egg antibodies is minimal, the majority of applications of egg antibodies are against enteric infections in both people and animals (Gadde et al., 2015). A recent meta-analysis study focusing on the therapeutic effects of IgY for enteric infections in domestic animals concluded that the administration of IgY significantly reduced the risk of infection in the treated groups compared with that in the untreated controls (Diraviyam et al., 2014).

In veterinary applications, IgY has been proved to prevent a number of bacterial pathogens and improve growth performance and feed efficiency in livestock. IgY produced against the porcine enterotoxigenic *E. coli* fimbrial antigens was demonstrated to protect piglets from *E. coli* infection by inhibiting the binding of *E. coli* to porcine epithelial cells and intestinal mucus (Yokoyama et al., 1992). More recently, it is found when IgY against enterotoxigenic *E. coli* was encapsulated in chitosan-alginate microparticles, it exerted antidiarrheal effects much

faster and led to increased weight gain when compared to pigs fed with non-encapsulated IgY (Li et al., 2009). In addition, IgY against the outer membrane proteins of *S. enterica* and *S. typhimurium* reduced *Salmonella* adhesion to intestinal epithelial cells *in vitro* (Chalghoumi et al., 2009). Feeding chickens egg powder containing such IgY was found to reduce fecal shedding, cecal colonization, and the rate of *Salmonella*-contaminated eggs in experimentally infected chickens (Gürtler et al., 2004; Rahimi et al., 2007). Along with preventing diseases, IgY targeting specific molecules involved in inflammation and animal growth has been reported to improve growth and feed efficiency in food animals. IgY directed against the gut neuropeptides cholecystokinin and neuropeptide Y, believed to be involved in regulating appetite and phospholipase A₂ (PLA₂), which plays a role in the production of the inflammatory mediators prostaglandins and leukotrienes, has been shown to improve growth and feed efficiency when fed to chickens (Cook, 2004; Kovacs-Nolan & Mine, 2012). Similarly, feeding cattle with egg yolk preparations containing IgY against *Fusobacterium necrophorum* and *Streptococcus bovis* in place of antibiotics could reduce ruminal counts of the target bacteria and improve feed efficiency and growth performance (DiLorenzo et al., 2008).

In human medicine, one of the most successful clinical applications of IgY is in the prevention of *P. aeruginosa* colonization in the airways of cystic fibrosis patients. In the trials of cystic fibrosis patients, a mouth rinse containing purified anti-*P. aeruginosa* IgY was given on a continuous basis and significantly reduced or prevented *P. aeruginosa* colonization, thereby reducing the need for antibiotics (Nilsson et al., 2008). Another promising clinical application of IgY in humans is for the prevention of *Helicobacter pylori* infection. IgY against *H. pylori* reduced bacterial adhesion, growth, and urease activity *in vitro* and decreased *H. pylori*-induced gastric mucosal injury and inflammation in an animal model (Shin et al., 2002). A functional

drinking yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. and supplemented with 1% anti-urease IgY was produced commercially and given to volunteers tested positive for *H. pylori*. After four weeks, urea breath test values and antigen detection in feces were significantly reduced, indicating suppression of *H. pylori* infection (Horie et al., 2004). The immediate protection afforded by antigen-specific IgY has also been suggested for protection against agents of bioterrorism. Purified IgY against *Clostridium botulinum* neurotoxins type A and B is reported to block the functional activity of the toxins both *in vitro* and *in vivo* in mice following preincubation of the toxin with toxin-specific IgY (Trott et al., 2009).

In aquaculture, passive immunization of IgY has also been applied to prevent infectious diseases causing significant economic loss. *Edwardsiella tarda* is a bacterial pathogen infecting the intestinal mucosa in fish, and Edwardsiellosis in Japanese eels constitutes a serious problem for the eel-farming industry. Eels challenged with *E. tarda* followed by administration of anti-*E. tarda* IgY survived without any symptoms of infection, in contrast to control eels that died within 15 days (Gutierrez et al., 1993). This antibody is now in commercial use, and a field test on nearly 2,400,000 tails of cultured eels confirmed the disease prevention effect of this anti-*E. tarda* IgY (Hatta et al., 2008). Enteric redmouth disease, caused by *Y. ruckeri*, is a systemic bacterial septicemia of salmonid fish. The persistence of *Y. ruckeri* in carrier fish and shedding of bacteria in feces can cause a continuing source of infection. Feeding anti-*Y. ruckeri* IgY before or after bacterial challenge resulted in lower mortality and reduced infection rates in rainbow trout (Lee et al., 2000). Similarly, IgY against *Vibrio anguillarum* protected rainbow trout against vibriosis for at least 14 days when given via intraperitoneal injection, oral intubation, or feeding (Arasteh et al., 2004).

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CHAPTER II: Characterization of the Enterobactin-specific Antibodies
Induced by a Novel Enterobactin Conjugate Vaccine

Abstract

Enterobactin (Ent)-mediated high affinity iron acquisition is critical for Gram-negative bacteria to survive in the host. Given the bacteriostatic effect of lipocalin resulting from its potent Ent-binding ability, immune intervention directly targeting Ent is promising for iron-dependent pathogen control. Recently, an Ent conjugate vaccine was reported but it still has several significant weaknesses. In this study, we aimed to develop an innovative Ent conjugate vaccine that can induce high level of antibodies directed against Ent, and to provide solid evidence demonstrating siderophore-binding capacity of Ent-specific antibodies. Using a simple method, we successfully conjugated purified Ent to different carriers including keyhole limpet hemocyanin (KLH), bovine serum albumin, and CmeC, a vaccine candidate for *Campylobacter* control. Subcutaneous immunization of rabbits with the KLH-Ent conjugate triggered strong systemic IgG immune response with up to 16,384 fold increase in IgG titer directed against whole conjugate and up to 4,096 fold increase in the level of specific anti-Ent IgG. To evaluate the binding ability of Ent-specific IgG to the Ent derivatives present *in vivo*, various Ent derivatives were chemically synthesized and a unique ELISA method was developed. The Ent-specific IgG also displayed exceptional reactivity to ferric Ent, linear trimer of Ent, and different salmochelins. Growth assays further demonstrated the Ent-specific antibodies significantly inhibited Ent-dependent growth of *Campylobacter* spp. and *E. coli*. Collectively, this study reports an efficient method to prepare new type of Ent conjugate vaccines for inducing high level of Ent-specific antibodies, which can bind to various Ent derivatives and display lipocalin-like bacteriostatic feature.

2.1 Introduction

All Gram-negative bacteria have an absolute requirement for iron in order to survive and colonize the host. In animal host, however, iron from various sources is not normally available to invading bacteria; the concentrations of free iron *in vivo* are well below the levels required for the growth of Gram-negative bacteria (Miethke & Marahiel, 2007). To obtain sufficient iron for survival and multiplication, Gram-negative bacteria have evolved complex and efficient genetic systems for iron uptake (Miethke & Marahiel, 2007). Of these systems, siderophore (iron chelator)-mediated high-affinity iron acquisition is the most efficient, widely prevalent, and highly conserved strategy used by Gram-negative bacteria to scavenge iron in the host (Miethke & Marahiel, 2007; Skaar, 2010). Enterobactin (Ent), a catecholate siderophore that has the highest affinity for ferric iron of all natural siderophore compounds, is an archetype for iron acquisition in Gram-negative bacteria (Raymond et al., 2003). In particular, Ent is produced by most members of *Enterobacteriaceae*, the only representative family in the order Enterobacteriales of the class γ -proteobacteria (Zeng & Lin, 2014). In addition, the hydrolyzed Ent derivatives (e.g. linear trimer) and the glycosylated Ent derivatives (named as “salmochelins”) can also be produced and utilized for iron uptake by intestinal commensals and pathogens (e.g., *Escherichia coli* and *Salmonella enterica*) (Miethke et al., 2011; Zeng & Lin, 2014). Therefore, Ent is likely to be used as a significant source of iron for Gram-negative pathogens during intestinal colonization (Zeng & Lin, 2014). It has been shown that a significant quantity of catecholate siderophores, primarily Ent, were produced in the intestine and Ent-mediated iron acquisition played a fundamental role in *E. coli* colonization in the gut (Pi et al., 2012).

Not surprisingly, siderophore-mediated iron acquisition, particularly that mediated through Ent, has been a historically important research area in bacterial pathogenesis and has been targeted for developing various iron-dependent pathogen control strategies. In the past decades, extensive efforts have been placed on development of specific siderophore biosynthesis inhibitors and the vaccines targeting surface-exposed iron-regulated outer membrane proteins (Lin et al., 2002; Miethke & Marahiel, 2007). However, these approaches have had only partial success, primarily due to complexity of siderophore synthesis and redundancy of systems for utilization of the ferric-siderophore complex in various Gram-negative pathogens. For example, there are up to five ferric Ent receptors in *E. coli* (FepA, Cir, Fiu, IroN, and Iha) that show different affinity to Ent and its derived products (Curtis et al., 1988; Hantke et al., 2003; Léveillé et al., 2006; Nikaido & Rosenberg, 1990; Pierce et al., 1983). Clearly, such redundancy together with other issues (e.g., antigenic variation) raises significant challenges for developing iron-dependent pathogen control by targeting outer membrane proteins.

Rather than focusing on iron acquisition-related large cellular targets (e.g., iron-regulated outer membrane proteins), an innovative ‘out-of-the-box’ strategy focusing on extracellular Ent siderophore has been inspired by a unique antibacterial function of lipocalins (Clifton et al., 2009; Flo et al., 2004; Santos et al., 2009; Wu et al., 2010; Xiao et al., 2017), and further supported by a recent study on evaluation of an Ent conjugate vaccine (Sassone-Corsi et al., 2016). Lipocalins are host acute phase proteins secreted by neutrophils and epithelial cells during inflammation and rapid cell growth. In addition to their important and pleiotropic functions in diverse cellular processes in the host, which are unrelated to antibacterial activity, lipocalins have been demonstrated to be involved in the antibacterial iron depletion strategy of the innate immune system (Clifton et al., 2009; Flo et al., 2004; Santos et al., 2009; Wu et al., 2010; Xiao et

al., 2017). Specifically, lipocalins inhibit *E. coli* and other Gram-negative infections by binding Ent with high affinity, thus functioning as bacteriostatic agents controlling the growth of bacteria in which Ent-mediated iron acquisition plays an important role in pathogenesis (Clifton et al., 2009; Flo et al., 2004; Santos et al., 2009; Wu et al., 2010; Xiao et al., 2017). The bacteriostatic function of lipocalins has provided a strong rationale for developing lipocalin-like Ent-binding protein to inhibit Ent-mediated iron acquisition in Gram-negative bacteria. It is widely accepted that huge repertoire of different antibodies can be generated in individual human beings or animals. For example, it has been estimated that humans are able to generate about 10 billion different antibodies, each capable of binding a distinct epitope of an antigen (Fanning et al., 1996). In addition, specific high-affinity antibodies could be produced against small molecule (called 'hapten') if hapten is conjugated with a carrier protein (Clementi et al., 1991). Therefore, specific high-affinity antibodies directed against Ent, a hapten with low molecular mass of 669 Da, could be theoretically obtained and such Ent-specific antibodies may display a bacteriostatic effect similar to that observed for lipocalins. This speculation has been partly supported by a recent publication showing an Ent conjugate vaccine inhibited *Salmonella* colonization in mice (Sassone-Corsi et al., 2016). Nevertheless, in this recent study, preparation of Ent conjugate was lengthy and complicated, which required addition of a linker to Ent for its conjugation with carrier protein (Sassone-Corsi et al., 2016). In addition, the Ent-specific immune response was weak upon immunization of mice with the Ent conjugate (< 4 fold increase in Ent-specific antibodies) (Sassone-Corsi et al., 2016), highlighting the needs to develop new type of Ent conjugate that is capable of inducing high levels of Ent-specific antibodies. Finally, Sassone-Corsi *et al.* (Sassone-Corsi et al., 2016) did not provide microbiological and biochemical features of anti-Ent antibodies in terms of their binding capability to catecholates and effectiveness for

iron sequestration when compared to lipocalins, a critical issue that needs to be addressed to test the hypothesis.

In this study, using a simple and efficient protocol, we successfully conjugated purified Ent to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), and CmeC, a promising subunit vaccine candidate for *Campylobacter* control (Zeng et al., 2010).

Immunization of rabbits with the KLH-Ent conjugate triggered strong immune response, leading up to 4,096-fold increase in the level of specific anti-Ent immunoglobulin G (IgG) in serum. For the first time, we also observed that the Ent-specific IgG displayed potent binding ability not only to both *apo* and ferric Ent (FeEnt) but also to other Ent derivatives including salmochelins, indicating the Ent-specific antibodies induced by the KLH-Ent conjugate vaccine overcome a significant pitfall of the host innate immune component lipocalin that has low affinity to salmochelins. In another word, the Ent antibodies have a significant advantage over lipocalins by cross-reacting to various Ent derivatives including salmochelins. Finally, we demonstrated that the rabbit Ent antiserum displayed significant inhibitory effect on Ent-dependent growth of *Campylobacter* and *E. coli*.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *Campylobacter* strains were routinely grown in Mueller-Hinton (MH) broth (Difco, Detroit, MI) or on MH agar (Difco) at 42 °C in a microaerophilic incubator (5% O₂, 10% CO₂, and 85% N₂). *E. coli* strains were grown in Luria-Bertani (LB) broth (Difco) or TSB broth (Difco, Detroit, MI) with shaking (200 rpm) or

on agar at 37 °C. When needed, culture media were supplemented with kanamycin (30 µg/ml) or tetracycline (15 µg/ml).

2.2.2 Purification of Ent

An Ent transport mutant of *E. coli* AN102 (JL122, Table 1) was generously provided by Dr. Sandra K. Armstrong (University of Minnesota) and used for Ent purification. The purification procedure was described previously (Anderson & Armstrong, 2004; Zeng & Lin, 2017). The purified Ent was dissolved in methanol or DMF, and stored at -20 °C until it was used.

2.2.3 Conjugation of Ent with carrier proteins

BSA and KLH were purchased from Thermo Fisher Scientific (Waltham, MA). Recombinant CmeC was purified as described in a previous study (Zeng et al., 2010). A standard protocol was developed by examining various conjugation parameters, such as concentration, temperature, organic solvents, and reaction time. Briefly, 45 µl BSA, KLH or CmeC (1.0 µg/ml) was dissolved in 360 µl sodium phosphate buffer (0.1 M, pH 8.0). To this solution, 45 µl of Ent (50 mM in DMF) was added. The mixture was incubated at 37 °C for 4 hrs, followed by buffer exchange using Amicon Ultra-4 Centrifugal Filter Units (MilliporeSigma, St. Louis, MO) with ddH₂O three times and PBS buffer (pH 7.4) six times. Each batch of Ent conjugates were subjected to SDS-PAGE analysis to validate the success of conjugation. Finally, Ent conjugates (~1 mg/ml in PBS) were stored at -20 °C prior to use.

2.2.4 Immunization of rabbits with the KLH-Ent conjugate

Two independent immunization trials (two rabbits per trial) were performed in 2013 and 2016, respectively, by Pacific Immunology Corp. (Ramona, CA). The vaccination regimen was same for the two trials except that a 4-week extension was added in the second trial to collect

more antisera. Briefly, adult New Zealand white rabbits received primary immunization via subcutaneous administration (in the area between the shoulders) with 100 µg of KLH-Ent conjugate emulsified with Freund's complete adjuvant, followed by three booster immunizations (100 µg of KLH-Ent conjugate emulsified with Freund's incomplete adjuvant) every 3-4 weeks. Blood samples were collected at different time points. Rabbits were euthanized at 14th week or 18th week for the first trial and second trial, respectively, after the primary immunization.

2.2.5 Immunoblotting

To validate production of rabbit polyclonal anti-Ent IgG, Western blotting and dot blotting were performed as described previously (Sahin et al., 2001; Zeng et al., 2009). For Western blotting, protein samples were first separated by SDS-PAGE with a 12% (w/v) polyacrylamide gel and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 90V for 1 hr. For dot blotting, approximately 1 µl of protein sample (1 mg/ml) was spotted onto nitrocellulose membranes and air dried. Subsequently, the nitrocellulose membrane was incubated with the blocking buffer (PBS containing 0.05% of Tween 20 and 5% of skim milk) at room temperature (22 °C) for 1 hr, prior to 1-hr incubation at room temperature with primary antibodies (1:100 diluted polyclonal rabbit anti-Ent serum in blocking buffer). After incubation, the membranes were washed four times with PBS containing 0.05% Tween 20 and then incubated with secondary antibody (goat anti-rabbit IgG-horseradish peroxidase, SeraCare, Milford, MA; diluted 1:5000 in blocking buffer) for 1 hr at room temperature. After incubation the membranes were washed four times with PBS containing 0.05% Tween 20. The membranes were then developed with the 4CN Membrane Peroxidase Substrate System (KPL, Gaithersburg, MD).

2.2.6 Purification of His-tagged recombinant proteins

The His-tagged recombinant IroE, IroB, human lipocalin-2, and CmeC were purified from JL633, JL636, JL1002, and JL243 (Table 1), respectively, as detailed in previous publications (Fischbach et al., 2005; Garénaux et al., 2013; Lin et al., 2005). Notably, the recombinant human lipocalin-2 was produced in an Ent deficient strain ($\Delta entD$), ensure that the purified lipocalin was not bound by endogenous Ent. The high purity of the recombinant proteins was confirmed by SDS-PAGE. The concentration of purified recombinant protein was determined using bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). The purified recombinant protein was aliquoted and stored at -20 °C prior to use. With respect to human lipocalin-2 used for growth assay described below, the protein was dialyzed against PBS, sterilized by membrane filtration (0.22- μ m filter), and stored at -20 °C prior to use.

2.2.7 Synthesis and purification of Ent derivatives

The ferric Ent complex was prepared by mixing Ent with 1.2 equivalent of $FeCl_3$ as described by Zeng *et al.* (Zeng et al., 2013). Linearized DHBS trimer was produced by hydrolysis of Ent by recombinant IroE followed by HPLC purification as described in our previous publication (Zeng et al., 2013). Salmochelins (MGE, DGE, and TGE) were synthesized by Ent glucosylation and purified with HPLC as described in our previous work (Fischbach et al., 2005; Lin et al., 2005) with slight modifications. Briefly, 500 μ l of reaction mix contains 75 mM Tris buffer (pH 7.5), 5 mM $MgCl_2$, 2.5 mM TCEP-HCl, 3 mM UPD-Glc, 500 μ M Ent, and 1 μ M IroB. At different time points, the reaction was stopped by adding 0.1% TFA and analyzed by HPLC using a gradient of 0-43.75% CH_3CN in 0.1% TFA water over 10 minutes. Fractions of MGE, DGE, and TGE were collected, lyophilized, and stored at -20 °C prior to use.

