Enterobactin-Specific Antibodies Induced by a Novel Enterobactin Conjugate Vaccine

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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. Recently, an Ent conjugate vaccine was reported, but it still has several significant weaknesses. In this study, we sought to develop an innovative Ent conjugate vaccine that can induce a 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 a strong systemic IgG immune response with an up to 16,384-fold increase in IgG titer directed against whole conjugate and an up to 4,096-fold increase in the level of specific anti-Ent IgG. To evaluate the ability of Ent-specific IgG to bind to the Ent derivatives present *in vivo*, various Ent derivatives were chemically synthesized and a unique enzyme-linked immunosorbent assay method was developed. The Ent-specific IgG also displayed exceptional reactivity to ferric Ent, a linear trimer of Ent, and different salmochelins. Growth assays further demonstrated that the Ent-specific antibodies significantly inhibited Ent-dependent growth of *Campylobacter* spp. and *Escherichia coli*. Collectively, this study reports an efficient method to prepare a new type of Ent conjugate vaccines for inducing a high level of Ent-specific antibodies, which can bind to various Ent derivatives and display lipocalin-like bacteriostatic features.

IMPORTANCE Ent-mediated high-affinity iron acquisition is a universal and critical contributor for Gram-negative pathogens to survive and infect hosts. Published information has supported an innovative immune intervention strategy that directly targets Ent to starve pathogens by limiting the availability of iron to be utilized. Compared to a recently published Ent conjugate, there are three advantages of the vaccine described in this study: ease of preparation, induction of high titer of anti-Ent IgG, and the ability of Ent-specific antibodies to bind various Ent derivatives, including the salmochelins that help enteric pathogens evade sequestration of siderophores by host lipocalins. In addition, the Ent-specific antibodies were demonstrated to function similarly to lipocalin to interfere with the Ent-dependent growth of *Campylobacter* and *E. coli* under iron-restricted conditions. This study has significant potential for broader applications to prevent and control various Gram-negative infections in humans and animals.

KEYWORDS conjugate vaccine, enterobactin, salmochelin, lipocalin

All Gram-negative bacteria have an absolute requirement for iron in order to survive and colonize the host. In animal hosts, however, iron from various sources is not normally available to invading bacteria; the concentrations of free iron *in vivo* are well

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below the levels required for the growth of Gram-negative bacteria (1). To obtain sufficient iron for survival and multiplication, Gram-negative bacteria have evolved complex and efficient genetic systems for iron uptake (1). 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 (1, 2). 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 (3). In particular, Ent is produced by most members of *Enterobacteriaceae*, the only representative family in the order *Enterobacterales* of the class *Gammaproteobacteria* (4). In addition, the hydrolyzed Ent derivatives (e.g., linear trimer) and the glycosylated Ent derivatives (named “salmochelins”) can also be produced and utilized for iron uptake by intestinal commensals and pathogens (e.g., *Escherichia coli* and *Salmonella enterica*) (4, 5). Therefore, Ent is likely to be used as a significant source of iron for Gram-negative pathogens during intestinal colonization (4). It has been shown that a significant quantity of catecholate siderophores, primarily Ent, was produced in the intestine, and Ent-mediated iron acquisition played a fundamental role in *E. coli* colonization in the gut (6).

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 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 (1, 7). However, these approaches have had only partial success, primarily due to the 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 affinities to Ent and its derived products (8–12). 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 the extracellular Ent siderophore has been inspired by a unique antibacterial function of lipocalins (13–17) and further supported by a recent study on the evaluation of an Ent conjugate vaccine (18). 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 (13–17). Specifically, lipocalins inhibit infections by *E. coli* and other Gram-negative bacteria 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 (13–17). 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 a 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 (19). In addition, specific high-affinity antibodies could be produced against a small molecule called “hapten” if hapten is conjugated with a carrier protein (20). Therefore, specific high-affinity antibodies directed against Ent, a hapten with a 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 that an Ent conjugate vaccine inhibited *Salmonella* colonization in mice (18). Nevertheless, in this recent study, the preparation of the Ent conjugate was lengthy and complicated and required

