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The Three Lipocalins of Egg-White: Only Ex-FABP Inhibits Siderophore-Dependent Iron Sequestration by *Salmonella* Enteritidis

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Salmonella Enteritidis is the most prevalent food-borne pathogen associated with egg-related outbreaks in the European Union. During egg colonization, *S. Enteritidis* must resist the powerful anti-bacterial activities of egg white (EW) and overcome ovotransferrin-imposed iron-restriction (the most important anti-bacterial mechanism of EW). Many pathogens respond to iron restriction by secreting iron-chelating chemicals called siderophores but EW contains a siderophore-sequestering “lipocalin” protein (Ex-FABP) that is predicted to limit the usefulness of siderophores in EW. *S. Enteritidis* produces two siderophores: enterobactin, which is strongly bound by Ex-FABP; and the di-glucosylated enterobactin-derivative, salmochelin (a so-called “stealth” siderophore), which is not recognized by Ex-FABP. Thus, production of salmochelin may allow *S. Enteritidis* to escape Ex-FABP-mediated growth inhibition under iron restriction although it is unclear whether its EW concentration is sufficient to inhibit pathogens. Further, two other lipocalins (Cal- γ and α -1-ovoglycoprotein) are found in EW but their siderophore sequestration potential remains unexplored. In addition, the effect of EW lipocalins on the major EW pathogen, *S. Enteritidis*, has yet to be reported. We overexpressed and purified the three lipocalins of EW and investigated their ability to interact with the siderophores of *S. Enteritidis*, as well as their EW concentrations. The results show that Ex-FABP is present in EW at concentrations (5.1 μ M) sufficient to inhibit growth of a salmochelin-deficient *S. Enteritidis* mutant under iron restriction but has little impact on the salmochelin-producing wildtype. Neither Cal- γ nor α -1-ovoglycoprotein bind salmochelin or enterobactin, nor do they inhibit iron-restricted growth of *S. Enteritidis*. However, both are present in EW at significant concentrations (5.6 and 233 μ M, respectively) indicating that α -1-ovoglycoprotein is the 4th most abundant protein in EW, with Cal- γ and Ex-FABP at 11th and 12th most abundant. Further, we confirm the preference (16-fold) of Ex-FABP for the ferrated form (K_d of 5.3 nM) of enterobactin over

the iron-free form (K_D of 86.2 nM), and its lack of affinity for salmochelin. In conclusion, our findings show that salmochelin production by *S. Enteritidis* enables this key egg-associated pathogen to overcome the enterobactin-sequestration activity of Ex-FABP when this lipocalin is provided at levels found in EW.

Keywords: salmochelin, enterobactin, Ex-FABP, Cal- γ , α -1-ovoglycoprotein, egg white, *Salmonella* Enteritidis

INTRODUCTION

From 2010 to 2013, the number of confirmed human salmonellosis cases in the European Union (EU) decreased by \sim 15% (Efsa Panel on Biological Hazards, 2019) but increased again after 2014 (Efsa Panel on Biological Hazards, 2019; ECDC and EFSA, 2017) with more than 750 strongly-evidenced food-borne *Salmonella* outbreaks reported between 2014 and 2016. Of these cases, eggs and egg products were identified as the main vehicle of infection (276 cases). In comparison, broiler meat (*Gallus gallus*) and derived meat products were responsible for only 46 such outbreaks (Efsa Panel on Biological Hazards, 2019). Together, *Salmonella enterica* serovars Enteritidis (SE) and Typhimurium were the most frequently detected in food-borne outbreaks linked with egg and egg products in the EU (92 and 5.9% of outbreaks, respectively), with SE being by far the most prevalent serovar (Efsa Panel on Biological Hazards, 2019). This indicates that, despite the current controls, SE infection of eggs remains a serious threat to health in the EU (Efsa Panel on Biological Hazards, 2014; ECDC and EFSA, 2017; Efsa Panel on Biological Hazards, 2019).

The two possible routes of egg contamination are horizontal and vertical transmission. The former refers to penetration through the eggshell while the latter results from the infection of reproductive organs (Gantois et al., 2009). During egg formation, SE contamination of both the yolk and the egg white (EW) have been reported (Gantois et al., 2009 for a review). Although there is no clear consensus in the literature, it has been suggested that the albumen is the more likely site of initial infection than the yolk (Gast and Beard, 1990; Humphrey et al., 1991; Keller et al., 1995). During horizontal transmission, colonization and survival in EW also seem crucial as SE penetrates through the shell and encounters this medium prior to the vitelline membrane and the yolk.

Egg white carries a powerful set of antimicrobial activities that SE must resist in order to mediate egg-linked infections (Clavijo et al., 2006; Vylder et al., 2013; Baron et al., 2016). The antibacterial properties of EW include its high pH and viscosity, and a diverse range of antibacterial proteins/peptide (Baron et al., 2016). However, the major factor limiting bacterial growth in EW is iron restriction (Schade and Caroline, 1944; Garibaldi, 1970; Lock and Board, 1992; Baron et al., 1997). Iron restriction arises from the high levels (\sim 170 μ M; second most abundant EW protein) of ovotransferrin (oTf; also known as conalbumin; Alderton et al., 1946), a powerful Fe^{3+} -chelating protein (binding constant of 10^{32} M^{-1} ; Chart and Rowe, 1993). OTf is closely related to serum Tf which delivers iron between tissues in man and other vertebrates (Williams, 1968). The iron-binding capacity of oTf outstrips the iron content of EW (0.1 mg iron per

100 g; Nys and Sauveur, 2004; USDA, 2010) by \sim 20-fold which suggests that there is virtually no “free” iron in EW (Julien et al., 2019). This high oTF:Fe ratio of EW resembles the conditions of serum where the partial iron saturation of circulating Tf (\sim 30%; Kelly et al., 2017) causes a powerful bacteriostatic iron-restriction effect (Bullen et al., 1978).

An important mechanism used by bacteria to overcome iron restriction involves the secretion of high-affinity ferric-iron-chelating molecules called siderophores (Andrews et al., 2003). SE produces two related siderophores, enterobactin (Ent) and salmochelin (Sal), both of which are catecholates (Pollack et al., 1970). Ent is a serine macrotrilactone and Sal is its glucosylated derivative. Hence the term Sal can refer to both mono-glucosylated Ent (MGE) and di-glucosylated Ent (DGE). Ent is reported as the strongest siderophore known, binding to ferric ion with an affinity constant of 10^{52} M^{-1} (Chart and Rowe, 1993). Because Ent has a higher affinity for iron than oTf/Tf, Ent-producing bacteria can acquire iron from both oTf and Tf (Ford et al., 1988; Carrano and Raymond, 1979; Chart and Rowe, 1993). The genetic locus responsible for this glucosylation of Ent in *Salmonella* is the *iroBCDEN* gene cluster (Bäumler et al., 1998). The di-glucosylation of Ent to generate Sal is catalyzed by the glucosyltransferase, IroB (Hantke et al., 2003; Bister et al., 2004). The resulting Sal is then exported across the cytosolic membrane by IroC (Crouch et al., 2008). Uptake and utilization are mediated by IroD, IroE, and IroN (Müller et al., 2009 for a review). Although the affinity of Sal for Fe^{3+} is not reported (Valdebenito et al., 2007; Watts et al., 2012), there is no indication that glucosylation significantly impacts Fe^{3+} ligation or affinity (Luo et al., 2006).