2.2.8 TLC-Immunostaining

The TLC of Ent was performed as described in our previous publication (Zeng et al., 2013). In brief, 1 μ l of the Ent solution (10 mM, dissolved in methanol) was spotted in TLC plate (Whatman PE silica gel with UV) and developed with benzene:acetic acid:water (110:85:5) (v/v). The immunostaining was performed by following the protocol described by Duvar *et al.* (Duvar et al., 1997). The silica gel was fixed with 0.5% polyisobutylmethacrylate (Polysciences, Inc., Warrington, PA), which is dissolved in chloroform:n-hexane (1:9), and dried in chemical fume hood. The silica gel was incubated in blocking solution (PBS containing 5% of skim milk) for 1 hr at room temperature, followed by incubation with anti-Ent antibodies (1:100 dilution of rabbit polyclonal Ent antiserum in blocking solution) with gentle shaking for 1 hr. Subsequently, the silica gel was washed five times with washing buffer (PBS containing 0.05% Tween 20), and incubated with secondary antibodies (goat-anti-rabbit IgG-HRP, Seracare; 1:2000 dilution in blocking solution) for 1 hr at room temperature. After incubation the silica gel was washed as described above. The silica gel was developed for visualization by using SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific).

2.2.9 ELISA

Indirect ELISA were conducted as described previously with modifications (Zeng et al., 2010) to examine IgG response in rabbit sera against KLH-Ent (to measure the general antibody response), BSA-Ent (to measure the Ent-specific antibody response), as well as different Ent-derivative compounds (to evaluate the binding specificity of Ent-specific IgG). The ELISA plates were coated differently depending on specific purpose. To determine the titer of general serum IgG directed against the KLH-Ent conjugate, microtiter plates (Thermo Fisher Scientific) were coated with 100 μ l of KLH-Ent (30 ng/well) in coating buffer (bicarbonate/carbonate

coating buffer, pH 9.6) overnight at room temperature. To measure the level of Ent-specific IgG in rabbit sera, 100 μ l of BSA-Ent (30 ng/well) was coated onto microtiter plates using the same coating method as described above.

To further validate the titer of anti-Ent IgG and particularly evaluate the binding ability of the Ent-specific IgG to different Ent derivatives, Ent or its derivatives was directly coated onto ELISA plates using a unique coating method based on an early publication (Bantroch et al., 1994) with modifications. In brief, Ent and its derivatives were diluted in methanol to final concentration of 0.5 mM and added in ELISA plate (20 μ l/well). Subsequently, the ELISA plates were placed on a hotplate (set at 60 °C) in chemical fume hood for 30 min for complete evaporation of methanol due to mild heat and airflow. Upon completion of specific coating procedure as described above, the dried ELISA plates with directly coated small compounds were subjected to standard ELISA test as described in a previous publication (Sahin et al., 2001) with slight modifications. Serum sample was serially diluted by up to 4,096-fold for ELISA analysis. The secondary antibody goat anti-rabbit IgG labeled with horseradish peroxidase (SeraCare) was diluted 1:1000 in blocking buffer and 100 μ l was added to each well. After 1-hr incubation, plates were washed three times. Then plates were developed using the ABTS Peroxidase Substrate Kit (KPL) and the reaction was stopped after 30 min using 100 μ l stopping solution (1X PBS, 1% SDS). Absorbance was measured at optical density of 405 nm (OD₄₀₅) using an ELX808 (BioTek Instruments, Inc., Winooski, VT) and data were collected using Gen5 software version 2.03.1 (BioTek Instruments, Inc.). The wells without addition of primary antibody served as background control. Endpoint titer was defined as the last dilution at which OD₄₀₅ of sample wells exceeded the cutoff value (mean OD₄₀₅ plus 3 $\times$ standard deviation of preimmune sera at 512-fold dilution). Titer was expressed as the reciprocal of the end point

dilution log₂. Duplicate or triplicate independent measurements were performed for each serum sample.

2.2.10 Ent growth promotion assay

The inhibitory effect of anti-Ent IgG on Ent-dependent growth of *Campylobacter* was evaluated using a modified growth promotion assay as described in previous publication (Zeng et al., 2009). The *C. jejuni* JL241 and *C. coli* JL170 (Table 1) were selected as representative strains in this assay. Prior to the assay, both rabbit control and Ent antisera were heat-inactivated at 56 °C for 30 min to abolish complement activity, followed by dialysis against PBS and sterilization via membrane filtration (0.22-µm filter). The sterile rabbit sera were stored at 4 °C. Briefly, *C. jejuni* log-phase cells grown in MH broth (~ 1 x 10⁷ cells) were mixed with melted MH agar (0.6% agar) supplemented with chelator Desferoxamine (DFO, final concentration of 20 µM), as well as PBS, control serum (final 5.6-fold dilution), Ent antiserum (final 5.6-fold dilution), or human lipocalin-2 (final concentration of 24 µg/ml). The mixture was immediately poured into small petri dishes (6 cm of diameter) for solidification. A sterile paper disc (6 mm diameter) containing 10 µl of Ent (10 mM) was placed onto the surface of each agar plate. Bacterial growth zones surrounding the disks were observed and measured after 24 hrs of incubation at 42 °C under microaerophilic condition. The experiment was performed in triplicate for each treatment.

2.2.11 In vitro growth assay

The inhibitory effect of anti-Ent IgG on the growth of *E. coli* K-12 strain MG1655 under iron-restricted condition was evaluated using a microtiter growth assay as described in previous publications (Garénaux et al., 2013; Lin et al., 1998) with slight modifications. Briefly, *E. coli* MG1655 was inoculated into the TSB broth supplemented with 0.6 mM of α,α'-dipyridyl. The

log-phase cells were washed two times with sterile PBS and subsequently diluted with RPMI medium (Gibco, Dublin, Ireland) to final concentrations about 5×10^5 CFU/ml. In microplate wells, 150 μ l of RPMI medium was mixed with 50 μ l of control serum, 50 μ l of Ent antiserum, or 50 μ l of control serum plus 10 μ l of stock human lipocalin-2 (0.8 mg/ml). Subsequently, 5 μ l of above bacterial inoculum cells were added into each well. The microplate was incubated at 37 °C for up to 24 hrs. At different time points (8 hrs, 18 hrs, and 24 hrs post-inoculation, respectively), 20 μ l of culture was taken from each well, serially diluted in ice-cold RPMI medium, and plated onto LB agar plates. The CFU number was enumerated after overnight growth and *in vitro* growth was expressed as $\log_{10}$ CFU/ml. Each treatment group was performed in duplicate.

2.2.12 Statistical analysis

Statistical analyses were conducted using a *t-test* with two-tailed distribution and paired sample. Data were presented as mean $\pm$ standard deviation. A probability level of $P < 0.05$ was considered as a statistically significant difference.

2.3 Results

2.3.1 Conjugation of Ent with carrier proteins

We first conjugated Ent to KLH and BSA, the two immunogenic and commonly used carrier proteins. Notably, these two carrier proteins display significant sequence divergence; therefore, corresponding antibodies lack any cross-reactivity between the two carrier proteins, providing an easier way to validate the production of Ent-specific IgG as described below.

Based on structural analysis and current knowledge in organic chemistry, we observed that Ent could be conjugated to the target carrier protein through one feasible chemical reaction.

Specifically, the ester groups in Ent theoretically could react with the Lys side chains in BSA and KLH if the carrier proteins were incubated with Ent under appropriate conditions; a linearized 2,3-dihydroxybenzoylserine (DHBS) trimer would be attached to the carrier protein (Figure 1A). We also speculated that the surface-exposed linear DHBS trimer on carrier protein might lead to effective antigen presentation upon immunization, consequently inducing high level of antibodies directed against Ent. Regarding BSA that contains 59 lysine residues in each molecule, approximately 35 of which have exposed primary amines that are capable of coupling Ent. Each molecule of KLH contains hundreds of primary amines available for coupling Ent. Therefore, under proper reaction conditions, Ent may directly react with amines in a carrier protein to form a covalent bond (Figure 1A).

The high-purity Ent was extracted from an Ent transport mutant of *E. coli* with yield about 100 mg of Ent/5 L culture using a standard protocol (Anderson & Armstrong, 2004; Zeng & Lin, 2017). The purity of Ent has been routinely validated by HPLC method as described in this study and in our previous publication (Zeng et al., 2013); no any organic molecules were detected to be co-purified along with Ent. We examined different parameters, such as Ent concentration, pH, temperature, and solvent, to optimize efficiency and consistency of conjugation of Ent to BSA and KLH. Initially, to conjugate Ent with BSA, we chose phosphate buffer (pH 8.0) as the reaction buffer. At 4 °C, the conjugation could not be completed even after overnight reaction (12 hrs). However, the labeling reaction worked well at 37 °C as indicated by the smear of bands with higher molecular mass (Figure 2A, lanes 2 & 3) than unlabeled BSA when less than 20% of methanol was used in reaction mix. To avoid potential cross conjugation, we intended to ensure saturated conjugation specifically by taking the ratio of Lys residue of BSA and Ent into account; the lowest ratio we tested (63:1) worked well (Figure 2A). Different

organic solvents, which included methanol, dimethyl sulfoxide (DMSO), and dimethylformamide (DMF), were used to dissolve Ent for preparation of stock solution; however, no obvious effect of solvent choice on the conjugation reaction was observed. Finally, we determined that 2-4 hrs of reaction time at 37 °C was the best condition for the conjugation of Ent to the carrier protein. Scale-up using the optimized conditions also led to successful conjugation of Ent to BSA and KLH (Figure 1B). Specifically, the 4-hr conjugation of Ent with KLH led to significant shift and smear when compared to control KLH (Figure 1B, left panel). A similar band shift was also observed for conjugation of Ent with BSA (Figure 1B, middle panel). To examine the commonality of the simple conjugation method when using different carrier protein, we chose CmeC, a subunit vaccine candidate that has 36 surface-exposed Lys residues (Su et al., 2014; Zeng et al., 2010) as a carrier. As shown in Figure 1B (right panel), a significant smear was also observed after the 4-hr conjugation of Ent with CmeC.

Successful conjugation of Ent to KLH (KLH-Ent), BSA (BSA-Ent), or CmeC (CmeC-Ent) was further validated by using the antiserum from rabbits immunized with the KLH-Ent conjugate. Both dot blot (Figure 3A) and standard Western Blot (Figure 3B) demonstrated successful conjugation of Ent to different carrier proteins using the same straightforward protocol. As expected, the antiserum could react with both KLH and KLH-Ent conjugate (Figure 3A). Due to significant sequence divergence between KLH and BSA, the antiserum raised by KLH-Ent did not cross-react with BSA (Figure 3A). However, the antiserum reacted with BSA-Ent vividly, demonstrating the presence of Ent-specific antibodies in the antiserum raised by KLH-Ent conjugate (Figure 3A). The finding from dot blot was further confirmed by Western blotting analysis of comparable amount of carrier protein and its corresponding Ent conjugate.

As shown in Figure 3B, the serum from the rabbit immunized with KLH-Ent failed to detect BSA or CmeC carrier proteins but reacted with both BSA-Ent and CmeC-Ent conjugates.

2.3.2 KLH-Ent conjugate vaccine elicited significant immune response and high level of Ent-specific IgG

Vaccination of rabbits with the KLH-Ent conjugate triggered strong systemic immune response, which was confirmed in two independent trials with two rabbits in each trial. With respect to IgG immune response against the whole KLH-Ent conjugate, upon primary and two boost immunizations, the ELISA titer of serum IgG directed against the KLH-Ent conjugate in each rabbit was drastically elevated by 11-14 log₂ units (or 2,048-16,384 fold increase) at week 7 postimmunization (Figure 3C). The high-level of IgG titer was maintained throughout the remaining period by week 10 postimmunization when the third boost immunization was performed (Figure 3C). However, the third boost immunization did not further enhance the titer of IgG directed against KLH-Ent (Figure 3C). The dynamic change and pattern of the titer of IgG directed against KLH-Ent were similar among the four individual rabbits (designated as R1, R2, R3, and R4; Figure 3C).

To determine specific immune response directed against the small Ent molecule, ELISA was performed by coating ELISA plates with the BSA-Ent conjugate. In general, immunization of rabbits with the KLH-Ent significantly induced Ent-specific IgG in each rabbit (up to 11 log₂ units or 2,048 fold increase); however, the dynamic change and pattern of anti-Ent IgG titer displayed great variation among the four individual rabbits (Figure 3D). For example, immunization of R2 elevated specific anti-Ent IgG in serum by 8 log₂ units at week 7 postimmunization; the subsequent third boost immunization at week 10 further increased level of anti-Ent IgG titer at week 13 postimmunization, leading to total 2,048 fold increase in the level

of Ent-specific IgG (Figure 3D). In other three rabbits (R1, R3, and R4), the magnitude of increase in the titer of anti-Ent IgG upon KLH-Ent immunization was smaller than that observed in R2 rabbit (Figure 3D). When increasing concentration of the coated BSA-Ent antigen from 30 ng/well to 2 µg/well for ELISA analysis, we observed more consistent basal IgG titer in preimmune sera (7 log₂ units) and larger magnitude of increase in the titers of Ent-specific IgG in postimmune sera in majority of rabbits, which were 5, 12, 8, and 5 log₂ units increase for R1, R2, R3, and R4, respectively (Figure 3E).

2.3.3 The anti-Ent IgG can bind to various Ent derivatives

Due to the high diversity and flexibility nature of antibodies, the Ent-specific antibodies triggered by the KLH-Ent conjugate may display broad binding spectrum not only for *apo* Ent but also for FeEnt complex as well as various Ent derivatives including salmochelins, the glycosylated Ent that helps enteric pathogens counteract sequestration of siderophores by host lipocalins (16). To test this, we synthesized a panel of Ent derivatives *in vitro* followed by successful validation of the binding ability of Ent antibodies to these catecholate compounds using a modified ELISA method.

Both FeEnt and linearized DHBS trimer were synthesized using a standard procedure as described in our previous publication (Zeng et al., 2013). To produce the three different salmochelins (mono-, di-, and tri-glucosyl Ent, denoted as MGE, DGE, and TGE, respectively), the high-purity IroB, a C-glycosyltransferase, was first purified from *E. coli* JL 636 strain (Table 1, Figure 4A). Subsequently, the three salmochelins were *in vitro* synthesized using Ent and IroB enzyme as described previously (Fischbach et al., 2005) followed by HPLC purification. As expected, HPLC displayed three characteristic peaks, representing three different salmochelin products (Figure 4B).

After obtaining the Ent derivatives, we initially performed Thin Layer Chromatography (TLC)-Immunostaining that was expected to provide direct and sensitive evidence showing interaction between specific catecholates and anti-Ent antibodies. However, in terms of positive control Ent, we failed to detect chemiluminescent signal although the cyclic Ent was separated well with TLC as observed in our previous study (Zeng et al., 2013). Given this technical issue, we subsequently developed an indirect ELISA to evaluate the binding ability of Ent-specific IgG to various Ent derivatives. Notably, the conventional coating solution for ELISA, such as the bicarbonate/carbonate buffer (pH 9.6), is not suitable to effectively coat Ent and its relevant catecholates, the hydrophobic compounds, on ELISA plate. Therefore, based on an early publication by Bantroch *et al.* (Bantroch et al., 1994), we developed a unique coating method by using organic solvent methanol as coating solution to coat catecholate siderophores on ELISA plate. As shown in Figure 5, the ELISA plates coated with Ent (10 nmol/well) or the BSA-Ent conjugate (2 µg/well) showed the same level of Ent-specific IgG for preimmune and postimmune sera, respectively; this finding is consistent and has been confirmed in at least two independent experiments. Thus, the modified method for direct coating of ELISA plate with Ent is reliable, simple, and straightforward to detect the binding ability of IgG against hydrophobic small compounds, such as catecholates, using indirect ELISA.

As shown in Figure 6, compared to preimmune serum, the rabbit Ent antiserum clearly displayed binding ability to Ent and various derivatives even if the serum was diluted by 4,096 folds. The rabbit antiserum showed comparable binding to *apo* Ent, linear Ent trimer, and salmochelins MGE and DGE (Figure 6). However, regarding TGE, the Ent modified with three glycosyls (Figure 7), the Ent antiserum showed reduced binding capability. Interestingly, the anti-Ent antibodies raised by the KLH-Ent conjugate in this study consistently showed enhanced

binding for FeEnt complex when compared to the binding for original hapten Ent (Figure 6). With respect to DHBA, a small siderophore considered as Ent precursor (Figure 7) (O'Brien et al., 1970), the anti-Ent antibodies failed to detect it even if the coating concentration of DHBA was increased by 3 fold accordingly due to monomer unit nature of DHBA, suggesting low binding of the anti-Ent antibodies to DHBA.

2.3.4 Ent-specific antibodies inhibited Ent-mediated growth promotion in *Campylobacter*

We speculated that Ent-specific antibodies could function as lipocalin-like agent to inhibit Ent-mediated iron uptake and bacterial growth. *Campylobacter* serves as an ideal organism to test this hypothesis because this organism lacks ability to synthesize any siderophores and shows low redundancy of iron acquisition systems (Zeng & Lin, 2014). Thus, using standard Ent growth promotion assay and a purified human lipocalin-2 (Figure 8A) as positive control, we initially examined the effect of Ent-specific antibodies on Ent-mediated growth promotion in JL170 and JL241 (Table 1), the representative *C. coli* and *C. jejuni* strains for FeEnt acquisition study in *Campylobacter* (Naikare et al., 2013; Xu et al., 2010; Zeng et al., 2013; Zeng et al., 2009).

As shown in Figure 9A, the Ent antiserum displayed significantly inhibitory effect on Ent-dependent growth of *C. coli* JL170; the diameter of growth zone for anti-Ent rabbit serum (3.32 ± 0.10 cm) is significantly smaller than those for control serum (3.85 ± 0.10 cm, $P=0.02$) and PBS control (3.68 ± 0.03 cm, $P=0.03$). As expected, the zone size (2.57 ± 0.03 cm) for the purified human lipocalin-2, the positive control, is significantly smaller ($P=0.01$) than those for any other treatments including Ent antiserum (Figure 9A). However, for *C. jejuni* NCTC 11168 (JL241), the zone size is not different between control serum and Ent antiserum (Figure 8B). Because the same concentration of human lipocalin-2 did not exert inhibitory effect on the growth of

NCTC11168 either (Figure 8B), we speculated *C. jejuni* NCTC 11168 was less sensitive than *C. coli* JL170 for the interference of iron acquisition resulting from Ent-binding proteins. Thus, the quantity of Ent-specific antibodies might not be high enough to exert inhibitory effect on FeEnt-mediated iron acquisition in *C. jejuni* NCTC 11168. To test this, we modified growth promotion assay by increasing the volume of control serum or Ent antiserum in soft agar, which showed the Ent antiserum significantly inhibited the growth of *C. jejuni* NCTC 11168 when compared to control serum ($P=0.03$) and PBS ($P=0.02$) (Figure 9B).