the addition of a linker to Ent for its conjugation with carrier protein (18). In addition, the Ent-specific immune response was weak upon immunization of mice with the Ent conjugate (i.e., a <4-fold increase in Ent-specific antibodies) (18), highlighting the need to develop new type of Ent conjugate that is capable of inducing high levels of Ent-specific antibodies. Finally, Sassone-Corsi et al. (18) did not provide microbiological and biochemical features of anti-Ent antibodies in terms of their capability to bind to catecholates and effectiveness for iron sequestration 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), bovine serum albumin (BSA), and CmeC, a promising subunit vaccine candidate for *Campylobacter* control (21). Immunization of rabbits with the KLH-Ent conjugate triggered a strong immune response, leading to an 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 ability to bind not only to both apo and ferric Ent (FeEnt) but also to other Ent derivatives, including salmochelins, indicating that 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 other words, the Ent antibodies have a significant advantage over lipocalins by cross-reacting with various Ent derivatives, including salmochelins. Finally, we demonstrated that the rabbit Ent antiserum displayed a significant inhibitory effect on the Ent-dependent growth of *Campylobacter* and *E. coli*.

RESULTS

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 (Fig. 1A). We also speculated that the surface-exposed linear DHBS trimer on the carrier protein might lead to effective antigen presentation upon immunization, consequently inducing a high level of antibodies directed against Ent. Regarding BSA that contains 59 lysine residues in each molecule, approximately 35 of these residues 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 (Fig. 1A).

The high-purity Ent was extracted from an Ent transport mutant of *E. coli* with a yield of about 100 mg of Ent/5-liter culture using a standard protocol (22, 23). The purity of Ent has been routinely validated by using high-performance liquid chromatography (HPLC) as described here and in our previous publication (24); no organic molecules were detected to be copurified along with Ent. We examined different parameters, such as Ent concentration, pH, temperature, and solvent, to optimize the 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 h). However, the labeling reaction worked well at 37°C, as indicated by the smear of bands with higher molecular mass (see Fig. S1A, lanes 2 and 3, in the supplemental material) than unlabeled BSA when less than 20% of methanol was used in the reaction mixture. To avoid potential cross-conjugation, we intended to ensure saturated conjugation specifically by taking the ratio of Lys residues of BSA and Ent into account; the lowest ratio we tested (63:1)

FIG 1 Ent conjugation with carrier proteins. (A) Schematic description of the conjugation of Ent to a carrier protein. (B) SDS-PAGE analysis of Ent conjugation with carrier protein KLH (left panel), BSA (middle panel), or CmeC (right panel).

worked well (Fig. S1A). Different organic solvents, which included methanol, dimethyl sulfoxide, 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 to 4 h 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 (Fig. 1B). Specifically, the 4-h conjugation of Ent with KLH led to a significant shift and smear compared to control KLH (Fig. 1B, left panel). A similar band shift was also observed for conjugation of Ent with BSA (Fig. 1B, middle panel). To examine the commonality of the simple conjugation method when using a different carrier protein, we chose CmeC, a subunit vaccine candidate that has 36 surface-exposed Lys residues (21, 25), as a carrier. As shown in Fig. 1B (right panel), a significant smear was also observed after a 4-h 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 antisera from rabbits immunized with the KLH-Ent conjugate. Both dot blot (Fig. 2A) and standard Western blot (Fig. 2B) analyses 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 (Fig. 2A). Due to significant sequence divergence between KLH and BSA, the antiserum raised by KLH-Ent did not cross-react with BSA (Fig. 2A). However, the antiserum reacted with BSA-Ent strongly, demonstrating the presence of Ent-specific antibodies in the antiserum raised by KLH-Ent conjugate (Fig. 2A). The finding from dot blot analyses was further confirmed by Western blotting of a comparable amount of carrier protein and its corresponding Ent conjugate. As shown in Fig. 2B, 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.