The host immune system can counter the action of bacterial siderophores through release of siderophore-binding proteins that remove ferri-siderophores from circulation (Clifton et al., 2009). These proteins belong to the lipocalin superfamily which includes members with a diverse range of functions beyond siderophore sequestration (Johnstone and Nolan, 2015). Human lipocalin 2 (LCN2) is the best-known example of a siderophore-binding lipocalin; it is able to sequester a variety of bacterial siderophores, including Ent (Goetz et al., 2002; Holmes et al., 2005). However, many bacteria can overcome the siderophore-sequestration activities of LCN2 by producing “stealth” siderophores, such Sal, which are not recognized by LCN2 (Fischbach et al., 2006; Valdebenito et al., 2007).

In EW, three lipocalin proteins have been identified: the extra fatty acid binding protein (Ex-FABP, or Ch21); the chondrogenesis-associated lipocalin (Cal- γ or prostaglandin D synthase); and α -1-ovoglycoprotein (Guérin-Dubiard et al., 2006; Mann, 2007; D’Ambrosio et al., 2008; Mann and Mann, 2011). Of these three EW proteins, siderophore-binding function has only

been explored for Ex-FABP. Ex-FABP was found to have similar function to LCN2 in sequestering ferric-Ent with high affinity (equilibrium dissociation constant, K_d , of 0.22 nM; Correnti et al., 2011). Ex-FABP also inhibits iron-restricted growth of a strain of *Escherichia coli* producing Ent as sole siderophore (Correnti et al., 2011), but not strains producing Sal (Garénaux et al., 2013). However, it is unclear whether Ex-FABP is present in EW at concentrations sufficient to inhibit growth of Ent-producing bacteria. Moreover, it is unclear whether the two other lipocalins of EW (Cal- γ and α -1-glycoprotein) might also sequester siderophores. Further, the capacity of Ex-FABP to inhibit the key EW-mediated pathogen, SE, under iron restriction has yet to be explored.

The aim of the research reported here was to explore whether the three lipocalins of EW can sequester the two siderophores produced by SE and to determine whether the EW lipocalins can inhibit SE growth under the iron-restricted conditions. The results show that only Ex-FABP inhibits iron restricted growth of SE and binds Ent with high affinity, and that it is present in EW at levels sufficient to inhibit growth when Ent is the sole siderophore produced. The results reported thus suggest that Ex-FABP is an effective component of the antibacterial repertoire of EW.

MATERIALS AND METHODS

Growth Media, Strains, and Plasmids

Lysogeny broth (LB) was used as the growth medium for *E. coli*, whereas either LB or Tryptone Soy Broth (TSB, Sigma-Aldrich) were used for the growth of *S. enterica*. M9 salts (Sigma-Aldrich) minimal medium (with 1 mM $MgSO_4 \cdot 7H_2O$, 0.2% w/v glucose, 20 μ M thiamine, 0.1 mM $CaCl_2$, 0.3% w/v casamino acids) and acid washed glassware were used to achieve iron-restricted growth. Ferric citrate (10 μ M) was added to achieve iron sufficiency. Growth in liquid medium was at 37°C with shaking generally at 250 rpm. *E. coli* TOP10 was used for cloning work and *E. coli* K-12 BW25113 Δ entB::kan (λ .DE3) was used as overexpression host. *S. enterica* serovar Enteritidis PT4-P125109 was used for growth tests and gene knockout. The bacterial strains and plasmids used and generated are listed in **Tables 1, 2**. Siderophore detection assay was performed using CAS (chrome azurol S) plates according to Loudon et al. (2011).

Inactivation of Genes Required for Enterobactin and Salmochelin Production/Utilization

The *entB* (enterobactin biosynthesis), *iroBC* (salmochelin uptake and synthesis), and *iroDEN* (salmochelin uptake and utilization) genes of SE were inactivated using the λ Red gene disruption system encoded by plasmid pKD46 (Datsenko and Wanner, 2000; **Supplementary Figure S1** and **Supplementary Table S1**). The chloramphenicol resistance cassette of pKD3 inserted in the genome was subsequently deleted using FLP recombinase encoded by the temperature-sensitive plasmid, pCP20 (Cherepanov and Wackernagel, 1995) to generate clean deletions. Mutants were confirmed by whole-genome sequencing

TABLE 1 | Bacterial strains used and their genetic features.

Strain name	Features	Source
<i>E. coli</i> Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80lacZ Δ M15 Δ lacX74 <i>recA1</i> <i>araD139</i> Δ (<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>	Invitrogen
BW25113	<i>E. coli</i> K-12	GE Healthcare
JW0587(λ .DE3)	BW25113 (Δ entB)::kan (λ .DE3)	This study
PT4-P125109	Wild type <i>Salmonella enterica</i> serovar Enteritidis PT4-P125109	McCusker Dublin, Ireland
PT4-P125109 Δ entB	<i>Salmonella enterica</i> serovar Enteritidis PT4-P125109 Δ entB	This study
PT4-P125109 Δ iroBC	<i>Salmonella enterica</i> serovar Enteritidis PT4-P125109 Δ iroBC	This study
PT4-P125109 Δ iroDEN	<i>Salmonella enterica</i> serovar Enteritidis PT4-P125109 Δ iroDEN	This study

(Illumina; MicrobesNG, Birmingham); corresponding genome sequences were deposited at the ENA database under the following project accession number: PRJEB36543. General molecular genetic methods were as previously described (Sambrook and Russell, 2001).

Cloning of Lipocalin Genes for Over-Production in *E. coli*

Nucleotide sequences for the human LCN2 and chicken Ex-FABP, α -1-ovoglycoprotein and Cal- γ genes were obtained from the NCBI GeneBank database using the ID numbers 3934, 396393, 395220, and 374110, respectively. The sequences were codon optimized for expression in *E. coli* (GeneArt optimisation portal;¹) (see **Supplementary Figure S2** for corresponding sequences) and then synthesized by GeneArt (Invitrogen, Germany) with flanking restriction sites to assist subsequent cloning from the pMK carrier plasmid together with an N-terminal signal sequence (except for LCN2, which does not have an N-terminal signal sequence) to allow secretion of the encoded hexa-His tagged proteins into the periplasm. The lipocalin-coding regions were released from the pMK plasmid using *NdeI* and *XhoI*, and then ligated into the corresponding sites of plasmid pET21a using the Rapid DNA Ligation kit (Thermo Fisher Scientific). The resulting constructs were confirmed by restriction digestion and nucleotide sequencing (Eurofins). *E. coli* K-12 BW25113 Δ entB::kan (JW0587) was converted to λ DE3-lysogen status using a Lysogenization kit (Novagen) and the resulting strain then acted as host for over-expression of the lipocalin genes from pET21a. This enterobactin-deficient host strain was used to avoid association of overexpressed lipocalins with host-specified enterobactin.