2.3.5 Ent-specific antibodies inhibited Ent-dependent growth of *E. coli*

The *E. coli* MG1655 was subsequently used as a representative Ent-producing strain to examine the inhibitory effect of the Ent-specific antibodies on bacterial growth under iron-restricted conditions. In the presence of control serum, the *E. coli* MG1655 strain displayed normal growth by reaching approximately 10 log units by 18 hrs (Figure 10). In contrast, the *E. coli* MG1655 barely grew in the presence of Ent antiserum; by 18 and 24 hrs, the concentration of the *E. coli* MG1655 was less than the original level at 0 hr (Figure 10). As expected, the lipocalin 2 also showed potent and similar bacteriostatic effect on the growth of the *E. coli* strain as Ent antiserum (Figure 10).

2.4 Discussion

Sassone-Corsi *et al.* (Sassone-Corsi et al., 2016) recently reported an Ent-based conjugate vaccine with the same concept but using different design and synthesis procedure. Specifically, a different carrier protein – cholera toxin subunit B (CTB) – was chosen for conjugation due to its adjuvant feature to induce mucosal immune response. In addition, with aid of a PEG₃ linker attached to Ent, which resulted from a stepwise chemical synthesis and modifications, the Ent was covalently attached to the surface-exposed Lys residues of CTB, leading to the CTB-Ent

conjugate that harbors the native Ent scaffold (Sassone-Corsi et al., 2016). Immunization of mice via intranasal route with the CTB-Ent conjugate was observed to generate mucosal IgA directed against Ent; however, the specific immune response was weak because anti-Ent IgA level was only slightly increased (< 4 fold) upon CTB-Ent vaccination (Sassone-Corsi et al., 2016). In addition, Ent-specific antibodies (e.g., anti-Ent IgG) were not detected in systemic system (blood and spleen) upon immunization of mice with the CTB-Ent. Thus, new type of conjugative Ent vaccine with simple synthesis procedure and capability to induce strong Ent-specific immune response is highly warranted. Finally, despite moderate protection conferred by the CTB-Ent conjugate against *Salmonella* colonization in mice, Sassone-Corsi *et al.* (Sassone-Corsi et al., 2016) did not provide any evidence demonstrating the induced anti-Ent antibodies could function similarly as lipocalin to interfere with Ent-dependent growth promotion in enteric bacteria under iron-restricted conditions, a critically important evidence required for validating the concept of the Ent conjugate vaccine. Here we reported a new type of Ent conjugate (Figure 1), which was synthesized by direct incubation of carrier protein with Ent for 2-4 hrs, can induce strong systemic immune response and increases specific anti-Ent IgG level by up to 4,096 fold (Figure 3). We also developed a unique coating method for ELISA and provided direct evidence that anti-Ent IgG has strong binding ability to various Ent derivatives (Figure 6). We further demonstrated that rabbit Ent antiserum did exert significant inhibitory effect on Ent-dependent growth of two representative Gram-negative organisms under iron-restricted conditions (Figure 9 and Figure 10).

In the study by Sassone-Corsi *et al* (Sassone-Corsi et al., 2016), it was noted that the modified cyclic Ent was further chelated with ferric iron prior to conjugation with CTB. Clearly, the FeEnt complex displays a distinct and more compact configuration when compared with *apo*

Ent (Figure 7). This evidence together with the modification of Ent with a PEG₃ linker likely interferes with proper antigen presentation to stimulate and select desired antibodies targeting Ent epitope, which may explain the weak Ent-specific immune response observed upon CTB-Ent vaccination (Sassone-Corsi et al., 2016) even if CTB has been widely demonstrated for its potent ability to induce a strong immune response as an adjuvant. In contrast, the Ent conjugate developed in this study is based on direct chemical reaction between surface exposed Lys of carrier protein and Ent (Figure 1); the Ent linked to the carrier is expected to be in the form of linear trimer (DHBS)₃ (Figure 1A and Figure 7). Based on such design, we have speculated that the Ent conjugate may trigger strong and specific immune response directed against the conserved Ent epitope(s) and such anti-Ent antibodies may even display broad spectrum to bind to various Ent derivatives, which have been tested and demonstrated in this study. Based on the straightforward principle of Ent conjugation adopted in this study (Figure 1A), it is important to mention that Ent is expected to be conjugated to diverse carrier proteins that have exposed Lys residues, such as subunit vaccine candidates and mucosal carrier/adjuvant, consequently generating innovative multivalent vaccines. This speculation has been partly tested and supported by the successful conjugation of Ent to CmeC, a promising subunit vaccine candidate for *Campylobacter* control (Figure 1B and 3B).

The finding from this work also strongly supports our hypothesis that the Ent antibodies may have a significant advantage over host lipocalins in terms of sequestration of various catecholate siderophores. The Ent antibodies elicited by the KLH-Ent have binding ability to a panel of structurally similar catecholates, which include FeEnt complex, linear Ent trimer, and salmochelins (MGE, DGE, and TGE). The anti-Ent IgG also displayed higher binding ability to FeEnt complex when compared to the binding to *apo* Ent (Figure 6), a noteworthy feature

resulting from our conjugation method (Figure 1). Recently, molecular structure of FeEnt was revealed by a Racemic crystallography (Johnstone & Nolan, 2017), which will help us understand the delicate interaction between Ent and its specific antibodies. Our study also showed that too many modifications on Ent could compromise the recognition of the Ent-specific antibodies, as reflected by the relatively low binding of ant-Ent IgG to TGE (Figure 6).

To develop effective Ent conjugate vaccine, it is important to put the bacteriostatic effects of the antiserum into biological context. In this proof-of-concept study, large quantity of Ent antisera was used in the *in vitro* growth assays, which may raise a concern regarding relative potency of the antisera compared to a biologically relevant concentration of lipocalin 2. However, the estimated concentration of specific anti-Ent IgG used in our assay is in fact much lower than the biologically relevant concentration of lipocalin 2, strongly suggesting the Ent-specific IgG induced by the new vaccine displays more potent bacteriostatic effect than lipocalin 2. This statement is based on following conservative calculation. It is a well-known fact that specific IgG directed against desired protective epitopes of a bacterial vaccine only accounts small proportion of total serum antibodies. For example, the concentration of the IgG directed against a highly immunogenic tetanus toxoid vaccine was 20 µg/ml in human serum (Gonzalez - Quintela et al., 2008; Lavinder et al., 2014). Given that total IgG in human serum was about 11 mg/ml (Gonzalez - Quintela et al., 2008; Lavinder et al., 2014), approximately only 0.2% of total serum IgG are the specific IgG directed against a highly immunogenic antigen (or against all epitopes of the immunogen). Assuming this is also the case for rabbit whose serum IgG concentration is approximately 14.5 mg/ml, the estimated concentration of specific IgG directed against whole Ent-KLH conjugate vaccine, which includes multiple KLH epitopes and limited Ent epitopes, is about 29 µg/ml. If simply ignoring anti-KLH IgG and assuming all KLH-Ent

conjugate-induced antibodies target Ent only, the Ent-specific IgG in rabbit serum should be less than 29 $\mu\text{g/ml}$ or 0.19 μM . Therefore, in our *Campylobacter* growth promotion assay, the estimated final concentration of anti-Ent IgG should be less than 0.07 μM , which is much lower than the concentration of lipocalin 2 used in the same growth assay (24 $\mu\text{g/ml}$ or 1.04 μM). This evidence together with the consistently high level of antibodies in serum and mucosa due to dynamic antibody production strongly supports that the Ent-specific antibodies induced by the Ent conjugate vaccine would function similarly as lipocalins to limit Gram-negative infections in animal host. In the future, the new conjugate vaccines developed in this study, such as KLH-Ent, need to be evaluated for their protective efficacy against Gram-negative infections using appropriate pathogen-animal model systems. Clearly, to improve protective efficacy of this new Ent conjugate vaccine, optimization of vaccination regimen is also highly warranted to further increase titer of the Ent-specific antibodies.

Because Ent and its related catecholate siderophores can be produced by a panel of Gram-negative bacteria in the intestine, we should consider potential effects of such Ent conjugate vaccine on host microbiome and physiology when performing animal studies. From standpoint of short-term therapeutic treatment against pathogens, the concern for potential detrimental effect of anti-Ent antibodies on microbiota should be mitigated due to wide acceptance of broad spectrum clinical antibiotics. For this purpose, production of Ent-specific hyperimmune egg yolk antibodies as immune therapeutics is enticing. Passive immunization with specific egg yolk antibodies is emerging as a potential alternative to antibiotics for the treatment and prevention of various animal and human diseases (Diraviyam et al., 2014). Laying hens are “small factories” for cost-efficient production of large quantities of high-quality polyclonal antibodies; the amount of egg yolk antibodies produced yearly by an immunized hen

is over 22,500 mg with up to 10% are antigen-specific antibodies (Xu et al., 2011). We speculate that immunization of laying hens with the Ent conjugate vaccine developed in this study can generate high titer of Ent-specific egg yolk antibodies, which would have preventive and/or therapeutic efficacy to control Gram-negative infections. This approach is being investigated in our laboratory.

From standpoint of long-term prevention of animals against Gram-negative infections, the anti-Ent antibodies induced by the Ent conjugate vaccine may reduce relative abundance of Ent-producing bacteria. However, the potential detrimental impact resulting from anti-Ent antibodies appears minimal. First, given the bacteriostatic nature of anti-Ent antibodies, the Ent-producing bacterial population should not be dramatically reduced upon vaccination with Ent conjugate. Second, based on recent study by Sassone-Corsi *et al.* (Sassone-Corsi et al., 2016), vaccination of mice with the Ent-CTB conjugate vaccine did not significantly affect composition of microbiota in the intestine. Following *Salmonella* challenge, *Enterobacteriaceae* population dramatically increased in the gut of control mice while *Lactobacillus* spp. rather than *Enterobacteriaceae* expanded significantly in the intestine of the mice immunized with Ent-CTB conjugate vaccine (Sassone-Corsi et al., 2016). Finally, based on recent extensive microbiome research, the Ent-based vaccine is likely even beneficial to host by limiting overpopulation of Ent-producing bacteria. Compelling evidence showed that the increased densities of various γ -proteobacteria species in the gut (e.g., *Enterobacteriaceae*) have detrimental effects on gut health (e.g., increased inflammation) (Bullock et al., 2004; Dicksved et al., 2008; Garrett et al., 2010; Jostins et al., 2012; Litvak et al., 2017; Winter & Bäumler, 2014). Notably, the γ -proteobacteria species are the major bacterial populations in the gut to produce Ent and relevant derivatives for effective iron acquisition and survival *in vivo* (Fischbach et al., 2006; Miethke & Marahiel, 2007;

Raymond et al., 2003). Therefore, the Ent conjugate vaccine may be beneficial for the host by controlling overpopulation of γ -proteobacteria species, consequently improving gut health. This hypothesis needs to be tested in the future.

The high-titer Ent specific antibodies developed in this study may also serve as a useful tool for various basic and translational studies. Although the importance of catecholates-mediated iron acquisition for bacterial pathogenesis has been widely recognized, there is still a significant knowledge gap in the *in vivo* status of such siderophores (Zeng & Lin, 2014). Detection and quantitative measurement of *in vivo* Ent and its derivatives, particularly those in the intestine, have been technically challenging. Limited studies using HPLC and LC/MS techniques have shown that a significant quantity of catecholates were produced in the intestine or air sacs (Caza et al., 2008; Pi et al., 2012). With aid of the anti-Ent antibodies, we can develop an easier and faster immune assay to quantify the level of intestinal Ent and its derivatives used by bacteria for effective iron acquisition. Such assays will be helpful for bacterial pathogenesis research focused on the role of high affinity siderophore-mediated iron scavenging in bacterial infection in host. Based on a recent report by Qi and Ban (Qi & Han, 2018), the anti-Ent antibodies may be used to study eukaryotic host iron homeostasis in cellular processes. Finally, the anti-Ent IgG-based immune assay may also serve as an innovative diagnosis tool to monitor gut health. Early diagnosis of gut health has enormous impacts on timely and effective control of many enteric diseases in animals and humans. As discussed above, the increased density of γ -proteobacteria species in the gut, the primary catecholates-producing population have been observed to exert detrimental effects on gut health (Bullock et al., 2004; Dicksved et al., 2008; Garrett et al., 2010; Jostins et al., 2012; Litvak et al., 2017; Winter & Bäumler, 2014). Therefore, the diverse γ -proteobacteria species may serve as a promising biomarker for gut health in animal

host. However, it is not feasible to use existing microbiological and molecular approaches to develop a single efficient diagnosis tool for evaluation of the level of diverse γ -proteobacteria populations in the intestine. With aid of the Ent antibodies developed in this study, we propose that specific anti-Ent antibodies may be used to monitor intestinal level of γ -proteobacteria species and serve as an early diagnosis tool to evaluate gut health in animals and humans.

In the context of holistic bacteria-host interaction, we should consider potential effect of anti-Ent antibody on eukaryotic host. Recently, using *Caenorhabditis elegans* model and *in vitro* human embryonic cell line, Qi and Ban (Qi & Han, 2018) reported that Ent is beneficial for host iron-uptake and development through the mitochondrial ATP synthase. However, at this stage, physiological relevance of this interesting finding in higher animal is still largely unknown. We cannot simply translate this *C. elegans* discovery into higher animals, such as mammals and other vertebrates. In particular, given the complexity of eukaryotic host and its associated microbiome, host cell iron homeostasis and development can be influenced by numerous factors, including various iron-binding compounds and proteins. *C. elegans* has only ~1,000 somatic cells and a few layers. Thus, the Ent produced by intestinal bacteria may penetrate through the intestinal cell wall and interact with other somatic cells in *C. elegans*. However, in higher animals, to date, there is no any evidence indicating that Ent can penetrate complex intestinal wall and subsequently reach different organs and tissues. Due to sterile nature of the environment of embryos, the mammalian embryo development and differentiation should not be affected by the bacteria-derived Ent. Upon establishment of microbiota after birth, as expected, Ent can be detected in specific niches in the host where Ent-producing bacteria reside, such as inside of the intestinal tract (Pi et al., 2012) and air sac (Caza et al., 2008). However, some studies have shown that Ent was not detected in the tissues of infected lambs

(Der Vartanian et al., 1992) and blood/liver of infected chickens (Caza et al., 2008). Therefore, it is still premature to speculate potential detrimental effect of ant-Ent antibody on iron homeostasis and development of higher animals. To ensure the finding reported by Qi and Ban (Qi & Han, 2018) is not an artificial scenario for higher animals, in the future, the effect of Ent on eukaryotic host iron homeostasis and development must be studied in well-controlled systems using appropriate animal model.

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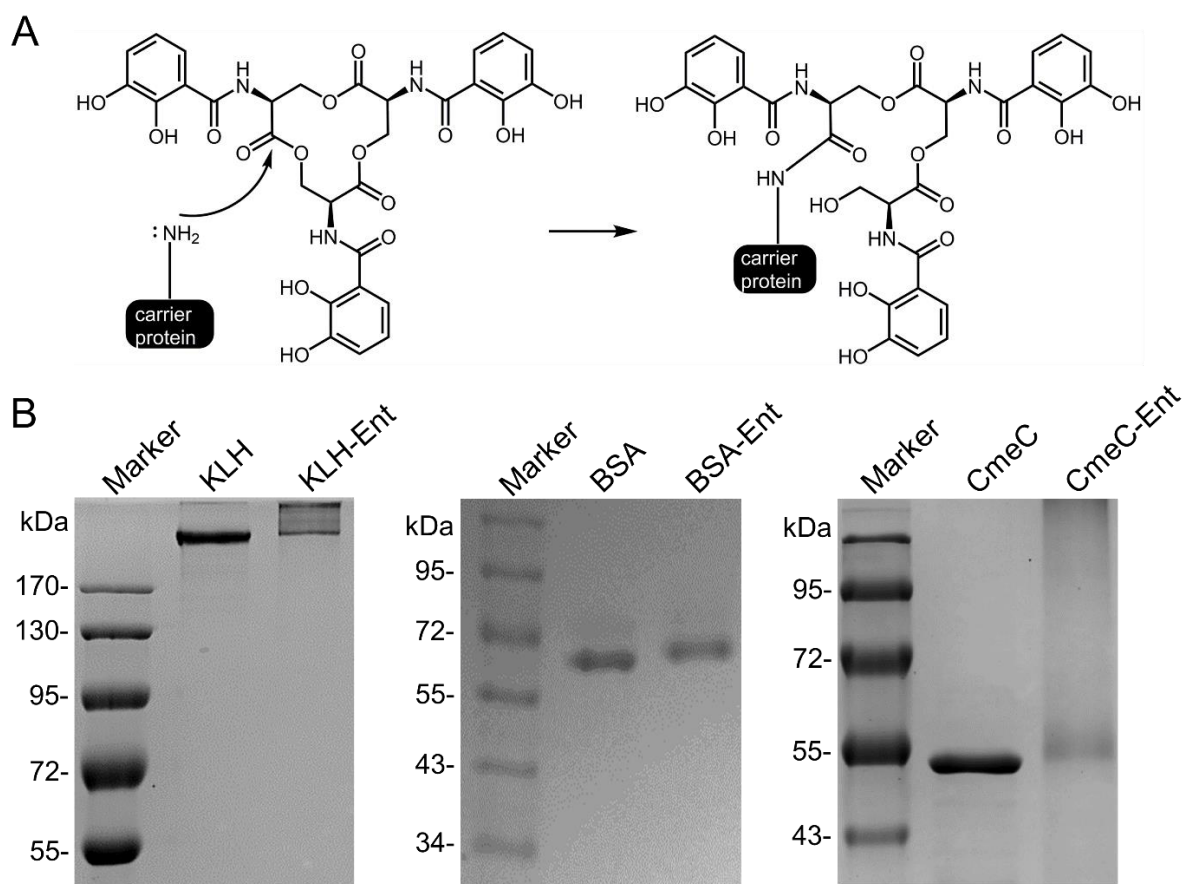
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Appendix I

Table 1. Major strains used in this study.