KLH-Ent conjugate vaccine elicited a significant immune response and a high level of Ent-specific IgG. Vaccination of rabbits with the KLH-Ent conjugate triggered a 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 enzyme-linked

FIG 2 The KLH-Ent conjugate vaccine elicited a 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 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 R1, R2, R3, and R4). Each point represents the average of duplicate measurements. (D) Serum IgG titer to the Ent molecule in each rabbit. Each point represents the average of duplicate measurements. (E) Effect of increased coating antigen BSA-Ent (2 μ g/well) on the measurement of the serum anti-Ent IgG titer. The sera in four rabbits (R1 to R4) collected at week 14 postimmunization and corresponding preimmune sera were serially diluted for ELISA analysis.

immunosorbent assay (ELISA) titer of serum IgG directed against the KLH-Ent conjugate in each rabbit was drastically elevated by 11 to 14 log₂ units (or a 2,048- to 16,384-fold increase) at week 7 postimmunization (Fig. 2C). The high-level of IgG titer was maintained throughout the remaining period by week 10 postimmunization, when the third boost immunization was performed (Fig. 2C). However, the third boost immunization did not further enhance the titer of IgG directed against KLH-Ent (Fig. 2C). The dynamic changes and patterns of the titer of IgG directed against KLH-Ent were similar among the four individual rabbits (designated R1, R2, R3, and R4; Fig. 2C).

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 a 2,048-fold increase); however, the dynamic change and pattern of anti-Ent IgG titer displayed great variation among the four individual rabbits (Fig. 2D). 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 a total 2,048-fold increase in the level of Ent-specific IgG (Fig. 2D). In the 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 the R2 rabbit (Fig. 2D). When we increased the concentration of the coated BSA-Ent antigen from 30 ng/well to 2 μ g/well for ELISA analysis, we observed a more consistent basal IgG titer in preimmune sera (7 log₂ units) and a larger increase in the titers of Ent-specific IgG in postimmune sera in the majority of the rabbits, i.e., 5-, 12-, 8-, and 5-log₂ unit increases for R1, R2, R3, and R4, respectively (Fig. 2E).

TABLE 1 Major strains used in this study

Strain	Description	Reference
JL122	<i>E. coli</i> AN102, Ent transport mutant for Ent purification	22
JL170	<i>C. coli</i> strain isolated from turkey	31
JL241	<i>C. jejuni</i> NCTC 11168, human isolate	52
JL633	<i>E. coli</i> BL21(DE3) recombinant strain producing C-terminal His-tagged IroE	49
JL636	<i>E. coli</i> BL21(DE3) recombinant strain producing C-terminal His-tagged IroB	26
JL999	<i>E. coli</i> K-12 MG1655	48
JL1002	<i>E. coli</i> Tuner, Δ entD derivative QT2908 producing His-tagged human lipocalin-2	48
JL243	<i>E. coli</i> JM109 recombinant strain producing N-terminal His-tagged CmeC	21

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

Both FeEnt and the linearized DHBS trimer were synthesized using a standard procedure, as described in our previous publication (24). To produce the three different salmochelins (mono-, di-, and triglucosyl Ent [denoted MGE, DGE, and TGE, respectively]), the high-purity IroB, a C-glycosyltransferase, was first purified from the *E. coli* JL636 strain (Table 1, Fig. 3A). Subsequently, the three salmochelins were *in vitro* synthesized using Ent and IroB enzyme as described previously (26), followed by HPLC purification. As expected, HPLC displayed three characteristic peaks, representing three different salmochelin products (Fig. 3B).

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 a chemiluminescent signal although the cyclic Ent was separated well with TLC, as observed in our previous study (24). Given this technical issue, we subsequently developed an indirect ELISA to evaluate the ability of Ent-specific IgG to bind to various Ent derivatives. Notably, the conventional coating

FIG 3 *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. The inset diagrams represent (from top to bottom) triglucosyl-Ent (TGE), diglucosyl-Ent (DGE), monoglucosyl-Ent (MGE), and Ent.