Lipocalin Purification

Escherichia coli JW0587(λ .DE3) transformants, carrying a corresponding lipocalin-expressing pET21a plasmid, were

¹<https://www.thermofisher.com/fr/fr/home/life-science/cloning/gene-synthesis.html>

TABLE 2 | Plasmid used and their features.

Plasmid	Size (bp)	Features	Resistance	Source
pMK-lcn2	2930	Encodes LCN2	Kan	GeneArt synthesis (Invitrogen)
pMK-Ex-FABP	2860	Encodes Ex-FABP	Kan	GeneArt synthesis (Invitrogen)
pMK-Cal- γ	2881	Encodes Cal- γ	Kan	GeneArt synthesis (Invitrogen)
pMK- α 1-glyco	2935	Encodes α 1-glycoprotein	Kan	GeneArt synthesis (Invitrogen)
pET21a	5443	Requires T7 RNA polymerase and under LacUV5 control	Amp	Novagen
pET-lcn2	5904	pET21a vector used for LCN2 over-production	Amp	This study
pET-Ex-FABP	5907	pET21a vector used for Ex-FABP over-production	Amp	This study
pET-Cal- γ	5928	pET21a vector used for CAL- γ over-production	Amp	This study
pET- α 1-glyco	5982	pET21a vector used for α 1-glycoprotein over-production	Amp	This study
pKD3	2804	λ red template vector	Cm, Amp	Datsenko and Wanner, 2000
pKD46	6329	T°C sensitive (30°C) λ red expressing vector encoding <i>exo</i> , <i>beta</i> and <i>gam</i> and under AraC control	Amp	Datsenko and Wanner, 2000
pCP20	9332	T°C sensitive (30°C) vector encoding FLP recombinase	Cm, Amp	H. Mori, Japan

grown in LB (supplemented with 10 μ M Fe-citrate) and expression was induced with 0.5 mM IPTG. Cells pellet were harvested by centrifugation (3000 \times g for 20 min at 4°C), resuspended in 1 mL of osmotic shock solution (20% sucrose, 30 mM Tris pH 8.0, 10 mM EDTA) per 50 mg of cells (~5 g in total). After 10 min at room temperature, the supernatant was harvested as above and dialysed thoroughly against binding buffer 1 (0.3 M NaCl, 50 mM Na₃HPO₄, 15 mM imidazole, pH 8.0) at 4°C. The C-terminally hexa-His-tagged lipocalin proteins were isolated from the retentate by Ni²⁺-affinity chromatography using a 5 mL Mini Nuvia IMAC Ni-charged cartridge (Bio-Rad) according to manufacturer's instructions. Eluted Ex-FABP and α 1-ovoglycoprotein were further purified using a 5 mL HiScreen Capto Diethylaminoethyl column (DEAE-sepharose, GE Healthcare) equilibrated in binding buffer 2 (20 mM Tris-HCl, pH 8) with elution achieved with a linear gradient of 1 M NaCl in the same buffer. LCN2 and Cal- γ were further purified using a 5 mL HiScreen Capto Multimodel Cation exchanger ImpRes column (MMC, GE Healthcare) equilibrated with binding buffer 3 (50 mM sodium acetate, pH 6); elution was achieved with a linear gradient of 1 M NaCl in the same buffer or in Tris-HCl at pH 8. The purity of the resulting proteins was determined by SDS-PAGE (Sambrook and Russell, 2001; **Figure 1A**) and identities were confirmed by electrospray-ionisation mass-spectrometry (ESI-MS). Bradford protein assay (Bio-Rad) and absorbance at 280 nm were used to estimate protein concentrations. Proteins were stored in phosphate-buffered saline (PBS, pH 7.4) at -80°C.

Electrospray Ionization – Mass Spectrometry

Isolated lipocalin proteins were dialysed against ultrapure water prior to analysis by MicroTOF-Q mass spectrometry (column Ace C8 50 \times 2.1, mass spectrometer Thermo Scientific LTQ Orbitrap XL with an ESI source *via* a Thermo

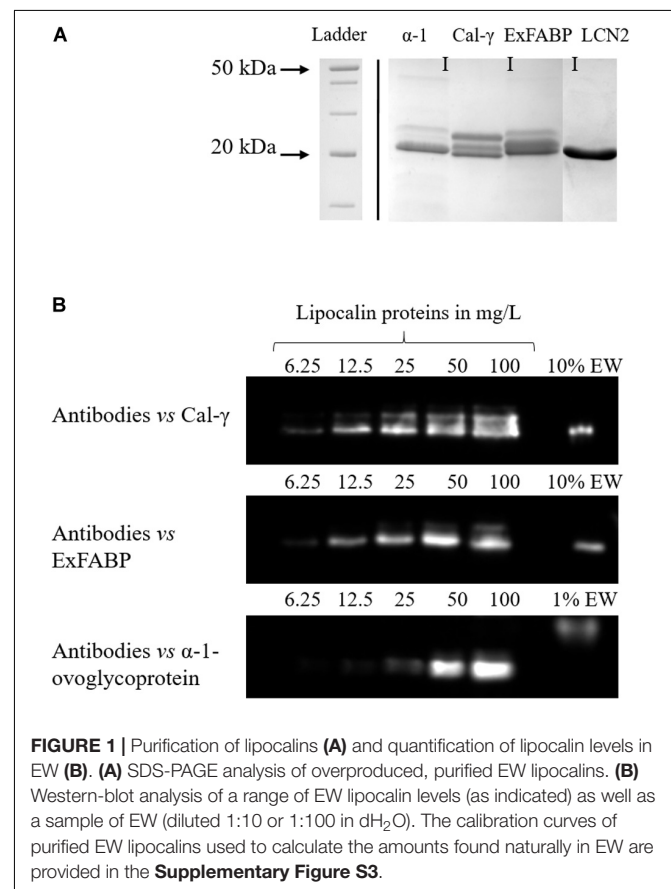


FIGURE 1 | Purification of lipocalins (A) and quantification of lipocalin levels in EW (B). (A) SDS-PAGE analysis of overproduced, purified EW lipocalins. (B) Western-blot analysis of a range of EW lipocalin levels (as indicated) as well as a sample of EW (diluted 1:10 or 1:100 in dH₂O). The calibration curves of purified EW lipocalins used to calculate the amounts found naturally in EW are provided in the **Supplementary Figure S3**.

Scientific Accela HPLC). Mass spectrum peaks were averaged and deconvolution to uncharged neutral mass was performed using Xtract within the Xcalibur software (the BioCentre, University of Reading).