Strains	Description	Source or Reference
JL122	<i>E. coli</i> AN102, Ent transport mutant for Ent purification	(Anderson & Armstrong, 2004)
JL170	<i>C. coli</i> strain isolated from turkey	(Xu et al., 2010)
JL241	<i>C. jejuni</i> NCTC 11168, human isolate	(Parkhill et al., 2000)
JL633	<i>E. coli</i> BL21 (DE3) recombinant strain producing C-terminal His-tagged IroE	(Lin et al., 2005)
JL636	<i>E. coli</i> BL21 (DE3) recombinant strain producing C-terminal His-tagged IroB	(Fischbach et al., 2005)
JL999	<i>E. coli</i> K-12 MG1655	(Garénaux et al., 2013)
JL1002	<i>E. coli</i> Tuner, $\Delta entD$ derivative QT2908 producing His-tagged human lipocalin-2	(Garénaux et al., 2013)
JL243	<i>E. coli</i> JM109 recombinant strain producing N-terminal His-tagged CmeC	(Zeng et al., 2010)



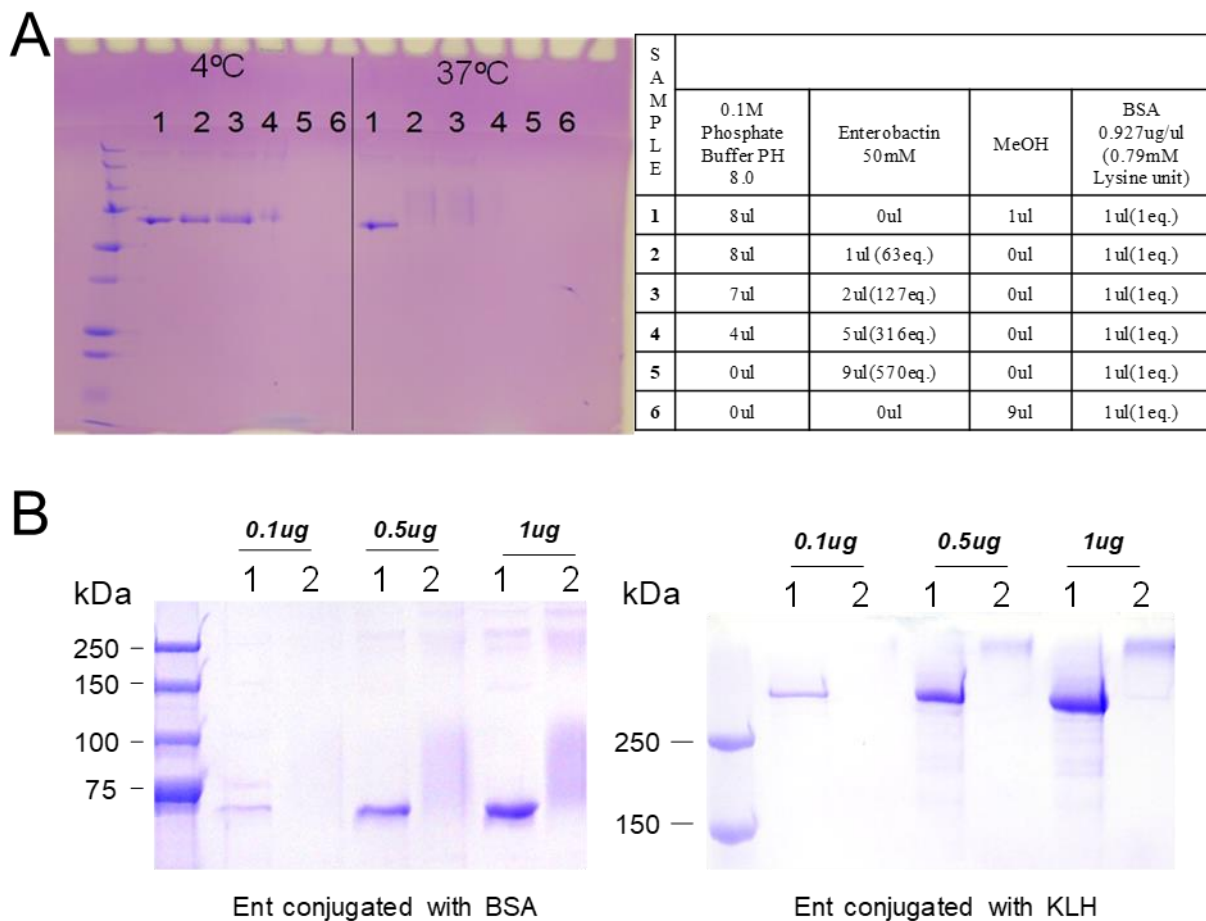


Figure 2. Optimization of conditions for conjugation of Ent to KLH and BSA. (A) Effects of Ent concentration and temperature. Left panel is SDS-PAGE for different sample mixes incubated at 4 °C or 37 °C for 12 hrs. Right table shows detailed recipe for all samples. Ent was dissolved in methanol (MeOH) to final stock concentration of 50 mM. Due to high volume of MeOH in sample 5 and 6, loading of protein samples to the gel did not work, leading to the lack of visible proteins in lanes 5 and 6. (B) SDS-PAGE analysis of conjugation of Ent to KLH and BSA. The same components described in Sample #2 above were scaled up for conjugation of Ent to BSA as well as KLH; the reaction mix was incubated at 37 °C for 12 hrs. Comparable amount of proteins from control and treatment were analyzed by SDS-PAGE. Lane 1, control containing carrier protein only; lane 2, reaction containing both Ent and carrier protein. (This work was performed by the collaborators Dr. Hening Lin and Dr. Bin He at Cornell University)

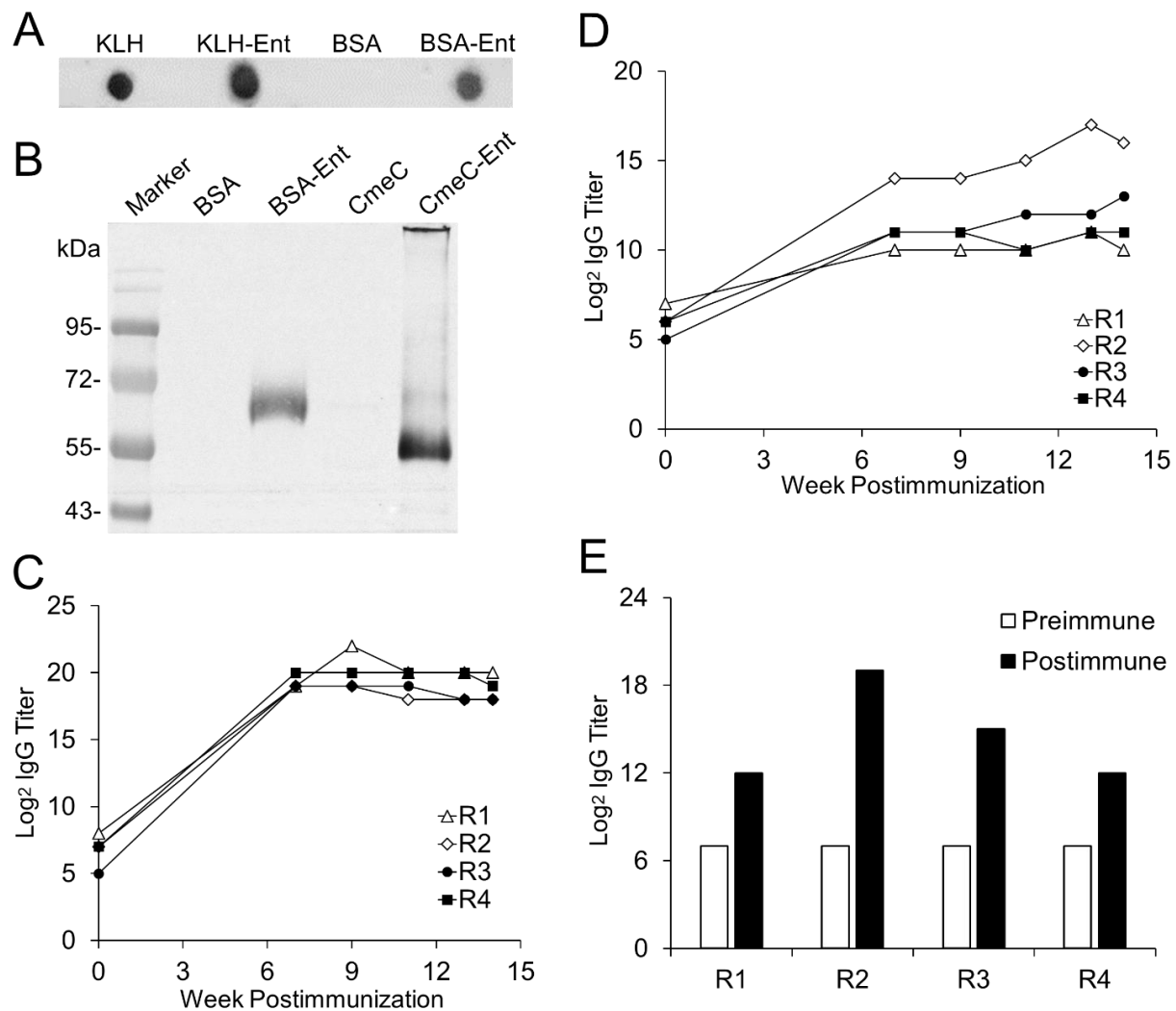


Figure 3. KLH-Ent conjugate vaccine elicited significant immune response and Ent-specific antibodies. (A) Dot blotting analysis of the reactivity of Ent antiserum to KLH, KLH-Ent, BSA, and BSA-Ent. (B) Western blotting analysis of the reactivity of Ent rabbit antiserum to BSA, BSA-Ent, CmeC, and CmeC-Ent. (C) Serum IgG titer to the KLH-Ent conjugate antigen in each rabbit (designated as R1, R2, R3, and R4). Each point represents the average of duplicate measurements. (D) Serum IgG titer to Ent molecule in each rabbit. Each point represents the average of duplicate measurements. (E) Effect of increased coating antigen BSA-Ent (2 µg/well) on measurement of serum anti-Ent IgG titer. The sera in four rabbits (R1-R4) collected at 14th week postimmunization and corresponding preimmune sera were serially diluted for ELISA analysis.

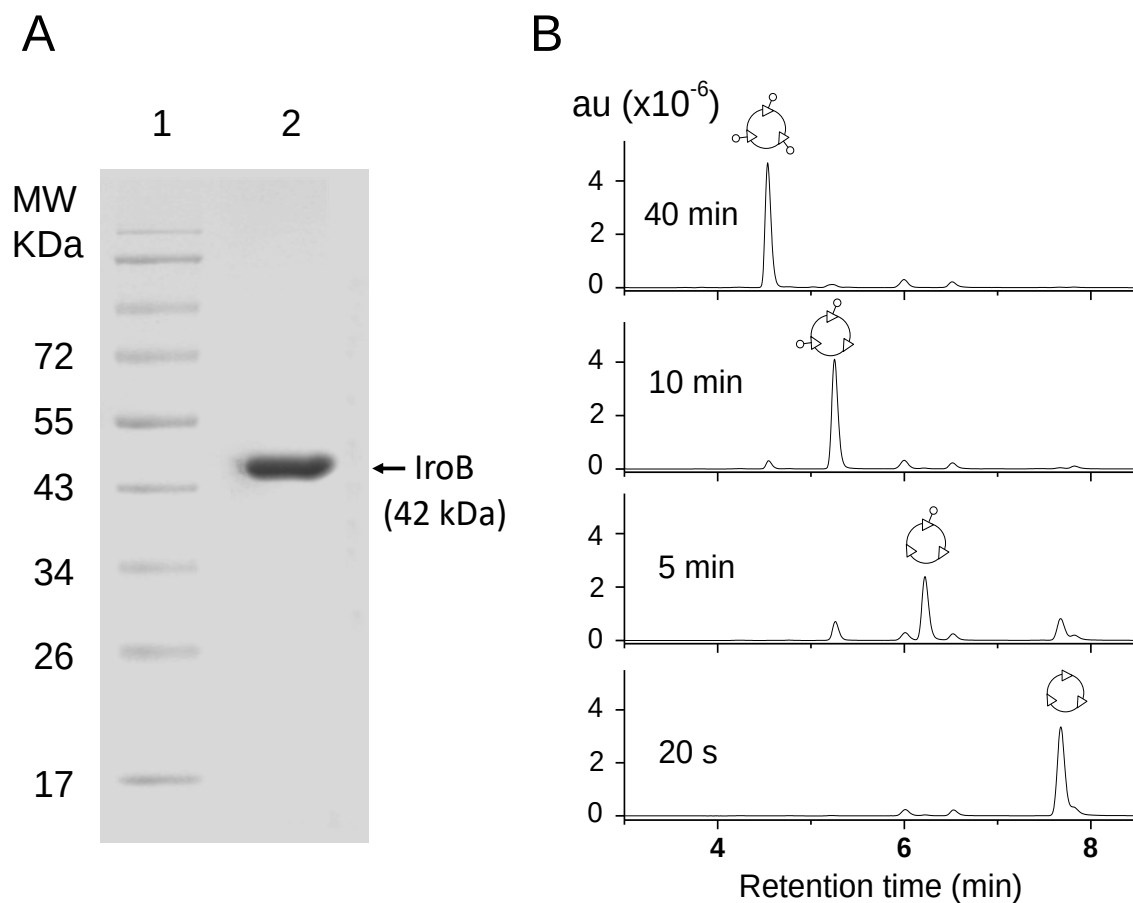


Figure 4. *In vitro* synthesis of salmochelins. (A) SDS-PAGE analysis of IroB purification. Lane 1, molecular weight marker (Bio-Rad); lane 2, purified recombinant IroB. (B) HPLC analysis of the IroB-catalyzed conversion of Ent to various salmochelins. , Ent; , monoglucosyl-Ent (MGE); , diglucosyl-Ent (DGE); , triglucosyl-Ent (TGE). (This work was performed by the previous postdoctoral researcher Dr. Yiming Mo)

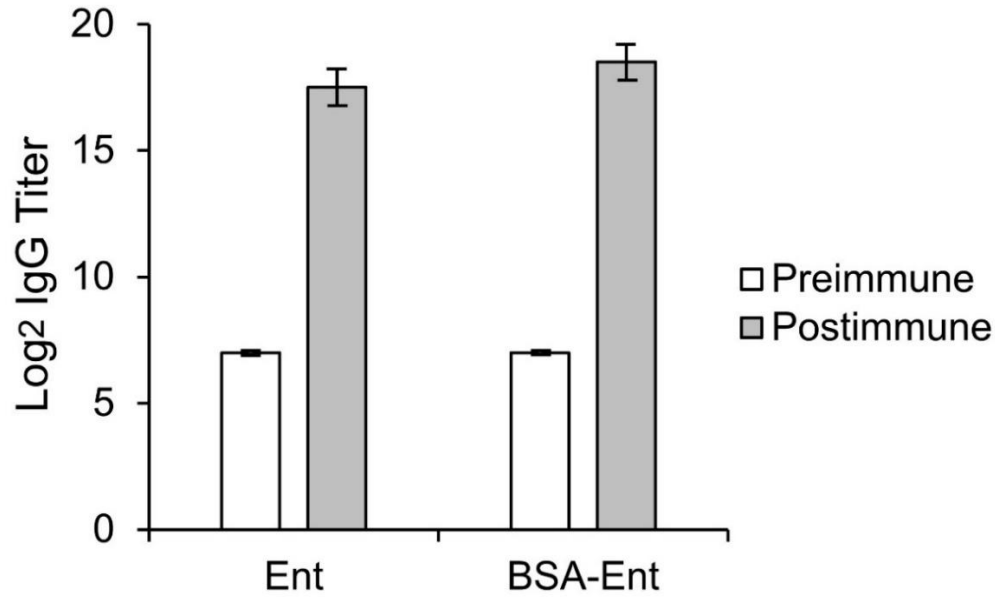


Figure 5. Validation of direct coating of Ent for indirect ELISA using a modified coating method. Ent was dissolved in methanol (10 nmol/well) for direct coating on ELISA plate as detailed in *Materials and Methods*. The preimmune serum and the postimmune serum (collected at 14th week) from Rabbit R2 were 2-fold serially diluted and subjected to ELISA analysis with the plates directly coated with Ent and the plates coated with BSA-Ent (2 µg/well). Titer was expressed as the reciprocal of the end point dilution log₂. The bars represent the means of log₂ IgG titer ± standard deviations of two independent experiments with duplicate measurements performed for each serum sample in each independent experiment.

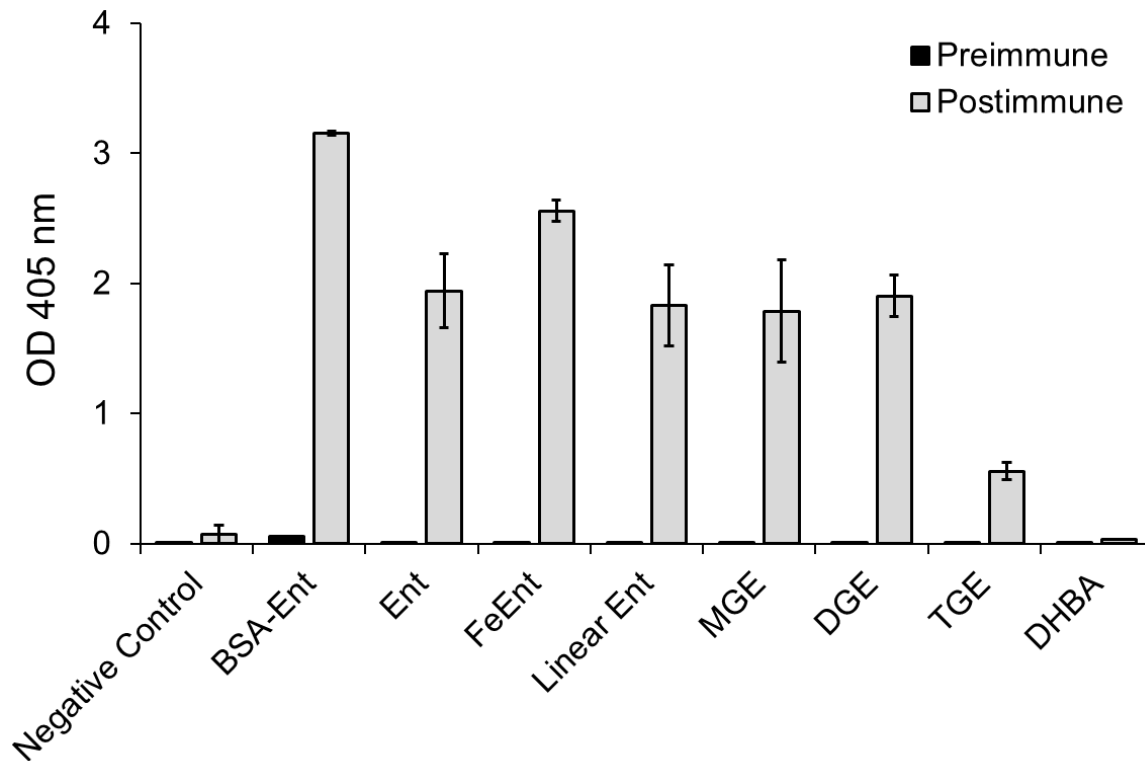


Figure 6. Binding specificity of anti-Ent antibodies to different Ent derivatives. Ent and its derivatives were directly coated on ELISA plate as detailed in *Materials and Methods*. Preimmune and postimmune (14th week) sera from Rabbit 2 were diluted by 4,096 folds and served as primary antibody in indirect ELISA. Each bar represents the average of triplicate measurements. Negative control, no antigen coated; BSA-Ent, BSA-Ent conjugate; Ent, *apo* Ent; FeEnt, ferric Ent complex; Linear Ent, linear trimer of *N*-(2,3-dihydroxybenzoyl) serine, or (DHBS)₃; MGE, monoglucosyl-Ent; DGE, diglucosyl-Ent; TGE, triglucosyl-Ent; DHBA, 2,3-Dihydroxybenzoic acid. Error bars represent standard deviation from at least two independent experiments with duplicate measurements performed in each independent experiment.