FIG 4 Specificity of binding of anti-Ent antibodies to different Ent derivatives. An ELISA plate was directly coated with Ent and its derivatives as detailed in Materials and Methods. Preimmune and postimmune (week 14) sera from rabbit 2 were diluted by 4,096-fold and served as the primary antibody in an indirect ELISA. Each bar represents the average of triplicate measurements. Negative control, no antigen coating; BSA-Ent, BSA-Ent conjugate; Ent, *apo* Ent; FeEnt, ferric Ent complex; Linear Ent, linear trimer of *N*-(2,3-dihydroxybenzoyl) serine [(DHBS)₃]; MGE, monoglucosyl-Ent; DGE, diglucosyl-Ent; TGE, triglucosyl-Ent; DHBA, 2,3-dihydroxybenzoic acid. Error bars represent the standard deviations from at least two independent experiments, with duplicate measurements performed in each independent experiment.

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 an ELISA plate. Therefore, based on an early publication by Bantroch et al. (27), we developed a unique coating method by using organic solvent methanol as coating solution to coat an ELISA plate with catecholate siderophores. As shown in Fig. S2, the ELISA plates coated with Ent (10 nmol/well) or the BSA-Ent conjugate (2 μ g/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 Fig. 4, compared to preimmune serum, the rabbit Ent antiserum clearly displayed ability to bind to Ent and various derivatives even if the serum was diluted by 4,096-fold. The rabbit antiserum showed comparable binding to *apo* Ent, linear Ent trimer, and salmochelins MGE and DGE (Fig. 4). However, regarding TGE, the Ent modified with three glycosyls (Fig. S3), 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 to the FeEnt complex ($P = 0.053$) compared to the binding to the original hapten Ent (Fig. 4). With respect to DHBA, a small siderophore considered an Ent precursor (Fig. S3) (28), the anti-Ent antibodies failed to detect it even if the coating concentration of DHBA was increased by 3-fold accordingly due to the monomer unit nature of DHBA, suggesting low binding of the anti-Ent antibodies to DHBA.

Ent-specific antibodies inhibited Ent-mediated growth promotion in *Campylobacter*. We speculated that Ent-specific antibodies could function as a 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 (4). Thus, using a standard, semiquantitative Ent growth promotion assay and a purified human lipocalin-2 (Fig. S4A) 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 the FeEnt acquisition study in *Campylobacter* (24, 29–31).

As shown in Fig. S4B, the Ent antiserum displayed a significantly inhibitory effect on Ent-dependent growth of *C. coli* JL170; the diameter of the growth zone for anti-Ent rabbit

FIG 5 Inhibitory effect of the rabbit Ent antiserum on the growth of *E. coli* under iron-restricted conditions. *E. coli* MG1655 cells were grown in RPMI medium supplemented with rabbit control serum (4-fold dilution), anti-Ent serum (4-fold dilution), or control serum (4-fold dilution) plus lipocalin-2 (final concentration, 38 μ g/ml). Each data point represents the mean value obtained from duplicate wells in the microtiter plate growth assay.

serum (3.32 ± 0.10 cm) is significantly smaller than those for control serum (3.85 ± 0.10 cm, $P = 0.02$) and the phosphate-buffered saline (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 (Fig. S4B). However, for *C. jejuni* NCTC 11168 (JL241), the zone size is not different between control serum and Ent antiserum (Fig. S4C). Because the same concentration of human lipocalin-2 did not exert an inhibitory effect on the growth of NCTC 11168 either (Fig. S4C), we speculated that *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 an inhibitory effect on FeEnt-mediated iron acquisition in *C. jejuni* NCTC 11168. To test this, we modified the growth promotion assay by increasing the volume of control serum or Ent antiserum in soft agar, which showed that the Ent antiserum significantly inhibited the growth of *C. jejuni* NCTC 11168 compared to control serum ($P = 0.03$) and PBS ($P = 0.02$) (Fig. S4D).