Western-Blot Analysis of Lipocalin Levels in EW

Polyclonal antibodies were raised in two New Zealand White rabbits by DC Biosciences (Dundee, United Kingdom) using 2–2.5 mg of each of the three purified chicken-EW lipocalin proteins. A 90-day protocol using Freud's adjuvant as a stimulating agent was followed. The levels of each lipocalin in EW were then determined by western blotting (Mahmood and Yang, 2012) using corresponding lipocalin standards (6.25; 12.5; 50; 100 mg/L) along with EW dilutions (1:10 or 1:100 in qH₂O). Immunodetection was achieved using the primary antibodies raised in rabbit, and secondary anti-rabbit IgG antibody raised in goat and HRP-conjugated (Sigma-Aldrich batch A-6154). Westar C 2.0 chemiluminescent substrate (Cyanagen) was used for signal development. The membrane was imaged using a Syngene G:BOX. Images were analyzed with ImageJ software in order to band determine signal intensities. The lipocalin quantification was repeated three times with eggs of three different egg brands (free range eggs, the Cooperative; free range eggs, Clarence Court; and free-range eggs, Happy Egg). All eggs were certified Class A and produced in the United Kingdom. From the three concentration values gathered, an average and standard error were calculated.

Isothermal Titration Calorimetry

Experiments were carried out with a GE Healthcare Microcal VP-ITC microcalorimeter according to the manufacturer's guidelines (Operating Instructions 28-9639-79 AA). The device consisted of a motorized injection syringe, in which the ligand was loaded, with two adiabatic cells set at 30 °C. The left cell was filled with the reference (TBS with 1.3% DMSO), whereas the right cell was filled with 5 μM lipocalin protein dissolved in the same buffer. Sequential 10 μL injections (29 in total) of ligand were applied consisting of either 50 μM enterobactin (Sigma-Aldrich) or 50 μM salmochelin (EMC siderophore) dissolved in TBS (pH 7.4) with 1.3% DMSO. Samples were degassed and pre-equilibrated at the desired temperature using a GE MicroCal ThermoVac degassing station before loading into the syringe, sample cell and reference cell.

Growth Tests in Liquid Medium

Growth comparison in iron-free M9 medium (with/without 10 μM ferric citrate) was performed in 100-deepwell Honeycomb plates incubated in a Bioscreen C (Lab System) apparatus (at 37°C with constant shaking at 200 rpm) for ~20 h. Growth comparison in LB medium (with/without 200 μM 2,2 dipyriddy; DIP; an iron chelator) was achieved using 96-well microplates incubated in a Spectrophotometer SpectraMax 340 PC (Molecular devices) at 37°C with 60 s shaking at 200 rpm every 15 min (for ~20 h).

RESULTS AND DISCUSSION

Isolation of EW Lipocalins

The genes encoding the three EW lipocalins (Ex-FABP, Cal-γ, and α1-glycoprotein) and human lipocalin 2 (LCN2) were

TABLE 3 | Comparison of the purification products detected through ESI-MS and the theoretical mass of the lipocalin-like proteins.

	Mass determined through ESI (Da)	Theoretical mass (Da)	Difference between the theory and the observed Mass (Da)
Mature α1-glyco	21,377.5	21,381.9	4.4
PelB-α1-glyco	23,586.7	23,592.7	5.9
Mature Ex-FABP	19,127.4	19,130.6	3.2
Mature Cal-γ	20,655.0	19,892.2	762.8
PelB-Cal-γ	22,866.2	22,103.0	763.2

codon-optimized (for *E. coli*) and overexpressed in *E. coli* to give C-terminal hexa-His-tagged recombinant proteins, as described in Experimental Procedures. Purification was then achieved using Ni²⁺-affinity and ion-exchange chromatography. SDS-PAGE analysis indicated a purity of ≥90% and polypeptides of the anticipated mobility (Figure 1A). ESI-MS of α-1-ovoglycoprotein and Ex-FABP showed that, in each case, the major polypeptide was a close match to the theoretical mass of the mature forms (Table 3), indicating cleavage of the PelB_{SS} had been achieved during secretion. However, lower levels of the immature form of α-1-ovoglycoprotein were recovered (Figure 1A and Table 1). For Cal-γ, ESI-MS indicated a mass that is 763 Da greater than that predicted for both the major mature and minor immature forms. ESI-MS analysis carried out under native and denaturing conditions showed that the additional mass was likely covalently associated, and MALDI-MS showed that the N- and C-terminal amino acid sequences (residues 1–36 and 161–173) of the mature form were as expected. Thus, the nature of the additional 763 Da component remains unclear.

EW Contains Micro-Molar Levels of All Three Lipocalins

The purified EW lipocalins were used to raise antibodies in rabbit to enable quantification of the lipocalins in EW by western blotting (Figure 1B). Western-blot analysis showed that all three lipocalins are indeed present in EW at detectable levels, with mobilities matching those of the purified proteins for Ex-FABP and Cal-γ (Figure 1B). However, α-1-ovoglycoprotein in EW showed a higher apparent mass (37.4 kDa) than that of the recombinant protein (22.7 kDa). Haginaka et al. (1995) also reported a higher mass for α-1-ovoglycoprotein in EW by MALDI-TOF mass spectroscopy (30 kDa). These discrepancies are presumed to arise from the N-type glycosylation of α-1-ovoglycoprotein within EW (30.3% total glycosylation; Ketterer, 1965) resulting in aberrant mobility of glycosylated proteins during SDS-PAGE (Guérin-Dubiard et al., 2006). The semi-quantitative western blots indicated EW concentrations of 232.9, 5.6, and 5.1 μM for α-1-ovoglycoprotein, Cal-γ, and Ex-FABP, respectively (Table 4). They are therefore the 4th, 11th, and 12th most abundant proteins in EW by mass (Kovacs-Nolan et al., 2005). The levels observed were relatively consistent between egg producers which suggests that the abundances reported here are likely to be generally reflective of those found in chicken eggs. Although the 2D-PAGE and MS analyses of previous workers (Guérin-Dubiard et al., 2006) indicated that Ex-FABP and Cal-γ

TABLE 4 | Concentration of lipocalin-like proteins found in EW (three trademarks coming from different providers in the United Kingdom) according to western-blot analysis.

	Ex-FABP		Cal- γ		α -1-ovoglycoprotein	
	g/L	μ M	g/L	μ M	g/L	μ M
Cooperative	0.079	4.1	0.095	4.8	2.976	139.1
Clarence court	0.115	6.0	0.123	6.2	6.497	303.6
Happy egg	0.098	5.1	0.113	5.7	5.480	256.1
Average	0.097	5.1	0.110	5.6	4.984	232.9
SD	± 0.018	± 1.0	± 0.014	± 0.7	± 1.812	± 84.7

To convert the concentration from g/L to molarity, the theoretical MW of the mature proteins was used: 19.1, 19.9, and 21.4 kDa for Ex-FABP, Cal- γ and α 1-ovoglycoprotein, respectively.

TABLE 5 | Lipocalins-binding affinities towards apo- and ferric-ligands obtained using isothermal titration calorimetry (in TBS pH 7.4 at 30°C) in this study and compared values from the literature. K_d is the equilibrium dissociation constant, n the binding stoichiometry, ΔS the entropy, and ΔH the enthalpy.