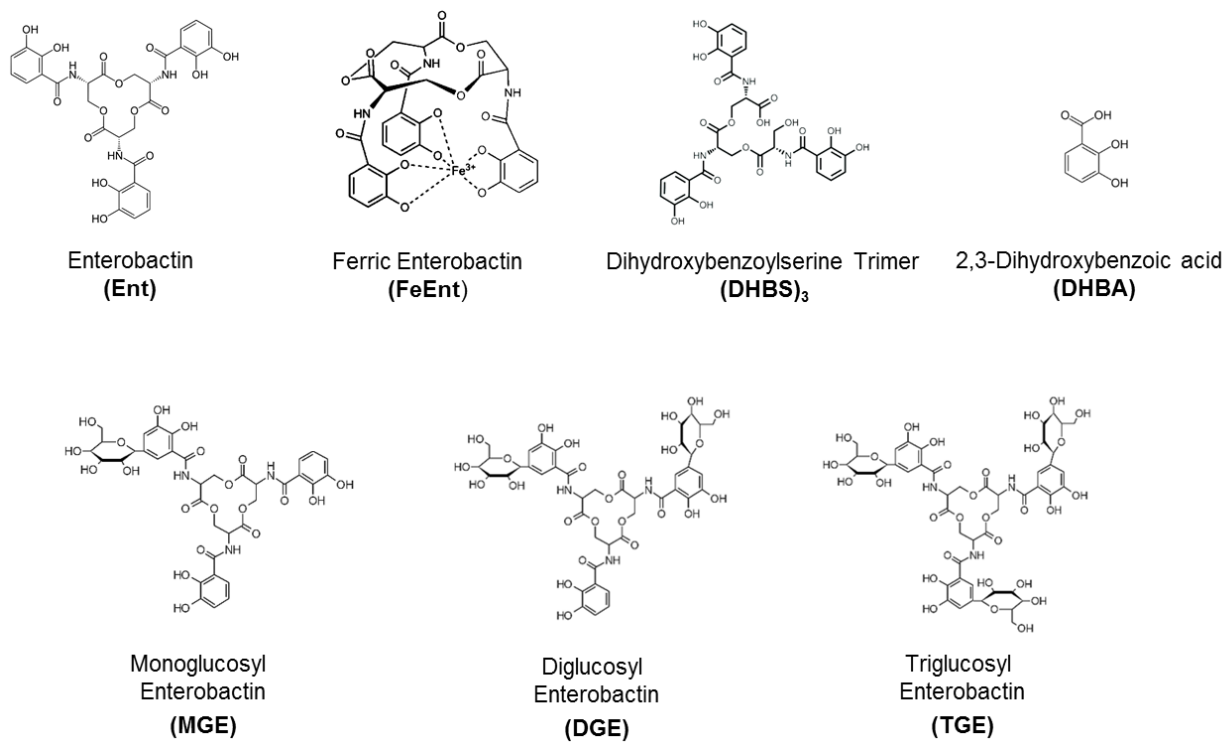


Figure 7. Structure of catecholate compounds.

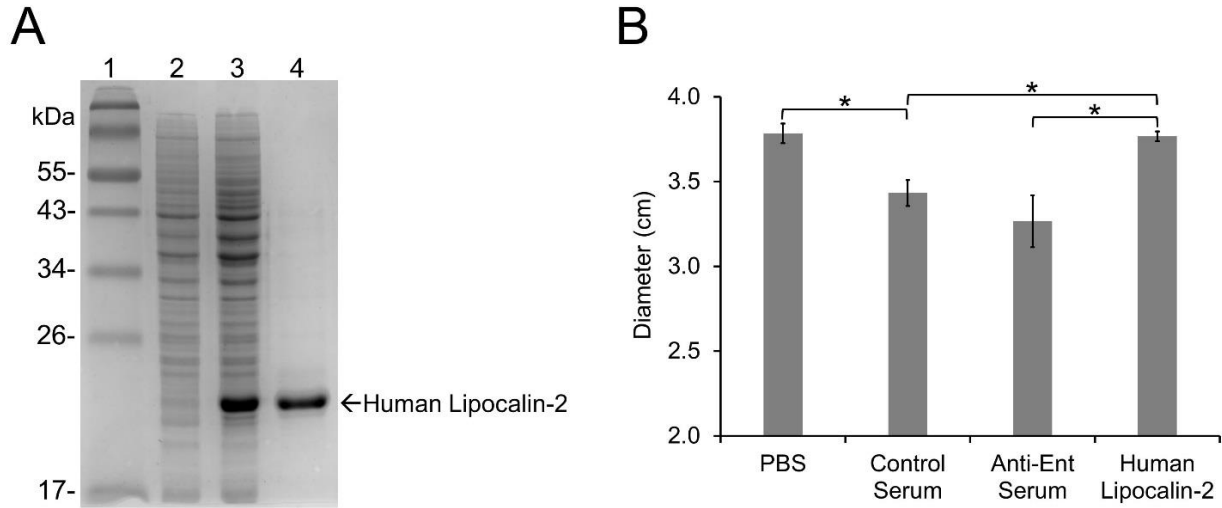


Figure 8. Effect of Ent-specific antibodies on Ent-mediated growth promotion in *Campylobacter*. (A) SDS-PAGE analysis for purification of human lipocalin-2. 1, Marker; 2, Protein fragments of recombinant strain before the induction of IPTG; 3, Protein fragments after the induction with overexpression of human lipocalin-2; 4, Human lipocalin-2 eluted through Ni-NTA agarose column; (B) The diameters of growth zones for *C. jejuni* JL241 (NCTC 11168) grown in iron-restricted MH agar supplemented with PBS, control serum (final 5.6-fold dilution), Ent antiserum (final 5.6-fold dilution), or human lipocalin-2 (final concentration of 24 $\mu\text{g/ml}$). The bars represent the means of growth zone diameter $\pm$ standard deviations of three independent measurements. The statistically significant differences between four groups are indicated with asterisks (*, $P < 0.05$).

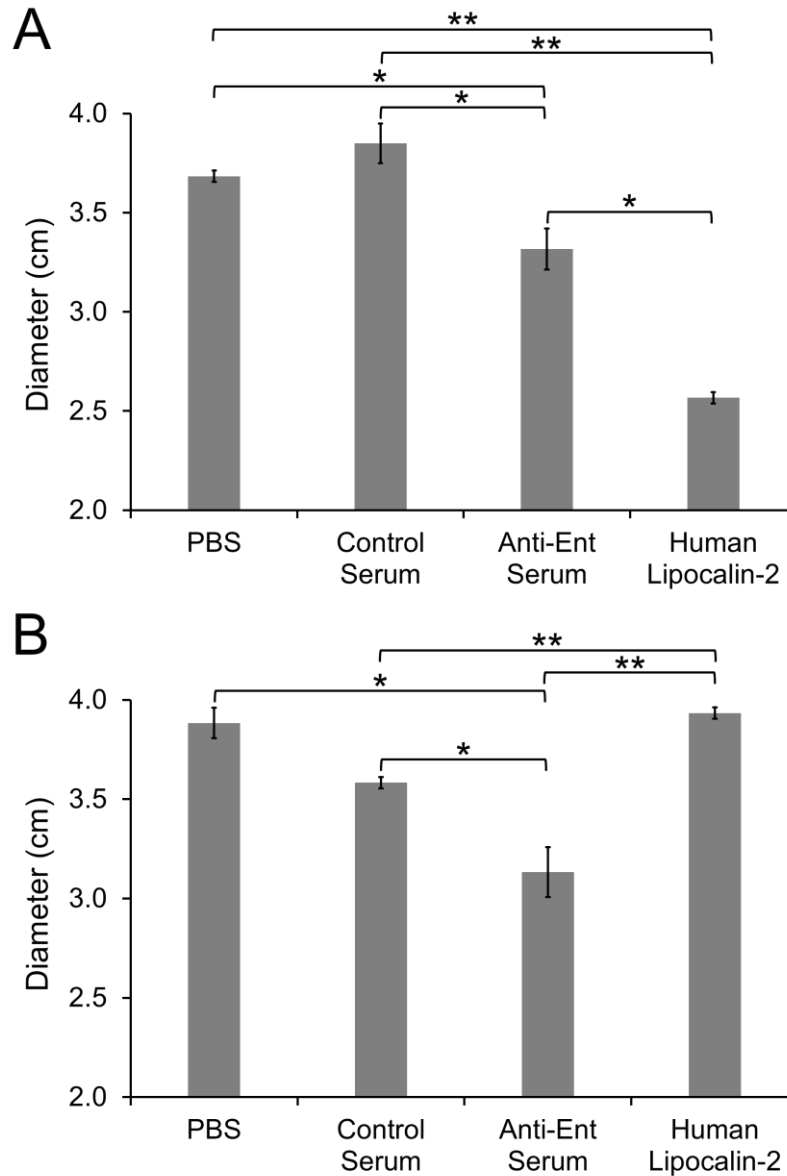


Figure 9. Inhibitory effect of the rabbit Ent antiserum on Ent-dependent growth promotion in *Campylobacter*. (A) The diameters of growth zones for *C. coli* JL170 grown in iron-restricted MH agar supplemented with PBS, control serum (final 5.6-fold dilution), Ent antiserum (final 5.6-fold dilution), or human lipocalin-2 (final concentration of 24 $\mu\text{g/ml}$). (B) The diameters of growth zones for *C. jejuni* 241 (NCTC 11168) grown in iron-restricted MH agar supplemented with PBS, control serum (final 2.9-fold dilution), Ent antiserum (final 2.9-fold dilution), or human lipocalin-2 (final concentration of 24 $\mu\text{g/ml}$). The bars represent the means of growth zone diameter $\pm$ standard deviations of three independent measurements. The statistically significant differences between four groups are indicated with asterisks (*, $P<0.05$; **, $P<0.01$).

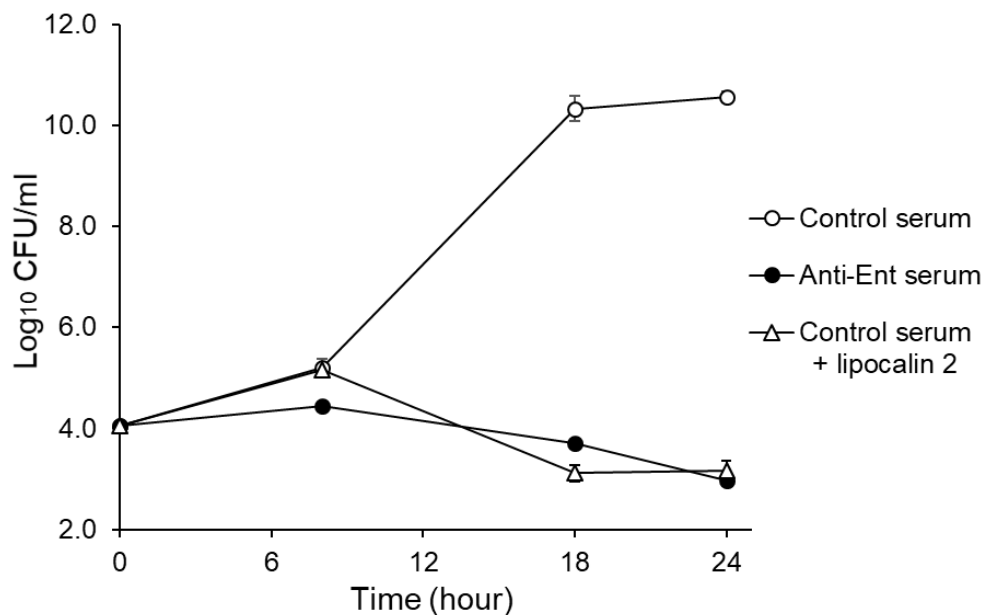


Figure 10. Inhibitory effect of the rabbit Ent antiserum on the growth of *E. coli* under iron-restricted condition. The *E. coli* MG1655 cells were grown in RPMI medium supplemented with rabbit control serum (4-fold dilution), anti-Ent serum (4-fold dilution), or the control serum (4-fold dilution) plus lipocalin 2 (final concentration of 38 µg/ml). Each data point represents the mean value obtained from duplicate wells in the microtiter plate growth assay.

CHAPTER III: Production of Egg Yolk IgY Directed against Enterobactin

Abstract

Passive immunization with pathogen-specific egg yolk antibodies (IgY) is emerging as a potential alternative to antibiotics to prevent and treat microbial infections. Previous findings have strongly supported that immune intervention by targeting enterobactin (Ent), a high-affinity archetype of siderophore, is a promising approach to control infections caused by Gram-negative bacterial pathogens. Recently, we have successfully developed a simple method to prepare Ent conjugate vaccines that can induce high level of Ent-specific antibodies in rabbit. In this study, we evaluated the capability of the Ent conjugate vaccines for production of hyperimmune egg yolk IgY directed against Ent in laying hens. Purified Ent was conjugated to three carrier proteins, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and CmeC (*Campylobacter* outer membrane protein). In the first trial, Barred Rock layers (n=3) were intramuscularly immunized with 100 µg of KLH-Ent conjugate, followed by three booster immunizations every two weeks. The KLH-Ent conjugate vaccine triggered significant immune response in layers, leading to moderately increased titers of Ent-specific IgY in serum (up to 8 folds) and yolk (up to 2 folds). In the second trial, Rhode Island Red layers were subcutaneously immunized with specific Ent conjugates (KLH-Ent, BSA-Ent, or CmeC-Ent; n=2 per conjugate), followed by three booster immunizations every 3-4 weeks. ELISA analysis indicated that anti-Ent IgY titers dramatically increased in serum (up to 2,048 fold) and yolk (up to 1,024 fold) of each individual layer upon vaccination with KLH-Ent or CmeC-Ent. However, the BSA-Ent conjugate failed to induce anti-Ent IgY. Together, this study determined optimal immunization regimen to produce high level of innovative Ent-specific egg yolk antibodies for prevention and control of bacterial infections.

3.1 Introduction

Antibiotics have long been used in food animal production for growth promotion (sub-therapeutic dose), disease prevention (prophylactic dose) and treatment (therapeutic dose) (Yegani & Korver, 2010). However, antibiotic usage in food animals has been linked to the emergence of antibiotic resistant bacteria. Antibiotic resistant bacteria as well as resistance determinants can therefore spread from animals to humans, compromising the effectiveness of antibiotics for treating human infections and posing a serious threat to public health (Marshall & Levy, 2011). Due to this concern and consumer's growing demand for livestock products from antibiotic-free systems, the alternatives to antibiotics in food animal production are in high demand and have become an important research area.

Immune intervention is a promising alternative approach to antibiotics to prevent and control bacterial infections. Vaccination has been a popular and cost-effective methods to combat bacterial infections in livestock production by enhancing disease resistance of animals (Cheng et al., 2014). Nevertheless, there are still some issues that have significantly impeded the development of antibacterial vaccines, especially the vaccines against enteric bacterial pathogens. The major drawbacks of current vaccines against enteric pathogens include but are not limited to: deficiency in triggering sufficient mucosal immune response, antigenic variation, and susceptibility to digestion in the gastrointestinal tract via oral administration (Lycke, 2012). Recently, administration of antibodies for passive immunity, has received increased attention due to the direct and rapid protection against infectious pathogens or toxins (Hedegaard & Heegaard, 2016). Because of transient nature of the protection provided by passively transferred antibodies, repeated or continuous antibody administration is necessary, and large amounts of antigen-specific antibodies are required, especially when used orally. To this end, hen's egg can be an

excellent source of large quantities of antibodies (IgY) used for passive immunization applications (Kovacs-Nolan & Mine, 2012).

IgY in chicken serum and egg yolk is equivalent to IgG in mammals. However, compared to the IgG in mammalian counterpart, production of hyperimmune egg yolk IgY from laying hens is much more efficient. It was shown that an immunized hen can produce approximately 1,500 mg of egg yolk IgY in one month, 2-10% of which is antigen-specific. However, an immunized rabbit produces 18 times less IgG from serum in the same time period (Gadde et al., 2015). Additionally, compared to blood collection in mammals, IgY production is less invasive and more sustainable, which only requires the daily collection of eggs and makes the large-scale production of IgY technically feasible (Karlsson et al., 2004). Moreover, IgY is fairly stable during storage and processing. IgY is also relatively stable between pH 4 and pH 11, especially with the addition of stabilizers (Kovacs-Nolan & Mine, 2012). Therefore, passive immunization with specific egg yolk antibodies is emerging as a potential alternative to antibiotics for the treatment and prevention of various bacterial infections in human and animal.

Enterobactin (Ent), a catecholate siderophore produced by most members of *Enterobacteriaceae* and displaying the highest affinity to ferric iron among all natural siderophores (Raymond et al., 2003), is a promising target of immune intervention as demonstrated in Chapter 2. In particular, the novel KLH-Ent conjugate vaccine could trigger high titer of Ent-specific IgG in rabbits (up to 4,096-fold increase). The anti-Ent IgG could cross-react with various Ent derivatives (such as linearized Ent and different salmochelins) and inhibit Ent-mediated growth promotion in both *Escherichia coli* and *Campylobacter* under iron-restricted conditions (Chapter 2). These data together with the promising features of egg yolk IgY as passive immune agent prompted us to hypothesize that Ent conjugate vaccines can induce

high level of anti-Ent egg yolk IgY in layers and facilitated us to produce sufficient Ent-specific antibodies for passive immune protection against Gram-negative bacterial infections.

In this study, in order to determine appropriate conditions to produce large amount of Ent-specific egg yolk IgY, laying hens were immunized with different Ent conjugate vaccines via different vaccination routes. Subcutaneous immunization of laying hens with KLH-Ent conjugate was found to induce the highest level of anti-Ent IgY in both serum and egg yolk. The high level of Ent-specific IgY in egg yolk was demonstrated to be maintained after the lyophilization process. This study lays a solid foundation for evaluating the passive immune protection of Ent-specific egg yolk IgY against different Gram-negative infections in the future.