Ent-specific antibodies inhibited Ent-dependent growth of *E. coli*. *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, *E. coli* MG1655 displayed normal growth by reaching approximately 10 log units by 18 h (Fig. 5). In contrast, *E. coli* MG1655 barely grew in the presence of Ent antiserum; by 18 and 24 h, the concentration of *E. coli* MG1655 was less than the original level at 0 h (Fig. 5). As expected, the lipocalin-2 also showed a potent and similar bacteriostatic effect on the growth of the *E. coli* strain as Ent antiserum (Fig. 5).

DISCUSSION

Sassone-Corsi et al. (18) recently reported an Ent-based conjugate vaccine with the same concept but using a 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 a mucosal immune response. In addition, with the aid of a polyethylene glycol (PEG₃) linker attached to Ent, which resulted from a stepwise chemical synthesis and modifications, Ent was covalently attached to the surface-exposed Lys residues of CTB, leading to the CTB-Ent conjugate that harbors the native Ent scaffold (18). Immunization of mice via the intranasal route with the CTB-Ent conjugate was observed to generate mucosal IgA directed against Ent; however, the specific immune response was weak because the anti-Ent IgA level was only slightly increased (<4-fold) upon CTB-Ent vaccination (18). In addition, Ent-specific antibodies (e.g., anti-Ent IgG) were not detected in the system (blood and spleen) upon immuni-

zation of mice with the CTB-Ent. Thus, a new type of conjugative Ent vaccine with a simple synthesis procedure and the capability to induce a 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. (18) did not provide any evidence demonstrating that the induced anti-Ent antibodies could function similarly to lipocalin to interfere with Ent-dependent growth promotion in enteric bacteria under iron-restricted conditions, critically important evidence required for validating the concept of the Ent conjugate vaccine. Here, we report a new type of Ent conjugate (Fig. 1), synthesized by direct incubation of carrier protein with Ent for 2 to 4 h, which can induce a strong systemic immune response and which increases the specific anti-Ent IgG level by up to 4,096-fold (Fig. 2). We also developed a unique coating method for ELISA and provided direct evidence that anti-Ent IgG has the ability to strongly bind to various Ent derivatives (Fig. 4). We further demonstrated that rabbit Ent antiserum did exert a significant inhibitory effect on the Ent-dependent growth of two representative Gram-negative organisms under iron-restricted conditions (Fig. 5 and Fig. S4).

Sassone-Corsi et al. (18) 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 compared to *apo* Ent (Fig. S3). This, together with the modification of Ent with a PEG₃ linker, likely interferes with proper antigen presentation to stimulate and select desired antibodies targeting the Ent epitope, which may explain the weak Ent-specific immune response observed upon CTB-Ent vaccination (18) even if the potent ability of CTB to induce a strong immune response as an adjuvant has been widely demonstrated. In contrast, the Ent conjugate developed in this study is based on a direct chemical reaction between surface-exposed Lys of the carrier protein and Ent (Fig. 1); the Ent linked to the carrier is expected to be in the form of linear trimer (DHBS)₃ (Fig. 1A and Fig. S3). Based on this 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 a broad spectrum to bind to various Ent derivatives, which has been tested and demonstrated in this study. Based on the straightforward principle of Ent conjugation adopted in this study (Fig. 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 (Fig. 1B and 2B).

The findings from this work also strongly support our hypothesis that 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 the ability to bind 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 ability to bind to the FeEnt complex compared to the binding to *apo* Ent (Fig. 4), a noteworthy feature resulting from our conjugation method (Fig. 1). Recently, the molecular structure of FeEnt was revealed by racemic crystallography (32), 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 anti-Ent IgG to TGE (Fig. 4).