Protein	Ligands	Reference	K_d nM	n	ΔS cal/mol, K	ΔH cal/mol
LCN2	apo-Ent	This study	58.4 \pm 12.5	0.872	-58.9	-2.78×10^4
		Abergel et al., 2006	3.57			
	Fe ³⁺ -Ent	This study	11.8 \pm 8.8	0.863	-50.2	-2.62×10^4
		Goetz et al., 2002	0.41			
Ex-FABP	apo-Sal (or DGE)	This study	Under threshold	0.617	-60.9	-2.83×10^4
		Fischbach et al., 2006	Under threshold			
	apo-Ent	This study	86.2 \pm 14.6	0.672	-26.1	-1.94×10^4
		Correnti et al., 2011	0.5 \pm 0.15			
	Fe ³⁺ -Ent	This study	5.3 \pm 3.8	0.22 \pm 0.06	-26.1	-1.94×10^4
		Correnti et al., 2011	0.22 \pm 0.06			
apo-Sal (or DGE)	This study	Under threshold	Under threshold	-26.1	-1.94×10^4	
	Correnti et al., 2011	Under threshold				
Cal- γ	apo-Ent	This study	Under threshold	Under threshold	-26.1	-1.94×10^4
	apo-Sal (or DGE)	This study	Under threshold			
α -1-ovoglycoprotein	apo-Ent	This study	Under threshold	Under threshold	-26.1	-1.94×10^4
	apo-Sal (or DGE)	This study	Under threshold			

are considerably less abundant in EW than α -1-ovoglycoprotein, no concentration values had been assigned to Ex-FABP and Cal- γ in EW, until the results reported herein.

The level of α -1-ovoglycoprotein in EW was previously estimated as ~ 1 g L⁻¹ (Ketterer, 1965), whereas here a considerably higher concentration of 232.9 μ M (or 5 g L⁻¹) is reported (Table 4). This difference may reflect the use of alcohol precipitation before quantification in the previous report (Ketterer, 1965), which could lead to incomplete protein recovery. Since in the work reported here quantification was achieved directly on EW, it seems reasonable to suggest that the estimation obtained is closer to the true value but might still represent an underestimate as the glycosylation of the native EW protein might reduce its immunoreactivity against the antibodies raised to the non-glycosylated form. Given the previous classification of EW proteins based on abundance (Kovacs-Nolan et al., 2005), α -1-ovoglycoprotein belongs to the high concentration group of “major EW protein” set along with ovalbumin (54 g L⁻¹), ovotransferrin (12.0 g L⁻¹), ovomucoid (11 g L⁻¹), globulin (4 g L⁻¹), ovomucin (3.5 g L⁻¹) lysozyme

(3.4 g L⁻¹), and ovoinhibitor (1.5 g L⁻¹). However, Cal- γ and Ex-FABP, present at 5.6 and 5.1 μ M (or 0.11 and 0.10 g L⁻¹), would belong to the “minor EW protein” set, which include ovoflavoprotein (0.8 g L⁻¹), ovostatin (0.5 g L⁻¹), cystatin (0.05 g L⁻¹), and avidin (0.05 g L⁻¹).

Ex-FABP Binds Enterobactin With High Affinity and Strong Preference for the Ferrated Form

The ability of the three EW lipocalins to specifically bind the iron-free forms of Ent (apoEnt) and Sal (diglycosylated form, apoDGE) was compared with that of LCN2, using isothermal titration calorimetry (ITC). LCN2 bound apoEnt in an exothermic reaction with a high affinity, giving a dissociation constant (K_d) of 58 \pm 12 nM (at pH 7.4; Table 5 and Supplementary Figure S4), which is 16-fold higher than the value previously reported using tryptophan quenching fluorescence (TQF; 3.57 nM; Abergel et al., 2006). No binding was observed with apoDGE (Supplementary Figure S4), which

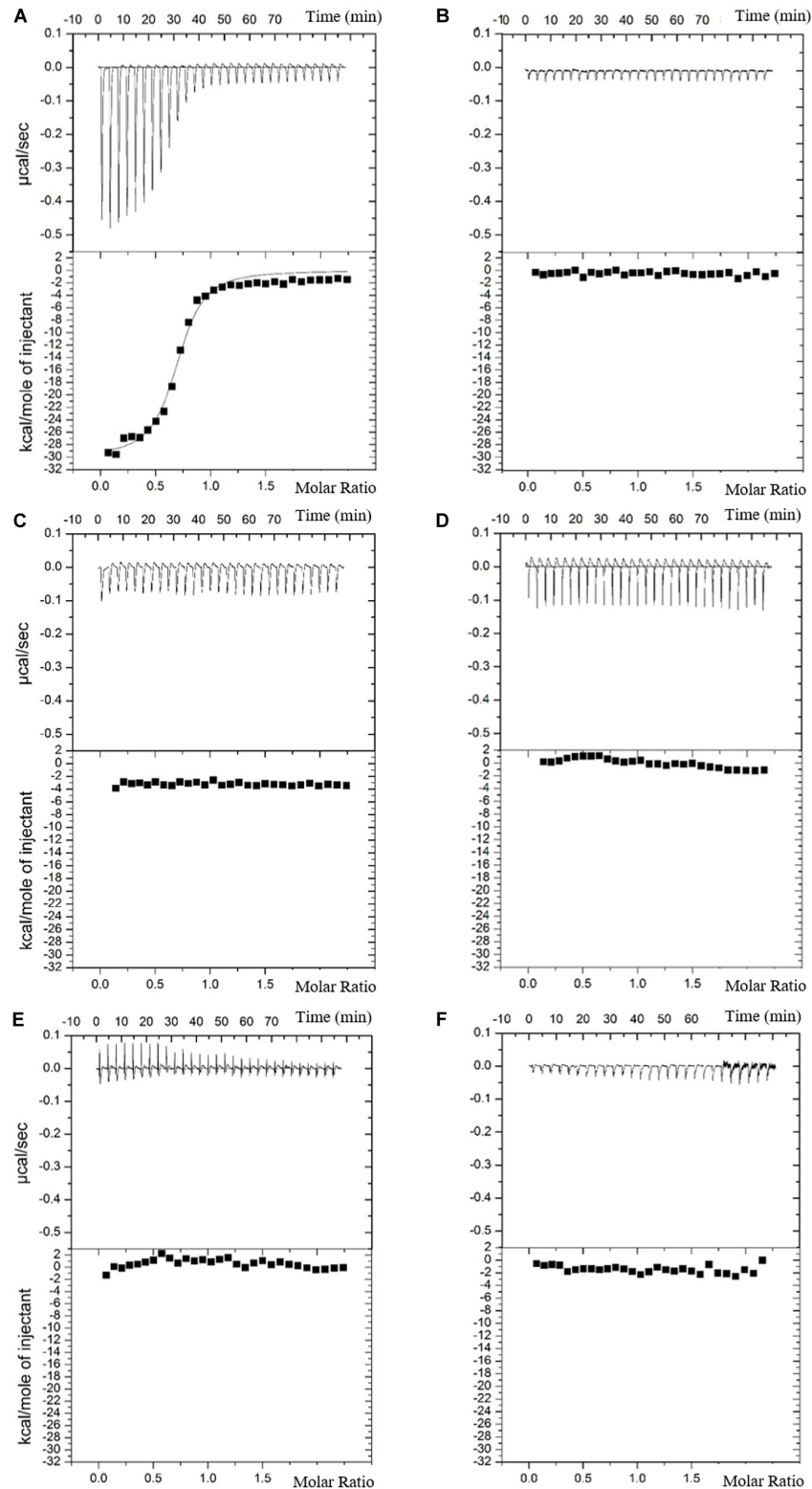
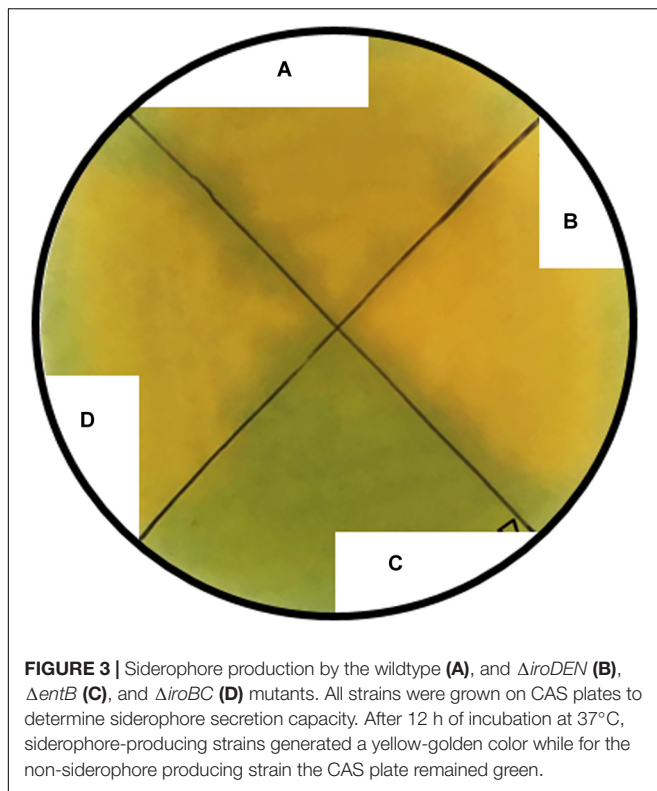