3.2 Materials and methods

3.2.1 Ent purification and conjugation with carrier proteins

Ent was purified from *E. coli* AN102, an Ent transport mutant, as detailed in Chapter 2. Carrier proteins BSA and KLH were purchased from Thermo Fisher Scientific (Waltham, MA). Recombinant CmeC was purified as described in a previous study (Zeng et al., 2010). Scale-up conjugation of Ent with BSA, KLH, or CmeC was performed using the same protocol as described in Chapter 2. Successful conjugation of Ent to KLH (KLH-Ent), BSA (BSA-Ent), or CmeC (CmeC-Ent) was confirmed by SDS-PAGE and further validated by immunoblotting as detailed in Chapter 2.

3.2.2 Immunization of layers with Ent conjugate vaccines

Two independent immunization trials were performed to explore the optimal immunization regimen to produce high levels of Ent-specific egg yolk IgY. The first trial was performed at Johnson Animal Research and Teaching Unit (JARTU) at the University of Tennessee using KLH-Ent conjugate, as outlined in Table 2. Six Barred Rock pullets were

purchased from McMurray Hatchery (Iowa) and raised by the age of 26 weeks when pullets started to lay eggs routinely. One group of layers (n=3) received primary immunization via intramuscular injection (in breast) with 100 µg of KLH-Ent conjugate emulsified with Freund's complete adjuvant, followed by three booster immunizations (100 µg of KLH-Ent conjugate emulsified with Freund's incomplete adjuvant) every 2 weeks. The other group of layers (n=3) that did not receive any vaccinations were served as non-immunized control. Blood samples of each layer were collected from the wing vein at different time points to monitor the systemic immune responses. All eggs along the whole trial were also collected and stored at -4 °C. Layers were euthanized at week 11 postimmunization. The second trial was performed by Pacific Immunology Corp. (Ramona, CA), as outlined in Table 3. Compared to the first trial, this trial used different conjugate vaccines (BSA-Ent, KLH-Ent, and CmeC-Ent) and immunization route (subcutaneous route). Briefly, six Rhode Island Red layers (two hens for each vaccine) received primary immunization via subcutaneous injection (under the neck skin near the shoulders) with 100 µg of Ent conjugates emulsified with Freund's complete adjuvant, followed by three booster immunizations (100 µg of Ent conjugate emulsified with Freund's incomplete adjuvant) every 3-4 weeks. Blood samples of each layers were collected from the wing vein at different time points to monitor the systemic immune responses. All eggs along the whole trial were also collected and stored at -4 °C for future analysis and processing.

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

The immune response in serum and egg yolk was measured by indirect ELISA using the same protocol detailed in Chapter 2 with minor modifications. For egg yolk, each sample was diluted with sterile PBS (1/1, v/v) prior to the assay. The rest of ELISA procedure for egg yolk was the same as that for serum. To examine the general antibody responses in serum and egg

yolk samples immunized with KLH-Ent, BSA-Ent, or CmeC-Ent, 100 µl of KLH-Ent, BSA-Ent, or CmeC-Ent was coated on microtiter plates (30 ng/well). To examine the level of Ent-specific antibody in serum and egg yolk samples, 100 µl of BSA-Ent was coated on microtiter plates (2 µg/well) for detection of Ent-specific IgY in the samples from layers immunized with KLH-Ent or CmeC-Ent, while 100 µl of KLH-Ent was coated (2 µg/well) for detection of Ent-specific IgY in the samples from layers immunized with BSA-Ent. In addition, to determine Ent-specific IgY in the serum for serum and egg yolk samples from layers immunized with BSA-Ent, 1% (w/v) fish gelatin (Sigma-Aldrich, MO) was used in blocking buffer to minimize the background due to non-specific binding.

3.2.4 Lyophilization of egg yolk

The pooled egg yolk was lyophilized using the freeze dryer (VirTis AdVantage Plus EL-85 Benchtop Lyophilizer, SP Scientific Inc., NY) for evaluating *in vivo* efficacy of Ent-specific IgY in the future. The egg yolks from control group or the group subcutaneously immunized with KLH-Ent conjugate were pooled and diluted with sterile PBS (1/1, v/v). Then the mixture was stirred evenly using a sterilized egg whisk and transferred into metal trays with approximate 500 ml per tray. The trays were subsequently wrapped by aluminum foil with several small pores for evaporation. The trays containing egg yolks were put in -80 °C freezer overnight for complete solidification of egg yolk mixture prior to lyophilization. Under manual mode of the lyophilizer machine, the egg yolk was processed for about 72 hours. Lastly, the freeze-dried egg yolk was pulverized into powder using a mortar and stored at -20 °C until use. To determine Ent-specific IgY level in lyophilized egg yolk, 0.1 g of immunized and non-immunized egg yolk powders were dissolved in 1 ml of sterile PBS. The solutions were subjected to indirect ELISA as described above.

3.3 Results

3.3.1 Conjugation of Ent with carrier proteins

A total of approximately 10 mg of KLH-Ent, 5 mg of BSA-Ent, and 5 mg of CmeC-Ent conjugates were prepared using the conjugation protocol described in Chapter 2. Successful conjugation of Ent with KLH, BSA, or CmeC was further validated using SDS-PAGE and Immunoblotting, leading to the same results as shown in Figure 1B and Figure 3B. These conjugates were subsequently used for immunization trials and ELISA assay in this study.

3.3.2 Immune responses induced by intramuscular vaccination with KLH-Ent conjugate

Intramuscular vaccination of Barred Rock layers with KLH-Ent conjugate vaccine triggered significant immune responses in serum and egg yolk, while there was no immune response in control layers. With regard to the IgY response against the whole KLH-Ent conjugate, in serum (Figure 11A), the ELISA titers of anti-KLH-Ent IgY were elevated by 1-3 $\log_2$ units (or 2- to 8-fold increase) upon primary immunization, and were further enhanced and maintained by booster immunizations with an increase of up to 4 $\log_2$ units; in egg yolk (Figure 11B), the IgY titers against KLH-Ent conjugate were elicited by 2-4 $\log_2$ units (or 4- to 16-fold increase) upon primary immunization, and were dramatically boosted and maintained by booster immunizations with an increase of up to 7 $\log_2$ units (or 128 folds). For the individual haying hen (Figure 12A and B), the IgY titers in serum or egg yolk showed the similar trend as in Figure 11 and there was minor variation within each group. With regard to the IgY response directed against Ent, serum anti-Ent IgY did not increase upon primary immunization but was subsequently induced and maintained due to booster immunizations with an increase of up to 3 $\log_2$ units (or 8 folds) (Figure 13A). In egg yolk, anti-Ent IgY level did not increase upon immunization of layers with KLH-Ent. The anti-Ent IgY level did not differ between vaccination

and control groups either (Figure 13B). For the individual laying hen (Figure 14A and B), there were generally similar levels of Ent-specific IgY in serum or egg yolk of six layers, which also confirmed the fairly low level of Ent-specific IgY.

3.3.3 Immune responses induced by subcutaneous vaccination with KLH-Ent conjugate

In contrast to the very strong anti-Ent IgG response (up to 12 log₂ units or a 4,096-fold increase) in rabbit sera induced by subcutaneous vaccination of KLH-Ent conjugate (Figure 3E in Chapter 2), only weak anti-Ent IgY response was induced in the serum and yolk of layers by intramuscular vaccination of layers with the same KLH-Ent conjugate. So we speculated that the choice of immunization route may play an important role in inducing desired level of specific immune response to Ent. To test this, we subsequently immunized two Rhode Island Red layers with KLH-Ent conjugate via subcutaneous route.

Subcutaneous vaccination of Rhode Island Red layers with the KLH-Ent conjugate triggered strong immune response in serum and egg yolk. With respect to the IgY titer against the whole KLH-Ent conjugate, the titers of anti-KLH-Ent IgY in serum (Figure 15A and B) were drastically elicited by 7-8 log₂ units (or 128- to 256-fold increase) by 9 weeks postimmunization, and were slightly dropped afterwards. Similarly, the IgY titers against KLH-Ent conjugate in egg yolk (Figure 15C and D) were dramatically increased about 128 folds by 9 weeks postimmunization and maintained at comparably high level through the remaining period. With respect to the Ent-specific IgY titer, the titers of anti-Ent IgY in serum (Figure 16A and B) were drastically induced by 7-8 log₂ units (or 128- to 256-fold increase) upon primary immunization and two booster immunizations, and were maintained through the rest of period. The titers of anti-Ent IgY in yolk (Figure 16C and D), were significantly elevated by 6-8 log₂ units (or 64- to

256-fold increase) upon primary immunization and two booster immunizations, and were generally maintained through the rest of period.

3.3.4 Immune responses induced by subcutaneous vaccination with BSA-Ent or CmeC-Ent conjugate

Comparing the quite different levels of Ent-specific IgY induced by intramuscular or subcutaneous vaccination with the same KLH-Ent conjugate vaccine, we concluded that immunization route could be an important factor to affect the immune response of Ent conjugate vaccine. Then we further speculated that the carrier protein conjugated to Ent may be another factor affecting the immune response to Ent. To test this, we immunized the Rhode Island Red layers with BSA-Ent and CmeC-Ent conjugate vaccines (two layers for each vaccine) using the similar immunization regimen as the above.

Subcutaneous vaccination of Rhode Island Red layers with the BSA-Ent conjugate triggered significant immune response in serum and egg yolk. As to the IgY titer against the whole BSA-Ent conjugate, the titers of anti-BSA-Ent IgY in serum (Figure 17A and B) were elicited by 3-8 $\log_2$ units (or 8- to 256-fold increase) after primary immunization and two booster immunizations, and were generally maintained throughout the rest of period. The IgY titers against BSA-Ent conjugate in egg yolk (Figure 17C and D) were dramatically triggered by 6-8 $\log_2$ units (or 64- to 256-fold increase) after primary immunization and two booster immunizations, and were maintained through the remaining period, except for a drop of 3 $\log_2$ units in one layer at week 12 postimmunization. However, as to the Ent-specific IgY titer, there was generally minor increase of anti-Ent IgY in serum (Figure 18A and B) and egg yolk (Figure 18C and D) along the whole immunization process, despite an increase of 4 $\log_2$ units (or 16-fold increase) in one egg yolk sample at week 7 postimmunization.

Subcutaneous vaccination of Rhode Island Red layers with the CmeC-Ent conjugate elicited strong immune response in serum and egg yolk. As for the IgY titer against the whole CmeC-Ent conjugate, the titers of anti-CmeC-Ent IgY in serum (Figure 19A and B) were drastically elicited by 5-8 log₂ units (or 32- to 256-fold increase) after primary immunization and two booster immunizations, and were maintained throughout the remaining process. Similarly, the IgY titers against CmeC-Ent conjugate in egg yolk (Figure 19C and D) were significantly triggered by 5 log₂ units (or 32-fold increase) after primary immunization and two booster immunizations, and were slightly lowered through the remaining process regardless of the third booster immunization at week 10 postimmunization. As for the Ent-specific IgY titer, in general, the titers of anti-Ent IgY were dramatically induced (up to 10 log₂ units or 1,024-fold increase) in both serum (Figure 20A and B) and egg yolk (Figure 20C and D) upon primary immunization and two booster immunizations, but there were significant variations in Ent-specific IgY level between two layers, which primarily resulted from the comparatively much lower level of IgY titer in both serum and egg yolk of layer 2, except for a drastic increase of 10 log₂ units in its egg yolk at week 7 postimmunization.

3.3.5 The titer of Ent-specific IgY in lyophilized egg yolk powder

Since the Rhode Island Red layers immunized with KLH-Ent conjugate vaccine displayed the highest and stable IgY response against Ent throughout the entire period (Figure 16), their immunized egg yolk could be used for future *in vivo* passive immunization assays. Meanwhile, egg yolk from non-immunized Rhode Island Red layers was served as control. The pooled egg yolks from each group were lyophilized into powder. To confirm the activity of Ent-specific IgY was not affected by lyophilization process, KLH-Ent-immunized and control egg yolk powder was dissolved in PBS to the concentration of 0.1 g/ml, and was subsequently

subjected to indirect ELISA. The IgY titer in KLH-Ent-immunized egg yolk powder was much higher than control by 7 log₂ units (Figure 21). This titer difference was generally consistent with the differences of average IgY titers in egg yolk between preimmune and postimmune (Figure 16C), demonstrating the lyophilization process did not affect the activity of Ent-specific IgY in egg yolk powder.

3.4 Discussion

Siderophore-mediated iron acquisition has been a historically important research area in bacterial pathogenesis and has been targeted for developing various pathogen control strategies. However, those strategies have had only partial success primarily due to complexity of siderophore synthesis and redundancy of siderophore utilization systems in various Gram-negative pathogens. In Chapter 2 study using rabbit as a model, a novel Ent conjugate vaccine was successfully developed for inducing high level of Ent-specific antibodies, which displayed significant inhibitory effect on Ent-dependent growth of *Campylobacter* and *E. coli*. The findings from Chapter 2 study laid a solid foundation for producing hyperimmune Ent-specific egg yolk IgY as explored in this study.

The promising applications of egg yolk IgY as passive immune agent against bacterial pathogens have been well demonstrated. For example, oral administration of egg yolk IgY against *Salmonella enteritidis* was found to reduce fecal shedding and cecal colonization of *Salmonella* and even the rate of *Salmonella*-contaminated eggs in experimentally infected chickens (Gürtler et al., 2004). Feeding broilers IgY-rich yolks from hens immunized with *C. jejuni* dramatically reduced both *C. jejuni* colonization in the ceca after challenge with the homologous strain and transmission to non-inoculated contact birds (Hermans et al., 2014). Egg yolk IgY against *Helicobacter pylori* could reduce bacterial adhesion, growth, and urease activity

in vitro and decrease *H. pylori*-induced gastric mucosal injury and inflammation in Mongolian gerbil (Shin et al., 2002). Together, such encouraging evidence strongly entices us to utilize Ent conjugate vaccines to develop sufficient level of Ent-specific egg yolk IgY for passive immune protection against Gram-negative pathogens.

In this study, we performed immunization trials using different Ent conjugate vaccines and vaccination routes in order to optimize the production of sufficient level of Ent-specific IgY in egg yolk. For Rhode Island Red layers subcutaneously immunized with KLH-Ent, BSA-Ent, or CmeC-Ent conjugate, immunization with KLH-Ent conjugate induced the highest level of anti-Ent IgY in both serum and egg yolk. Immunization with CmeC-Ent conjugate also triggered high level of anti-Ent IgY in serum and egg yolk but with significant variation between two layers within the group. Immunization with BSA-Ent conjugate only elicited minor immune response directed against Ent, despite of strong IgY response against the whole BSA-Ent conjugate in serum and egg yolk. Such evidence indicates that different carrier proteins coupled to Ent can affect the level of Ent-specific IgY in serum and egg yolk and KLH is currently the most effective carrier protein that can help to induce sufficient levels of Ent-specific egg yolk IgY in layers. This may be due to multiple reasons. First, KLH is isolated from keyhole limpets that are evolutionarily distant from vertebrates, so it is very "foreign" for laying hens to effectively recognize the antigen and produce antibodies. It also has very large size (400 kDa) and complex structure, leading to high immunogenicity. Third, each KLH molecule contains hundreds of surface lysine groups covalently coupled to Ent, therefore each KLH can present more Ent haptens to host immune system. Last, KLH is also a naturally occurring immunoadjuvant that can prolong the T-cell dependent antibody response (Kantele et al., 2011;

Swaminathan et al., 2014). These features make KLH a highly immunogenic and effective carrier protein for inducing higher level of Ent-specific IgY in layers than BSA and CmeC.

Moreover, comparing the layers either subcutaneously or intramuscularly immunized with the same KLH-Ent conjugate, we found subcutaneous immunization dramatically elevated Ent-specific IgY in serum and egg yolk while intramuscular immunization triggered only moderate levels of Ent-specific IgY, in spite of significant levels of IgY against the whole conjugate, in serum and egg yolk. This implies that subcutaneous immunization can be a more efficient route than intramuscular immunization in eliciting high level of Ent-specific IgY in layers. We reason this phenomenon is probably due to that subcutaneous tissue is characterized as poor vascularity and a tendency to retain injected material (Chowdhury & Meyer, 2002), therefore subcutaneously administrated KLH-Ent conjugate may be more slowly exposed to host immune system and prolong the stimulation of Ent-specific immune response in laying hen.

Notably, for ELISA titers of preimmune or non-immune samples, comparatively high background titers (i.e., $\geq 12 \log_2$ units) were observed in serum or egg yolk samples from Trial 1 (Figure 11-14). We speculate that the laying hens may be colonized by Ent-producing *Enterobacteriaceae* prior to the beginning of immunization trial. To establish intestinal colonization before either a commensal or pathogenic interaction with the host, Ent-producing *Enterobacteriaceae*, like *Salmonella* or *E.coli*, will counteract iron-limited niches by secreting Ent to compete for iron from host or normal microbiota (Nagy et al., 2013; Pi et al., 2012). Therefore, the preexistence of Ent in chicken's intestine, as an external compound, will elicit certain level of Ent-specific IgY, leading to increased background titers in preimmune or non-immune serum or egg yolk. Despite of the high background titer, since there was no significant elevation (i.e., $\geq 2 \log_2$ units of increase) of Ent-specific IgY titer in postimmune serum or egg

yolk (Figure 13), we can still confirm there was only moderate effect of intramuscular immunization of KLH-Ent conjugate in inducing Ent-specific IgY in laying hens.

Since we have utilized Ent conjugate vaccine to successfully produce high level of Ent-specific IgY in serum and egg yolk of layers, it may pave the way for broad applications of large scale of Ent-specific egg yolk IgY against Gram-negative pathogens. In a recent work, intranasal administration of another Ent conjugate vaccine (CTB-Ent) elicited Ent-specific IgA in the intestinal mucosa of mice and reduced the colonization of *Salmonella* (Sassone-Corsi et al., 2016). Nevertheless, there was quite limited level of induced Ent-specific IgA, regardless of widely demonstrated adjuvant feature of CTB to induce strong mucosal immune response. From this perspective, oral intake of egg yolk containing high level of Ent-specific IgY can be advantageous since continuous administration of such hyperimmune egg yolk may provide constantly high level of Ent-specific antibodies, which therefore may better inhibit the enteric colonization of Gram-negative pathogens. Additionally, for immunodeficient humans or animals (e.g., neonates), they are more susceptible to enteritis or diarrhea caused by enteric Gram-negative pathogens, and normal vaccinations are insufficient to induce active immune protection against the corresponding pathogens. To this end, continuous administration of hyperimmune egg yolk containing high level of Ent specific IgY can present steady passive immune protection against the pathogens.

Since high titer of Ent-specific IgY in hen's serum can be transferred into the laid eggs, in poultry industry, hens can be immunized with Ent conjugate vaccines, conferring sufficient maternal Ent-specific IgY for newly hatched chicks against Gram-negative pathogens. This maternal immunization strategy overcomes some significant challenges in poultry immunization. Namely, underdeveloped immune function and certain suppressive maternal antibodies in chicks

within the age of 2 weeks result in ineffective vaccination; broilers reach slaughter weight by the age of 5-6 weeks, inadequate time period for induction of immunity (Hodgins et al., 2015).