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, a 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 concentrations 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 that the Ent-specific IgG induced by the new vaccine

displays a more potent bacteriostatic effect than lipocalin-2. This statement is based on the following conservative calculation. It is a well-known fact that specific IgG directed against desired protective epitopes of a bacterial vaccine accounts for only a small proportion of the total serum antibodies. For example, the concentration of the IgG directed against a highly immunogenic tetanus toxoid vaccine was 20 $\mu\text{g/ml}$ in human serum (33). Given that total IgG in human serum was about 11 mg/ml (34), approximately only 0.2% of the total serum IgG is the specific IgG directed against a highly immunogenic antigen (or against all epitopes of the immunogen). Assuming this is also the case for rabbits, 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 $\mu\text{g/ml}$. If we simply ignore the anti-KLH IgG and assume 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 the hypothesis that the Ent-specific antibodies induced by the Ent conjugate vaccine would function similarly to lipocalins to limit Gram-negative infections in an 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 the vaccination regimen is also highly warranted to further increase the titers 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 an Ent conjugate vaccine on host microbiome and physiology when performing animal studies. From the standpoint of short-term therapeutic treatment against pathogens, concern for the potential detrimental effect of anti-Ent antibodies on microbiota should be mitigated due to the wide acceptance of broad-spectrum clinical antibiotics. For this purpose, the 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 (35). 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 >22,500 mg, with up to 10% being antigen-specific antibodies (36). We speculate that immunization of laying hens with the Ent conjugate vaccine developed in this study can generate high titers 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 the standpoint of long-term protection of animals against Gram-negative infections, the anti-Ent antibodies induced by the Ent conjugate vaccine may reduce the relative abundance of Ent-producing bacteria. However, the potential detrimental impact resulting from anti-Ent antibodies appears to be 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 a recent study by Sassone-Corsi et al. (18), vaccination of mice with the Ent-CTB conjugate vaccine did not significantly affect the composition of microbiota in the intestine. Following a *Salmonella* challenge, the *Enterobacteriaceae* population dramatically increased in the guts of control mice, while *Lactobacillus* spp. rather than *Enterobacteriaceae* increased significantly in the intestines of the mice immunized with Ent-CTB conjugate vaccine (18). Finally, based on recent extensive microbiome research, the Ent-based vaccine is likely even beneficial to the host by limiting the

overpopulation of Ent-producing bacteria. Compelling evidence showed that the increased densities of various gammaproteobacterial species in the gut (e.g., *Enterobacteriaceae*) have detrimental effects on gut health (e.g., increased inflammation) (37–42). Notably, gammaproteobacterial species are the major bacterial populations in the gut to produce Ent and relevant derivatives for effective iron acquisition and survival *in vivo* (1, 3, 43). Therefore, the Ent conjugate vaccine may be beneficial for the host by controlling overpopulation of gammaproteobacterial 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 catecholate-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 (4). 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 liquid chromatography-mass spectrometry techniques have shown that a significant quantity of catecholates is produced in the intestine or air sacs (6, 44). 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 the host. Based on a recent report by Qi and Ban (45), 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. An early diagnosis of gut health has an enormous impact on the timely and effective control of many enteric diseases in animals and humans. As discussed above, the increased density of gammaproteobacterial species in the gut, the primary catecholate-producing population, has been observed to exert detrimental effects on gut health (37–42). Therefore, diverse gammaproteobacterial species may serve as a promising biomarker for gut health in an 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 gammaproteobacterial 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 gammaproteobacterial species and serve as an early diagnosis tool to evaluate gut health in animals and humans.

In the context of holistic bacterium-host interaction, we should consider the potential effect of an anti-Ent antibody on a eukaryotic host. Recently, using a *Caenorhabditis elegans* model and an *in vitro* human embryonic cell line, Qi and Ban (45) reported that Ent is beneficial for host iron uptake and development through the mitochondrial ATP synthase. However, at this stage, the physiological relevance of this interesting finding in higher animals is still largely unknown. We cannot simply translate this *C. elegans* discovery to higher animals, such as mammals and other vertebrates. In particular, given the complexity of the 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 the intestinal cell wall and interact with other somatic cells in *C. elegans*. However, in higher animals, to date, there is no evidence indicating that Ent can penetrate a complex intestinal wall and subsequently reach different organs and tissues. Due to the sterile nature of the environment of embryos, mammalian embryo development and differentiation should not be affected by the bacterium-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 the intestinal tract (6) and air sacs (44). However, some studies have shown that Ent was not detected in the tissues of infected lambs (46) and in blood and livers of infected chickens (44). Therefore, it is still premature to speculate on the potential detrimental effect of anti-Ent antibody on iron