FIGURE 2 | Interactions between lipocalins and SE siderophores. Isothermal titration calorimetry was achieved with 29 injections (10 μ L) of 50 μ M ligands (Ent and Sal) in an adiabatic well containing 5 μ M lipocalins. Proteins and ligands were both dissolved in TBS (20 mM Tris, 500 mM NaCl, 1.3% DMSO, pH 7.4) and experiments were achieved at 30°C. No interactions could be measured between Ex-FABP and apo-Sal (**B**), whereas a K_d of 86 ± 5 nM was measured between Ex-FABP and apo-Ent (**A**). No interactions could be measured between Cal- γ and enterobactin (**C**), Cal- γ and salmochelin (**D**), α -1-glycoprotein and enterobactin (**E**) and α -1-glycoprotein and salmochelin (**F**). Data for LCN2, Fe-Ent are shown in the **Supplementary Figure S2**.



matches previous findings showing that LCN2 does not bind DGE (Fischbach et al., 2006). Ex-FABP also bound apoEnt at high affinity, giving an exothermic reaction with a dissociation constant (K_d) of 86 ± 5 nM at pH 7.4 (Table 5 and Figure 2A) and likewise failed to interact with apoDGE, as observed previously (Correnti et al., 2011). It should be noted that the Ex-FABP binding affinity for apoEnt found here is 172-fold weaker than that determined previously (0.5 ± 0.15 nM) using TQF under similar (TSB buffer, pH 7.4) conditions (Correnti et al., 2011). This discrepancy could result from the difference in the techniques used (ITC vs TQF), differences in reaction conditions (e.g. Ex-FABP and DMSO concentrations) or differences in protein preparation. For both LCN2 and Ex-FABP, the apoEnt molar-binding stoichiometry was close to 1:1 at 0.872 and 0.617, respectively (Table 5), which again is consistent with previous reports (Goetz et al., 2002; Correnti et al., 2011).

Since a previous TQF study indicated that Ex-FABP binds Fe^{3+} -Ent with a higher (2.3-fold) affinity than apoEnt (Correnti et al., 2011), and similar findings have been made with LCN2 (8.7-fold; Abergel et al., 2006), the interaction between Fe^{3+} -Ent and Ex-FABP was tested here by ITC. The results show a 16-fold higher affinity (K_d 5.3 ± 3.8 nM) for Fe^{3+} -Ent than apoEnt (Table 5 and Supplementary Figure S4) which indicates a higher preference for the ferric- over the apo-form than was previously suggested (Correnti et al., 2011). The ITC data also indicates that LCN2 possesses a 4.9-fold higher affinity (K_d 11.8 ± 8.8 nM) constant for Fe^{3+} -Ent than for apoEnt (Table 5 and Supplementary Figure S2); a similar higher affinity (8.7-fold) was reported by Abergel et al. (2006), and

Bao et al. (2010) also showed that Fe^{3+} dramatically enhances the affinity of LCN2 for catechols (by at least 95-fold). It should be noted that the ITC-determined binding affinities of LCN2 for Fe^{3+} -Ent reported here (11.8 nM) and previously (240 nM; Li et al., 2015) are both considerably weaker than those determined by TFQ (0.41 and 0.43 nM; Goetz et al., 2002; Fischbach et al., 2006) indicating that differences in published affinity values for LCN2 (and Ex-FABP) are to a large degree related to the methods employed. ITC indicated that neither Cal- γ nor α -1-ovoglycoprotein bind either apoEnt or apoDGE (Table 5 and Figure 2) which suggests that these lipocalins do not act as “siderocalins,” although it remains possible that they could have affinity for siderophores not considered here. It is also important to note that α -1-ovoglycoprotein is glycosylated in EW and such post-translational modifications were not achieved during over-production in *E. coli*. This lack of glycosylation might have influenced the observed bioactivity of the recombinant protein.

Ex-FABP Inhibits Growth of Salmochelin-Deficient SE Mutants in Iron-Restricted Medium When Provided at Concentrations Found in EW

In order to determine whether EW lipocalins can cause growth inhibition of an SE strain producing Ent, but not Sal, a mutant (*ΔiroBC*) lacking the ability to produce and export Sal was generated. The *iroB* gene encodes the glucosyltransferase that converts Ent into Sal, and *iroC* encodes the transporter required for export of Sal across the cytosolic membrane (Hantke et al., 2003; Bister et al., 2004; Crouch et al., 2008); thus, the *iroBC* mutant can neither produce nor release Sal, but should retain the capacity to produce and utilize Ent. Furthermore, two other mutants were generated as controls: *ΔiroDEN* and *ΔentB*. The former should be able to produce both Ent and Sal but was not expected to utilize Sal due to absence of the IroN OM receptor and IroD/IroE esterases (Müller et al., 2009). The latter (*ΔentB*) is unable to convert isochorismate to 2,3-dihydro-2,3-dihydroxybenzoate (an Ent precursor; Gehring et al., 1998) and therefore can produce neither Ent nor its derivative, Sal. The genotype of the mutants was confirmed by genome sequencing and propagation on CAS plates (Louden et al., 2011) showed that, as expected, the *ΔentB* (Ent⁻ Sal⁻) mutant does not produce siderophore whereas both the *ΔiroBC* (Ent⁺ Sal⁻) and *ΔiroDEN* (Ent⁺ Sal⁺) mutants are siderophore producing (Figure 3).