To conclude, this study, through different immunization trials, has identified the optimal immunization regimen to produce high level and large quantity of Ent-specific egg yolk IgY, i.e., subcutaneous immunization of layers with KLH-Ent conjugate vaccine. At this stage, we have lyophilized egg yolk containing high level of Ent-specific IgY into powder, and confirmed the activity of Ent-specific IgY was maintained after the process. In the future study, we will utilize the hyperimmune egg yolk powder as feed additive, feed model animals with such additive, and test the passive immune protection of anti-Ent egg yolk against enteric bacterial pathogens (e.g., *Campylobacter*, *E. coli*, or *Salmonella*). These studies will significantly contribute to the development of hyperimmune egg yolk IgY as an effective alternative to antibiotics for prevention and control of various bacterial pathogens.

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Appendix II

Table 2. Immunization of Barred Rock layers with KLH-Ent conjugate (Trial 1).

Group	No. of Chickens	Immunogen	Immunization Route	Primary Immunization (week) ^a	Booster Immunization (week) ^b	Sample Collections (week)
1	3	None	N/A	26	28, 30, 32	Blood: 26, 28, 30, 32, 37 Eggs: along the whole trial
2	3	KLH-Ent (100 µg/bird)	Intramuscular			

a. Freund's complete adjuvant was used (1/1, v/v).

b. Freund's incomplete adjuvant was used (1/1, v/v).

Table 3. Immunization of Rhode Island Red layers with KLH-Ent, BSA-Ent, or CmeC-Ent conjugate (Trial 2).

Immunogen (100 µg/bird)	No. of Chickens	Immunization route	Primary Immunization (week) ^a	Booster Immunization (week) ^b	Sample Collections (week)
BSA-Ent	2	Subcutaneous	26	29, 32, 36	Blood: 33, 35, 38 Eggs: along the whole trial
KLH-Ent	2	Subcutaneous			
CmeC-Ent	2	Subcutaneous			

a. Freund's complete adjuvant was used (1/1, v/v).

b. Freund's incomplete adjuvant was used (1/1, v/v).

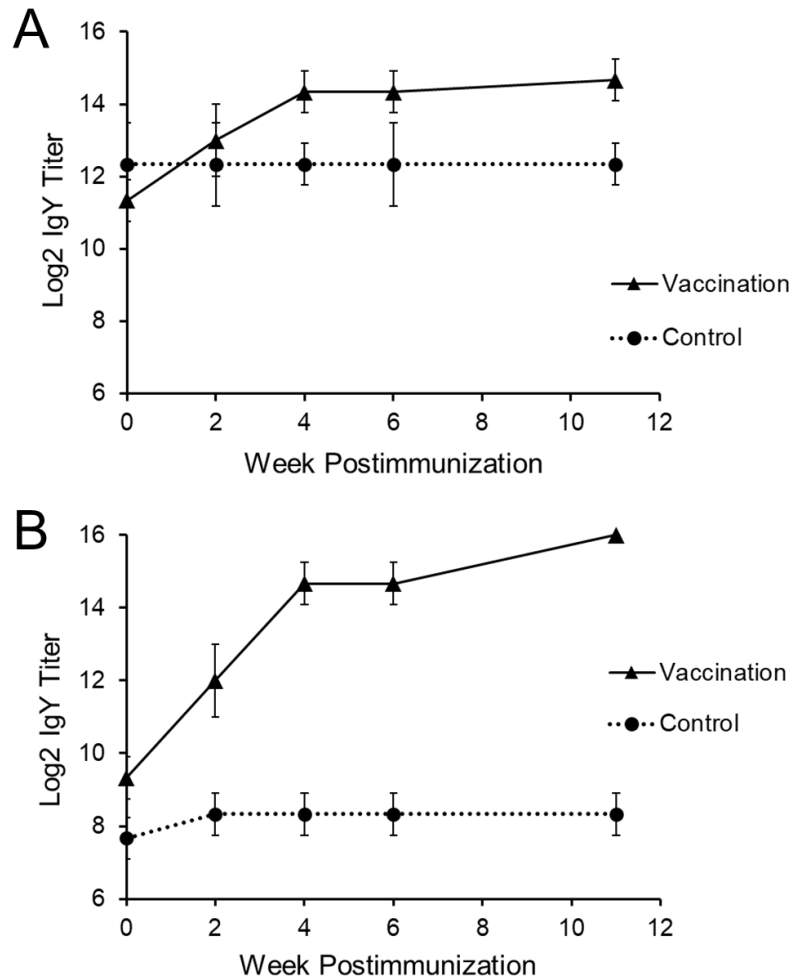


Figure 11. General immune response (anti-KLH-Ent IgY) in the Barred Rock layers in vaccination and control groups. (A) Serum IgY titers against the KLH-Ent conjugate. (B) Egg yolk IgY titers against the KLH-Ent conjugate. Each point represents the average IgY titer of three layers in vaccination or control group.

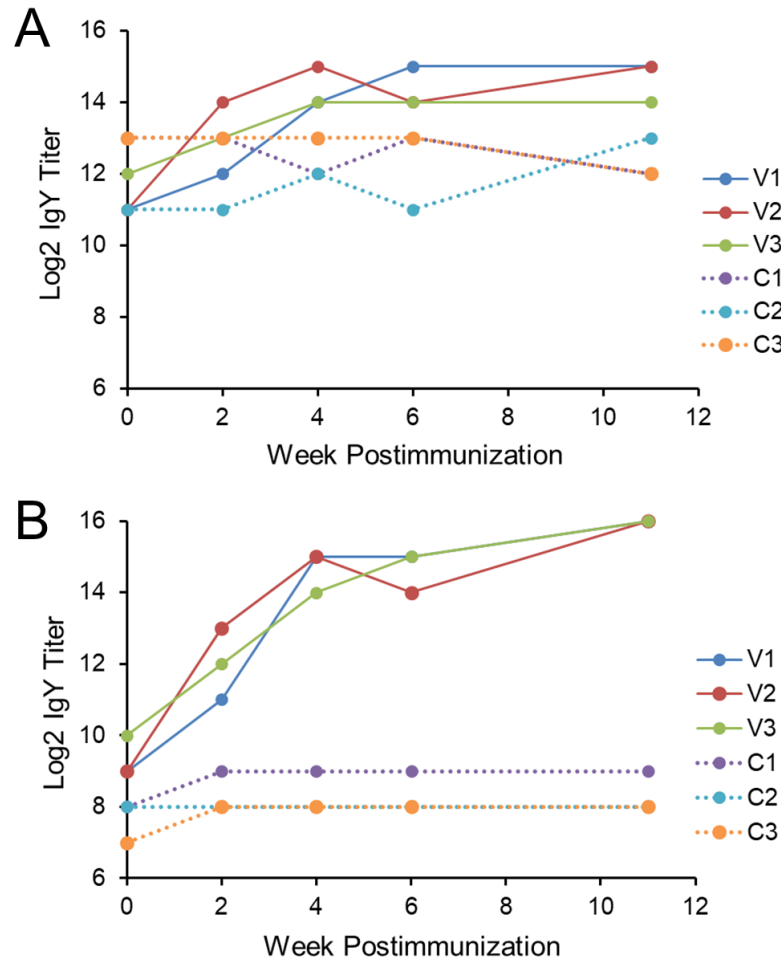


Figure 12. General immune response (anti-KLH-Ent IgY) in the individual Barred Rock layer in control group (C1-C3) and vaccination group (V1-V3). (A) Serum IgY titers against the KLH-Ent conjugate in each laying hen. (B) Egg yolk IgY titers against the KLH-Ent conjugate in each laying hen. Each point represents mean IgY titer from duplicate measurement.

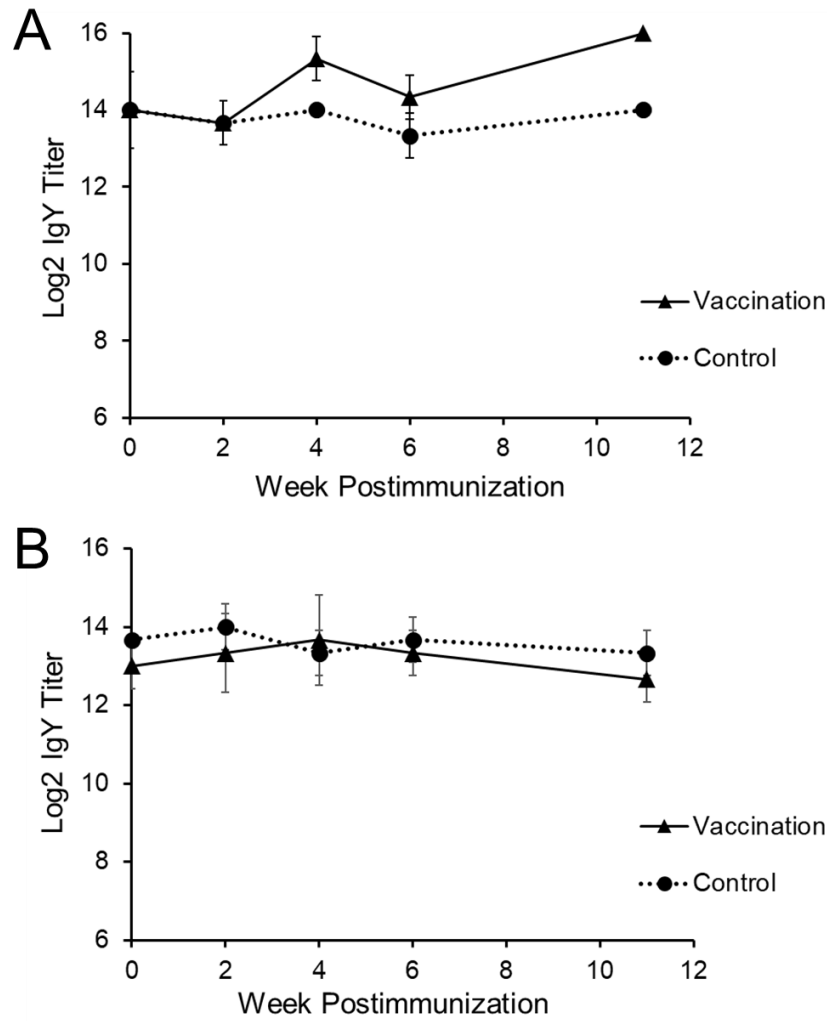


Figure 13. Ent-specific IgY immune response in the Barred Rock layers in vaccination and control groups. (A) Serum IgY titers directed against Ent. (B) Egg yolk IgY titers directed against Ent. Each point represents the average IgY titer of three layers in vaccination or control group.

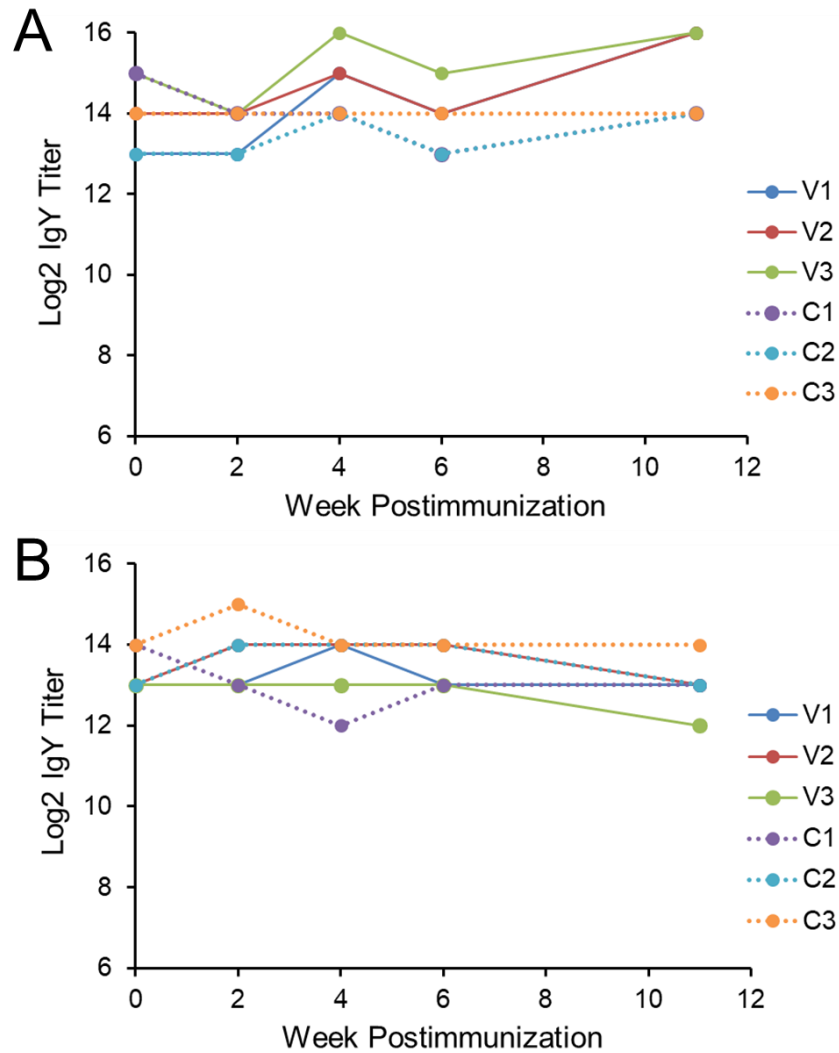


Figure 14. Ent-specific IgY immune response in the individual Barred Rock layer in control group (C1-C3) and vaccination group (V1-V3). (A) Serum IgY titers directed against Ent in each laying hen. (B) Egg yolk IgY titers directed against Ent in each laying hen. Each point represents mean IgY titer from duplicate measurement.

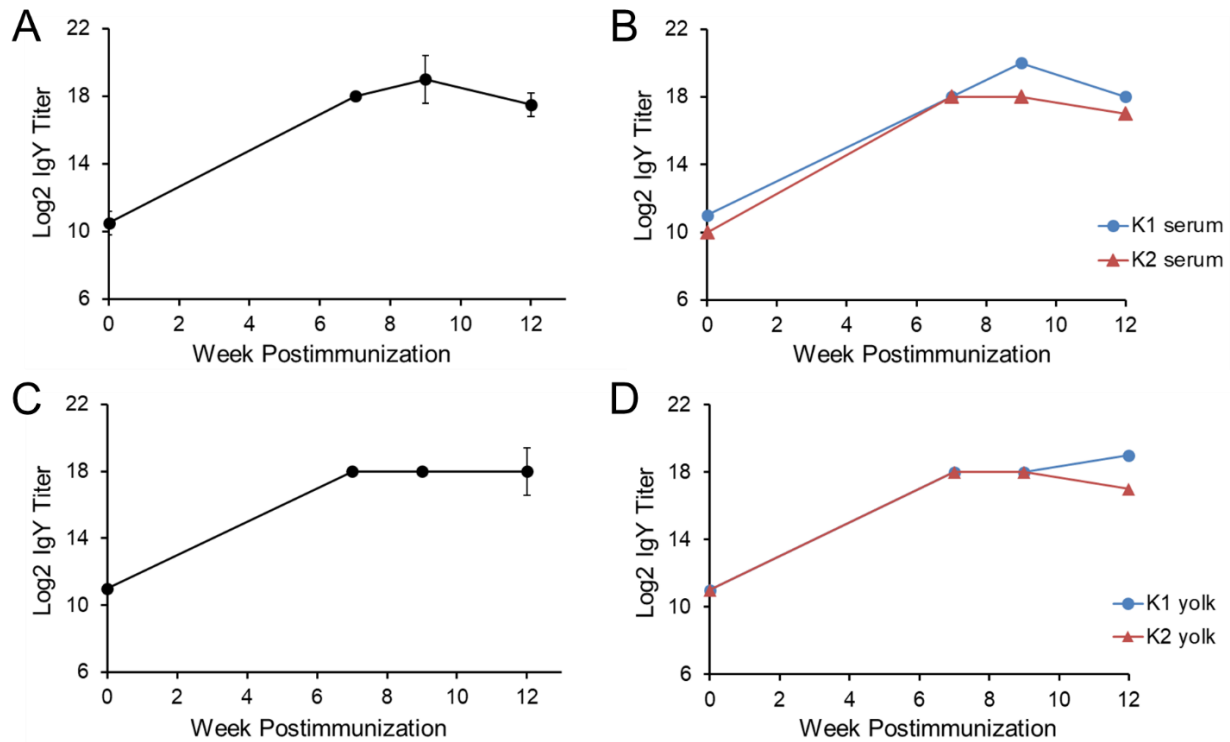


Figure 15. General immune response (anti-KLH-Ent IgY) in the two Rhode Island Red layers (K1, K2) immunized with KLH-Ent conjugate. (A) The average serum IgY titers against the KLH-Ent conjugate in two layers. (B) The individual serum IgY titers against the KLH-Ent conjugate in two layers. (C) The average egg yolk IgY titers against the KLH-Ent conjugate in two layers. (D) The individual egg yolk IgY titers against the KLH-Ent conjugate in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

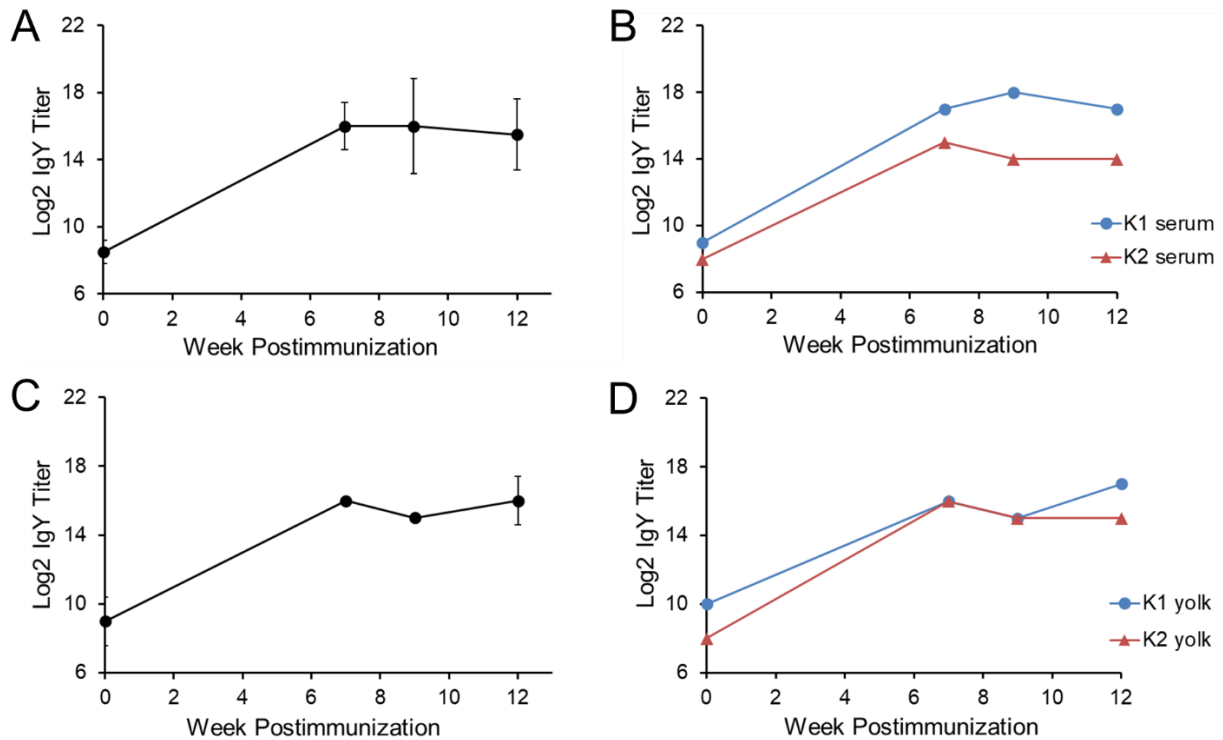


Figure 16. Ent-specific immune response (anti-Ent IgY) in the two Rhode Island Red layers (K1, K2) immunized with KLH-Ent conjugate. (A) The average serum IgY titers directed against Ent in two layers. (B) The individual serum IgY titers directed against Ent in two layers. (C) The average egg yolk IgY titers directed against Ent in two layers. (D) The individual egg yolk IgY titers directed against Ent in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