homeostasis and development of higher animals. To ensure the finding reported by Qi and Ban (45) is not an artificial scenario for higher animals, the effect of Ent on eukaryotic host iron homeostasis and development must be studied in well-controlled systems using an appropriate animal model.

MATERIALS AND METHODS

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 Trypticase soy broth (TSB) (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).

Purification of Ent. An Ent transport mutant of *E. coli* AN102 (JL122, Table 1) was generously provided by Sandra K. Armstrong (University of Minnesota) and used for Ent purification. The purification procedure was described previously (22, 23). The purified Ent was dissolved in methanol or DMF and stored at −20°C until it was used.

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 (21). A standard protocol was developed by examining various conjugation parameters, such as concentration, temperature, organic solvents, and reaction time. Briefly, 45 µl of BSA, KLH, or CmeC (1.0 µg/ml) was dissolved in 360 µl of 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 h, followed by buffer exchange using Amicon Ultra-4 centrifugal filter units (MilliporeSigma, St. Louis, MO) with double-distilled water (ddH₂O) three times and PBS (pH 7.4) six times. Each batch of Ent conjugates was 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.

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 the 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 complete adjuvant, followed by three booster immunizations (100 µg of KLH-Ent conjugate emulsified with Freund incomplete adjuvant) every 3 to 4 weeks. Blood samples were collected at different time points. Rabbits were euthanized at weeks 14 and 18 for the first and second trials, respectively, after the primary immunization.

Immunoblotting. To validate production of rabbit polyclonal anti-Ent IgG, Western blotting and dot blotting were performed as described previously (30, 47). For Western blotting, protein samples were first separated by SDS-PAGE with a 12% (wt/vol) polyacrylamide gel and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 90 V for 1 h. 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% Tween 20 and 5% skim milk) at room temperature (22°C) for 1 h prior to 1-h 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 1:5,000 in blocking buffer) for 1 h at room temperature. After incubation, the membranes were washed four times with PBS containing 0.05% Tween 20. The membranes were then developed with a 4CN membrane peroxidase substrate system (KPL, Gaithersburg, MD).

Purification of His-tagged recombinant proteins. The His-tagged recombinant IroE, IroB, human lipocalin-2, and CmeC were purified from strains JL633, JL636, JL1002, and JL243 (Table 1), respectively, as detailed in previous publications (26, 48, 49). Notably, the recombinant human lipocalin-2 was produced in an Ent-deficient strain ($\Delta entD$) to 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 a bicinchoninic acid 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-µm-pore-size filter), and stored at −20°C prior to use.

Synthesis and purification of Ent derivatives. The ferric Ent complex was prepared by mixing Ent with a 1.2 equivalent of FeCl₃ as described by Zeng et al. (24). Linearized DHBS trimer was produced by hydrolysis of Ent by recombinant IroE, followed by HPLC purification as described in our previous publication (24). Salmochelins (MGE, DGE, and TGE) were synthesized by Ent glucosylation and purified using HPLC as described in our previous work (26, 49), with slight modifications. Briefly, 500 µl of the reaction mixture contained 75 mM Tris buffer (pH 7.5), 5 mM MgCl₂, 2.5 mM TCEP-HCl, 3 mM UPD-Glc, 500 µM Ent, and 1 µM IroB. At different time points, the reaction was stopped by adding 0.1% trifluoroacetic acid (TFA) and analyzed by HPLC using a gradient of 0 to 43.75% CH₃CN in 0.1% TFA-water over 10 min. Fractions of MGE, DGE, and TGE were collected, lyophilized, and stored at −20°C prior to use.