To assess whether exposure to EW lipocalins negatively impacts SE growth through Ent sequestration, growth of the siderophore-defective SE mutants and wildtype was monitored in the presence and absence of each of the EW lipocalins and LCN2 (at 5 μM), under low- and sufficient-iron conditions (Figure 4). Of the lipocalins tested, only Ex-FABP and LCN2 had any impact on iron-deficient growth (Figure 4), and none had any impact under iron sufficiency. The *ΔiroBC* mutant (Ent⁺ Sal⁻) was particularly affected with a 2.5-fold growth reduction (at 6 h; Supplementary Figure S5) under

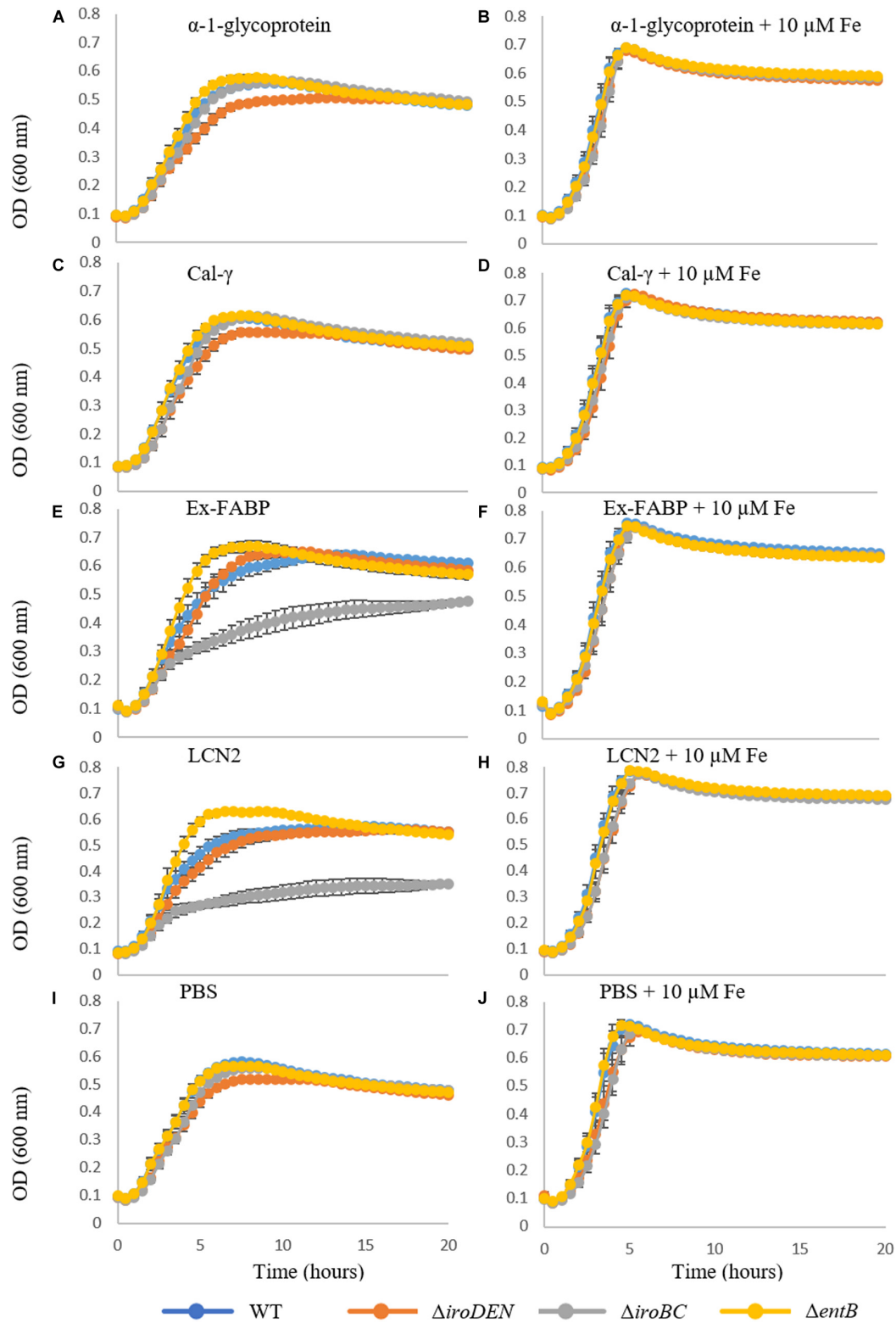


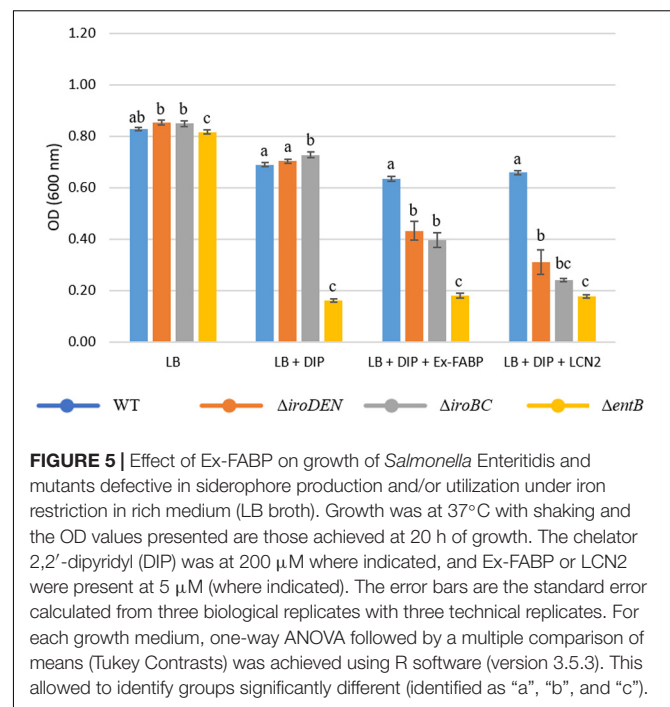
FIGURE 4 | Effect of EW lipocalins on growth of *Salmonella* Enteritidis and mutants defective in siderophore production and/or utilization under iron restriction. Growth of the WT and the Δ iroBC, Δ iroDEN, and Δ entB mutants was compared in M9 medium with (right) or without (left) 10 μM ferric citrate at 37°C and 200 rpm, for 20 h. The medium included 5 μM of either α -1-glycoprotein (A,B), Cal- γ (C,D), Ex-FABP (E,F), LCN2 (G,H) or \sim 100 μL of PBS (I,J). Error bars indicate standard error from three biological replicates with two technical replicates.

low-iron with Ex-FABP or LCN2 (Figures 4E,G). This reduced growth is fully consistent with the Ent sequestering activities of Ex-FABP and LCN2, and the dependence of the $\Delta iroBC$ mutant on Ent as the sole siderophore supporting its low-iron growth. The wildtype also displayed a reduced growth with Ex-FABP or LCN2 under low iron, but this was far more modest (at just 1.25-fold; at 6 h; Supplementary Figure S5) than that seen for the $\Delta iroBC$ mutant. This mild reduction in iron-restricted growth is consistent with the co-dependence of the wildtype on Ent and Sal under iron deficiency. Thus, production of Sal by the wildtype appears to largely overcome the impact of Fe-Ent sequestration by Ex-FABP or LCN2, as reported previously for other bacteria (Fischbach et al., 2006; Correnti et al., 2011).