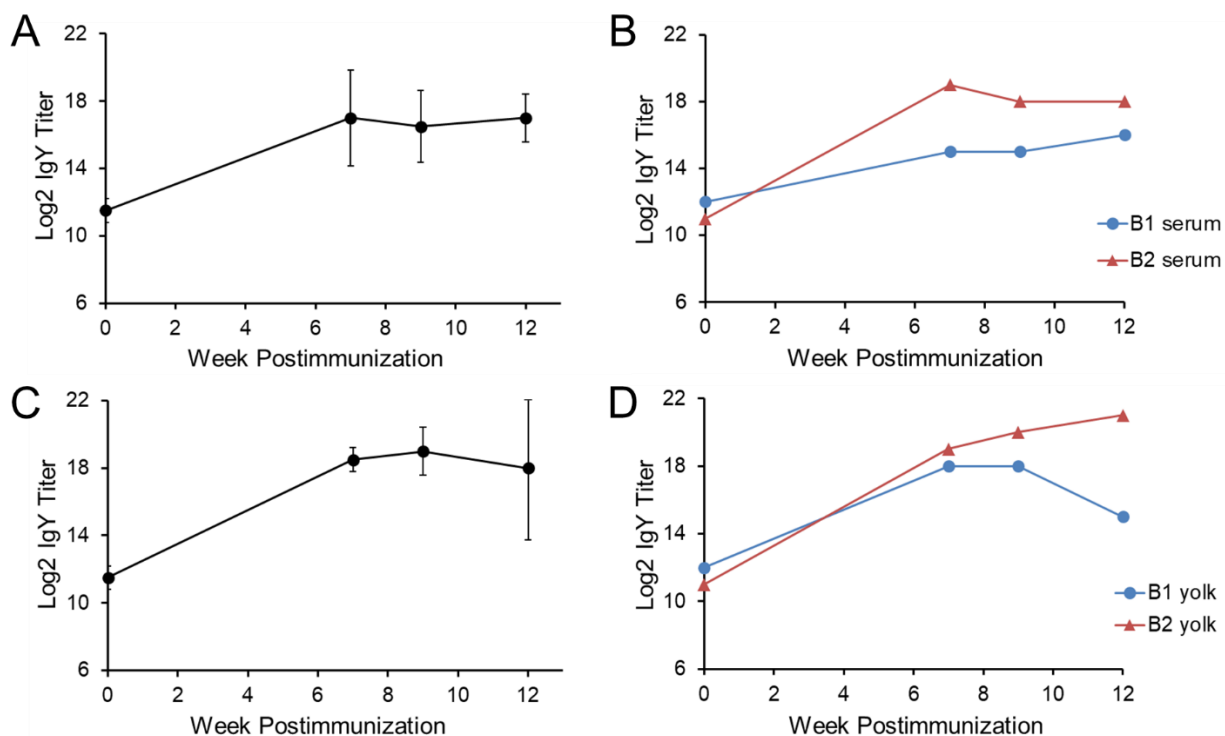


Figure 17. General immune response (anti-BSA-Ent IgY) in the two Rhode Island Red layers (B1, B2) immunized with BSA-Ent conjugate. (A) The average serum IgY titers against the BSA-Ent conjugate in two layers. (B) The individual serum IgY titers against the BSA-Ent conjugate in two layers. (C) The average egg yolk IgY titers against the BSA-Ent conjugate in two layers. (D) The individual egg yolk IgY titers against the BSA-Ent conjugate in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

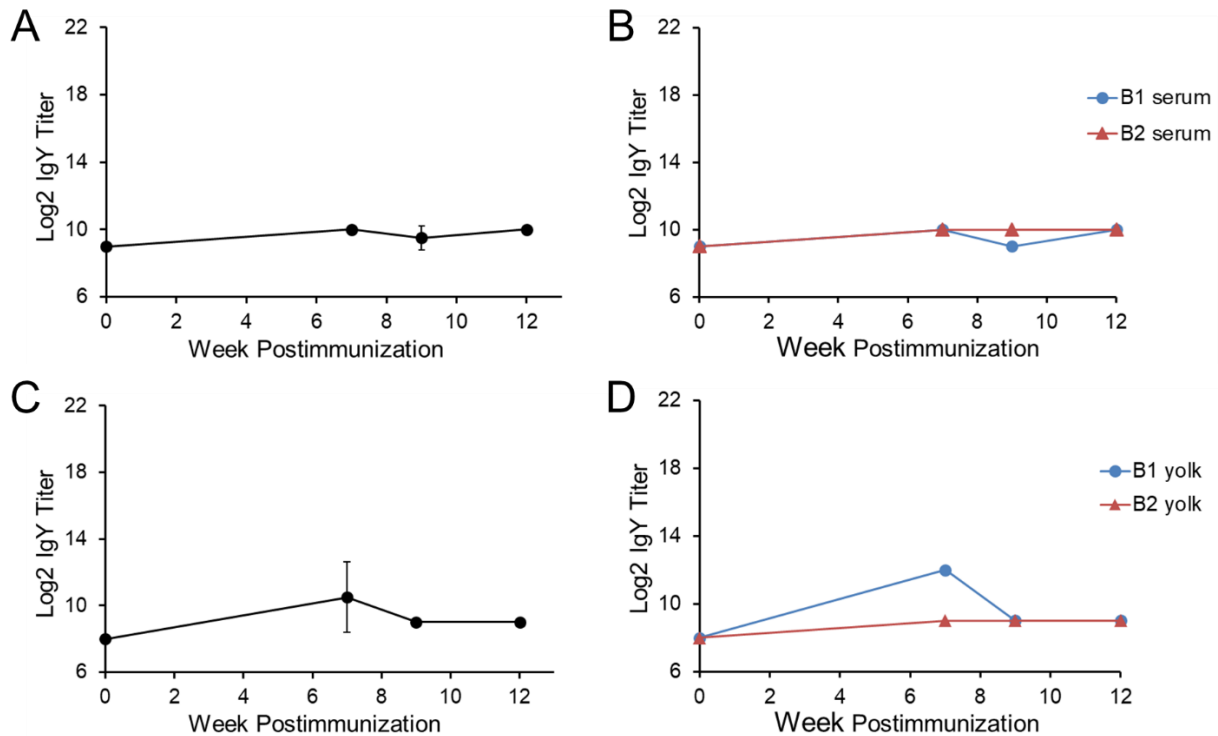


Figure 18. Ent-specific immune response (anti-Ent IgY) in the two Rhode Island Red layers (B1, B2) immunized with BSA-Ent conjugate. (A) The average serum IgY titers directed against Ent in two layers. (B) The individual serum IgY titers directed against Ent in two layers. (C) The average egg yolk IgY titers directed against Ent in two layers. (D) The individual egg yolk IgY titers directed against Ent in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

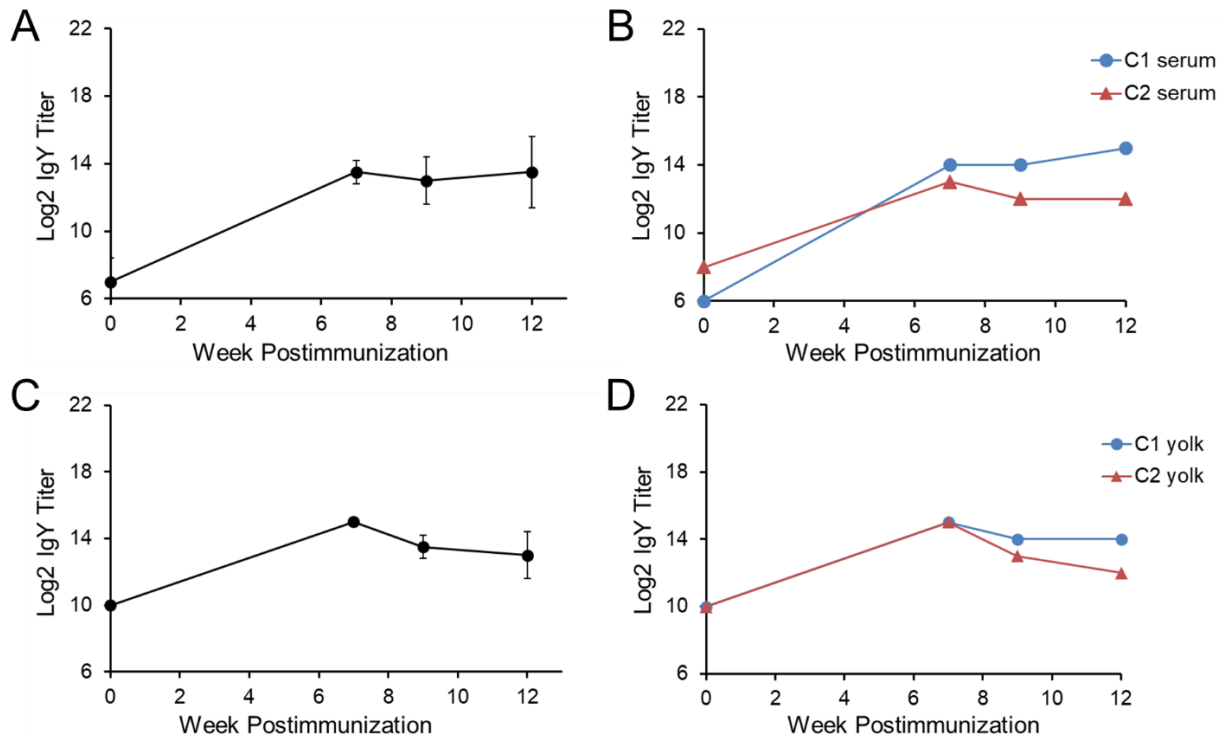


Figure 19. General immune response (anti-CmeC-Ent IgY) in the two Rhode Island Red layers (C1, C2) immunized with CmeC-Ent conjugate. (A) The average serum IgY titers against the CmeC-Ent conjugate in two layers. (B) The individual serum IgY titers against the CmeC-Ent conjugate in two layers. (C) The average egg yolk IgY titers against the CmeC-Ent conjugate in two layers. (D) The individual egg yolk IgY titers against the CmeC-Ent conjugate in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

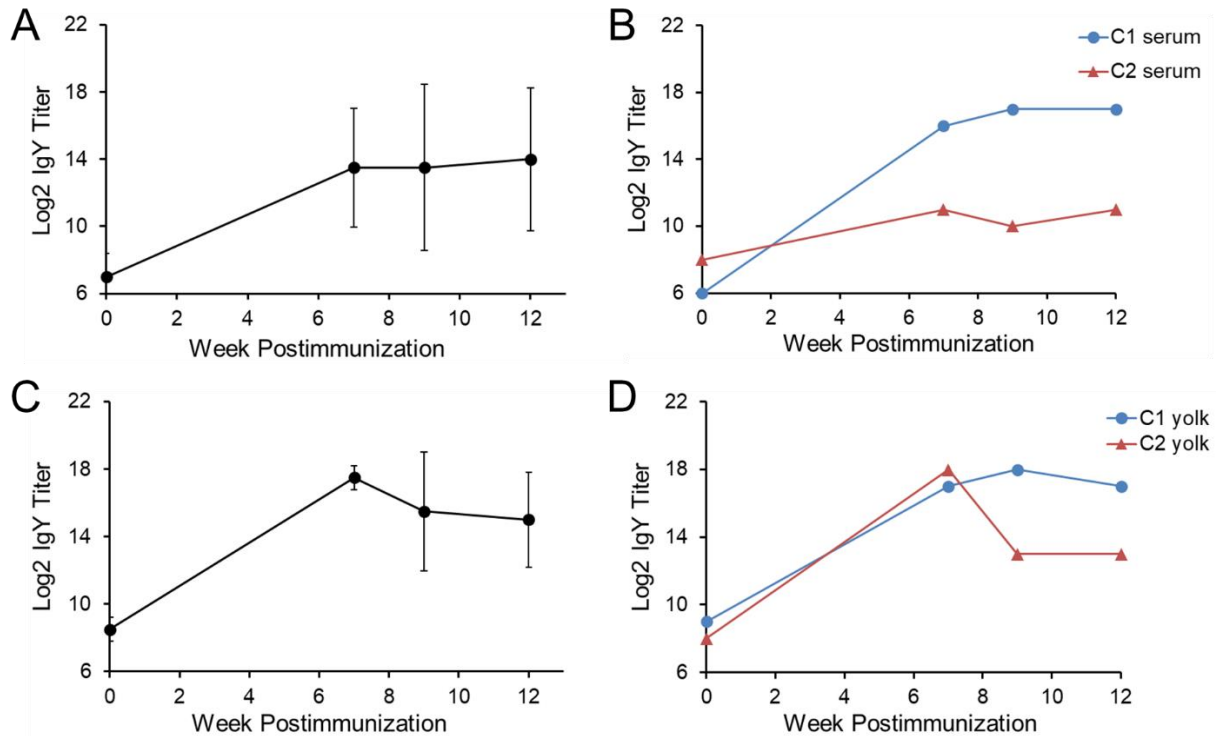


Figure 20. Ent-specific immune response (anti-Ent IgY) in the two Rhode Island Red layers (C1, C2) immunized with CmeC-Ent conjugate. (A) The average serum IgY titers directed against Ent in two layers. (B) The individual serum IgY titers directed against Ent in two layers. (C) The average egg yolk IgY titers directed against Ent in two layers. (D) The individual egg yolk IgY titers directed against Ent in two layers. In (A) and (C), each point represents the average IgY titer of two layers. In (B) and (D), each point represents mean IgY titer from duplicate measurement.

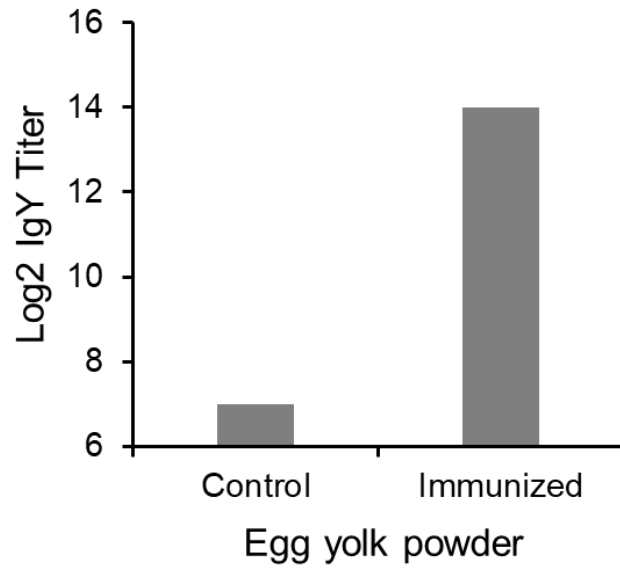


Figure 21. The titer of Ent-specific IgY in the lyophilized egg yolk powder. Immunized egg yolk powder was from Rhode Island Red layers vaccinated with KLH-Ent conjugate while control egg yolk powder was from non-vaccinated Rhode Island Red layers. Each bar represents the average of duplicate measurement of ELISA.

CHAPTER IV: Conclusion

Ent-mediated high affinity iron acquisition is critical for Gram-negative bacteria to survive in the host. Given the bacteriostatic effect of lipocalin resulting from its potent Ent-binding ability, immune intervention directly targeting Ent is promising for iron-dependent pathogen control. Recently, an Ent conjugate vaccine was reported but it still has several significant weaknesses. In the first study, using a simple biochemical method, we successfully conjugated purified Ent to different carriers including KLH, BSA, and CmeC, a vaccine candidate for *Campylobacter* control. Subcutaneous immunization of rabbits with the KLH-Ent conjugate triggered strong systemic IgG immune response with up to 16,384 fold increase in IgG titer directed against whole conjugate and up to 4,096 fold increase in the level of specific anti-Ent IgG. To evaluate the binding ability of Ent-specific IgG to the Ent derivatives present *in vivo*, various Ent derivatives were chemically synthesized and a unique ELISA method was developed. The Ent-specific IgG also displayed exceptional reactivity to ferric Ent, linear trimer of Ent, and different salmochelins. Growth assays further demonstrated the Ent-specific antibodies significantly inhibited Ent-dependent growth of *Campylobacter* spp. and *E. coli*.

In the second study, using different vaccination regimens, we immunized laying hens with different Ent conjugate vaccines to produce Ent-specific egg yolk IgY. In the first trial, Barred Rock layers (n=3) were intramuscularly immunized with the KLH-Ent conjugate vaccine, which triggered significant immune response in layers, leading to moderately increased titers of Ent-specific IgY in serum (up to 8 folds) and yolk (up to 2 folds). In the second trial, Rhode Island Red layers were subcutaneously immunized with specific Ent conjugates (KLH-Ent, BSA-Ent, or CmeC-Ent; n=2 per conjugate). ELISA analysis indicated that anti-Ent IgY titers dramatically increased in serum (up to 2,048 fold) and yolk (up to 1,024 fold) of each individual layer upon vaccination with KLH-Ent or CmeC-Ent. However, the BSA-Ent conjugate failed to

induce anti-Ent IgY. The hyperimmune egg yolks were pooled and lyophilized for future evaluation of *in vivo* passive immune protection.

Collectively, these studies have demonstrated the feasibility of developing Ent-specific antibodies as innovative and effective immune interventions and especially laid solid foundation for producing hyperimmune Ent-specific egg yolk IgY as a passive immune agent against Gram-negative bacterial pathogens.

VITA

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