TLC-immunostaining. TLC of Ent was performed as described in our previous publication (24). In brief, 1 µl of the Ent solution (10 mM, dissolved in methanol) was spotted into a TLC plate (Whatman PE silica gel with UV) and developed with benzene-acetic acid-water (110:85:5 [vol/vol/vol]). The immuno-

staining was performed using the protocol described by Duvar et al. (50). The silica gel was fixed with 0.5% polyisobutylmethacrylate (Polysciences, Inc., Warrington, PA), which was dissolved in chloroform-*n*-hexane (1:9), and then dried in a chemical fume hood. The silica gel was incubated in blocking solution (PBS containing 5% skim milk) for 1 h at room temperature, followed by incubation with anti-Ent antibodies (a 1:100 dilution of rabbit polyclonal Ent antiserum in blocking solution) with gentle shaking for 1 h. 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], a 1:2,000 dilution in blocking solution) for 1 h 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).

ELISA. Indirect ELISAs were conducted as described previously, with modifications (21), to examine the IgG response in rabbit sera against KLH-Ent (to measure the general antibody response) and 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 the 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 used to coat microtiter plates using the coating method described above.

To further validate the titer of anti-Ent IgG and in particular to evaluate the ability of the Ent-specific IgG to bind to different Ent derivatives, Ent or its derivatives were used to directly coat ELISA plates using a unique coating method based on an early publication (27), with modifications. In brief, Ent and its derivatives were diluted in methanol to a final concentration of 0.5 mM and added to an ELISA plate (20 μ l/well). Subsequently, the ELISA plates were placed on a hot plate (set at 60°C) in a chemical fume hood for 30 min for complete evaporation of methanol due to mild heat and airflow. Upon completion of the specific coating procedure, as described above, the dried ELISA plates with directly coated small compounds were subjected to standard ELISA, as described in a previous publication (47), with slight modifications. The 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 1:1,000 in blocking buffer, and 100 μ l was added to each well. After 1 h of incubation, the plates were washed three times. The plates were then developed using an ABTS [2,2'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate kit (KPL), and the reaction was stopped after 30 min using 100 μ l of stop solution (1 $\times$ PBS, 1% SDS). The absorbance was measured at an 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 the addition of primary antibody served as a background control. The endpoint titer was defined as the last dilution at which the OD₄₀₅ of sample wells exceeded the cutoff value (mean OD₄₀₅ plus 3 $\times$ the standard deviation of the preimmune sera at a 512-fold dilution). The titer was expressed as the reciprocal of the endpoint dilution log₂. Duplicate or triplicate independent measurements were performed for each serum sample.

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 a previous publication (30). *C. jejuni* JL241 and *C. coli* J1170 (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- μ m-pore-size filter). The sterile rabbit sera were stored at 4°C. Briefly, *C. jejuni* log-phase cells grown in MH broth ($\sim 1 \times 10^7$ cells) were mixed with melted MH agar (0.6% agar) supplemented with chelator desferoxamine (DFO; final concentration, 20 μ M), as well as PBS, control serum (final 5.6-fold dilution), Ent antiserum (final 5.6-fold dilution), or human lipocalin-2 (final concentration, 24 μ g/ml). The mixture was immediately poured into small petri dishes (6 cm in diameter) for solidification. A sterile paper disc (6 mm in diameter) containing 10 μ l 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 h of incubation at 42°C under microaerophilic conditions. The experiment was performed in triplicate for each treatment.

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 (48, 51) with slight modifications. Briefly, *E. coli* MG1655 was inoculated into TSB supplemented with 0.6 mM 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 of 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 portions of the above bacterial inoculum cells were added to each well. The microplate was incubated at 37°C for up to 24 h. At different time points (8, 18, and 24 h postinoculation, 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 the log₁₀ CFU/ml. Each treatment group was performed in duplicate.

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

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00358-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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