In contrast to the $\Delta iroBC$ mutant, the $\Delta entB$ mutant (Ent⁻ Sal⁻) exhibited better low-iron growth (1.2-fold at 6 h; Supplementary Figure S5) than the wildtype in the presence of Ex-FABP or LCN2 (Figures 4E,G), with no apparent reduction in growth exhibited in the presence of these lipocalins. The failure of Ex-FABP or LCN2 to inhibit the $\Delta entB$ mutant is consistent with the ability of this mutant to escape competition for iron by LCN2 or Ex-FABP due its lack of Ent production. Thus, the results indicate that production of Ent as sole siderophore is deleterious to growth under low-iron conditions in the presence of Ex-FABP or LCN2. The $\Delta iroDEN$ mutant showed reduced growth with respect to the other three strains (~1.1-fold at 6 h) under low-iron conditions in the absence of Ex-FABP or LCN2 (Figure 4I). This indicates that the capacity to secrete Sal, but not utilize it, results in deficient growth under iron restriction, which presumably arises due to sequestration of extracellular iron by secreted Sal. Cal- γ and α -1-ovoglycoprotein had no apparent effect on the growth of SE or its mutants under low- or sufficient-iron conditions (Figure 4). This is consistent with the ITC data which indicate that these lipocalins do not bind Ent or Sal.

In summary, Ex-FABP at 5 μ M caused a major inhibition of iron-restricted growth for an SE strain relying on Ent as sole siderophore but had only a modest impact on the wildtype deploying Sal alongside Ent. Since the western blotting data indicate that Ex-FABP is present in EW at 5.1 μ M, these results suggest that Ex-FABP concentrations in EW are sufficient to cause inhibition of iron-restricted growth for an SE strain, and potentially other bacterial species, relying on Ent as sole siderophore.

Although no iron-restricted growth defect was observed for the $\Delta entB$ mutant in minimal (M9) medium, when growth tests were performed in rich (LB) medium with the iron chelator DIP, a major reduction in growth (~4-fold), with respect to the wildtype, was observed (Figure 5). No growth defect was seen in the absence of DIP indicating that the phenotype obtained is related to iron restriction imposed by DIP chelation. This observation matches previous work (Liu et al., 2015) and likely arises from the inability of the $\Delta entB$ mutant to effectively compete with DIP for iron due to its lack of Ent (and Sal) production. In contrast to the $\Delta entB$ mutant, the $\Delta iroDEN$ and $\Delta iroBC$ strains showed no growth defect in LB with DIP (Figure 5), presumably because they retain the ability



to produce and utilize Ent, and can thus compete with DIP for iron.

As expected, the provision of either Ex-FABP or LCN2 had little impact on the weak growth of the $\Delta entB$ mutant in LB with DIP (Figure 5). However, the $\Delta iroDEN$ and $\Delta iroBC$ mutants showed a significant growth defect (~1.5–2-fold reduction) compared to the wildtype in LB with DIP when 5 μ M Ex-FABP or LCN2 were provided (Figure 5). This is consistent with the ability of Ex-FABP and LCN2 to sequester Ent, but not Sal. LCN2 caused a greater growth reduction than Ex-FABP (2-fold cf. 1.5-fold, respectively) for the $\Delta iroDEN$ and $\Delta iroBC$ mutants, and the growth of $\Delta iroBC$ mutant was slightly more reduced than that of the $\Delta iroDEN$ mutant (Figure 5).

The findings above are fully consistent with the Ent-sequestering roles of LCN2 and Ex-FABP, as shown here and previously (Goetz et al., 2002; Fischbach et al., 2006; Correnti et al., 2011). The results above are also in accordance with previous work showing that mutations in *iroBC* (Sal production and export) and *iroN* (Sal uptake) do not impair growth of *S. Typhimurium* in rich (LB) medium (Raffatellu et al., 2009) but do significantly reduce growth in (low iron) tissue culture medium (DMEM) containing LCN2. The present study also shows that Ex-FABP is present at sufficient concentration in EW to cause inhibition of iron-restricted growth for an SE strain relying on Ent as sole siderophore, but not for SE deploying Sal alongside Ent. These results reflect previous findings showing that 2.5 and 10 μ M Ex-FABP cause iron-restricted growth inhibition of Ent-producing *E. coli* (Correnti et al., 2011; Garénaux et al., 2013) and that complementation with *iroBCDEN* reverses inhibition by Ex-FABP (Garénaux et al., 2013).

CONCLUSION

The results presented here show for the first time that SE, the major pathogen associated with egg-linked food-borne outbreaks, is resistant to the enhanced iron-restriction imposed by the EW-lipocalin, Ex-FABP. This resistance is due to the ability of SE to produce and utilize the siderophore, Sal. This Sal-resistance property would be expected to contribute to survival of SE in the iron-restricted conditions of EW and would thus be likely to support the capacity of SE to infect eggs (Clavijo et al., 2006; Gantois et al., 2009; Vyllder et al., 2013; Baron et al., 2016). Furthermore, the results provide the first immuno-quantification of the three EW lipocalins in eggs and reveal that Ex-FABP is present at concentrations (5.1 μM) sufficient to inhibit iron-restricted, Ent-dependent growth. In contrast, neither Cal- γ nor α -1-ovoglycoprotein influenced the growth of SE under the conditions tested here, indicating that these two EW lipocalins do not bind the siderophores produced by SE and do not contribute directly to iron restriction; the ITC data fully support the lack of Ent or Sal binding activity by these lipocalins. ITC also showed that both Ex-FABP and LCN2 bind Ent (but not Sal) with high affinity and with a preference for Fe-Ent (Kd of 5.4 and 11.8 nM, respectively) over apo-Ent (Kd of 86.2 and 58.4 nM, respectively). These binding patterns are consistent with previous work (Goetz et al., 2002; Abergel et al., 2006; Fischbach et al., 2006; Bao et al., 2010; Correnti et al., 2011; Li et al., 2015) although the affinity value ranges reported here and elsewhere show wide variation, which largely correlate with the methodology (ITC or TFQ) employed. A key finding of the results provided here is that the natural concentration of Ex-FABP in EW appears sufficient to play a biological role in limiting bacterial growth through sequestration of the siderophore, Ent. However, this effect is overcome by SE through its ability to deploy a Sal as second, “stealth” siderophore.

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DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

LJ, FB, SB, SJ, and SA designed the experiments. LJ and CF performed the experiments and analyzed the corresponding results. LJ wrote the manuscript with FB, SB, CG-D, FN, MG, KK, SJ, and SA.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.00913/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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