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O-antigen chain-length distribution in *Salmonella enterica* serovar Enteritidis is regulated by oxygen availability

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ABSTRACT

Lipopolysaccharide (LPS) consists of three covalently linked domains: the lipid A, the core region and the O antigen (OAg), consisting of repeats of an oligosaccharide. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) produces a LPS with two OAg preferred chain lengths: a long (L)-OAg controlled by Wzz_{SE} and a very long (VL)-OAg controlled by Wzz_{fepE}. In this work, we show that OAg produced by *S.* Enteritidis grown in E minimal medium also presented two preferred chain-lengths. However, a simultaneous and opposing change in the production of L-OAg and VL-OAg was observed in response to oxygen availability. Biochemical and genetics analyses indicate that this process is regulated by transcriptional factors Fnr and ArcA by means of controlling the transcription of genes encoding Wzz_{SE} and Wzz_{fepE} in response to oxygen availability. Thus, our results revealed a sophisticated regulatory mechanism involved in the adaptation of *S.* Enteritidis to one of the main environmental cues faced by this pathogen during infection.

1. Introduction

Infections caused by *Salmonella* constitute a severe health problem, particularly in developing countries. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is considered the primary cause of food-borne salmonellosis worldwide [1,2]. In humans, *S.* Enteritidis causes a syndrome characterized by diarrhea, fever and abdominal pain [2]. The mechanisms involved in *S.* Enteritidis pathogenicity are poorly understood, and most knowledge is based on studies conducted in *S.* Typhimurium, which causes a similar disease in humans. However, it is known that *S.* Enteritidis genes involved in lipopolysaccharide (LPS) synthesis and modification are essential for systemic colonization in mice [3] and hens [4], and also for resistance to serum [5] and survival in egg albumen [4].

The LPS consists of three structural domains: the lipid A region embedded in the outer membrane, the core oligosaccharide, and the O antigen (OAg) exposed on the bacterial surface [6]. The number of O antigen units that are attached to the lipid A-core region is regulated by the chain-length determinants Wzz_{SE} and Wzz_{fepE}. We described that *S.* Enteritidis NCTC13349 produces LPS with two preferred OAg chain lengths: a long (L)-OAg controlled by Wzz_{SE} and a very long (VL)-OAg controlled by Wzz_{fepE} [5].

To date, not much is known about the environmental regulation of OAg production and chain-length distribution. A previous study in *S.* Anatum showed that the growth temperature affects the size and distribution of the OAg molecules [7]. Also, Murray et al. [8] reported that *S.* Typhimurium incubated in heat-inactivated guinea pig serum or grown under iron-limiting conditions resulted in an increased production of VL-OAg. More recently, Delgado et al. [9] demonstrated that expression of the OAg chain-length determinant wzz_{ST} in *S.* Typhimurium was induced in defined medium containing low Mg^{2+} plus Fe^{3+}, in a pathway that requires the PhoP/PhoQ and PmrA/PmrB two-component systems. In addition, we have shown that production of OAg by *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis is growth phase-regulated in a RfaH-dependent manner [5,10].

One of the conditions that *Salmonella* encounters during infection is low oxygen availability in the ileum and within host cells

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[11,12]. Adaptation to oxygen deprivation involves the global regulators Fnr and ArcA, which control the expression of a large number of genes whose products are mainly involved in adaptation to anaerobiosis [13–18]. In addition, transcriptomic analyses have shown that Fnr and ArcA also regulate the expression of several genes linked to *S.* Typhimurium virulence. However, oxygen-dependent regulation of OAg production and chain-length distribution has not been addressed. In this work, we show that the chain-length distribution of OAg in *S.* Enteritidis is under oxygen-dependent regulation mediated by Fnr and ArcA, which control the expression of the genes encoding Wzz_{SE} and Wzz_{fepE} chain-length determinants.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Cultures were performed in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L NaCl and 5 g/L yeast extract) or E minimal medium (0.2 g/L $MgSO_4 \times 7H_2O$, 2 g/L citric acid monohydrate, 13.1 g/L $K_2HPO_4 \times 3H_2O$, 3.3 g/L $NaNH_4HPO_4 \times 4H_2O$) supplemented with 0.2% glucose as carbon source (E-glucose). For aerobic or anaerobic cultures, LB-grown overnight cultures were diluted 1:100 in E-glucose. Aerobic cultures were incubated at 37 °C with shaking and anaerobic cultures were incubated at 37 °C in an anaerobic jar with the AnaeroGen system (Oxoid). Where appropriate, media were supplemented with ampicillin (Amp, 100 μg/mL), chloramphenicol (Cam, 20 μg/mL) or kanamycin (Kan, 50 μg/mL). Media were solidified by the addition of 15 g/L agar.

2.2. Mutagenesis and standard DNA techniques

Total genomic DNA was obtained from overnight cultures using the "GenElute Bacterial Genomic DNA Kit" (Sigma-Aldrich) according to the manufacturer's instructions. Plasmid DNA was obtained from overnight cultures using the "QIAprep Spin Miniprep Kit" (QIAGEN), according to the manufacturer's instructions. When required, PCR products were purified using the "QIAquick PCR Purification Kit" (QIAGEN).

S. Enteritidis Δ*arcA* and Δ*fnr* strains were constructed by allelic exchange as described [19], using plasmid pCLF4 as template and primers listed in Table S1. The mutant alleles were transduced to the wild-type background using phage P22 HT105/1 *int*-201. The Δ*arcA* Δ*fnr* mutant was generated by transduction of the Δ*fnr*::Kan allele into a Δ*arcA*::FRT recipient strain. A mutant strain expressing a Wzz_{fepE}-3xFLAG protein fusion was constructed by allelic exchange as described [20], using plasmid pSUB11 as template and primers listed in Table S1. The corresponding mutant allele was transduced to the wild-type, Δ*arcA* and Δ*fnr* backgrounds using phage P22 HT105/1 *int*-201. When required, the antibiotic-resistance cassettes inserted during mutagenesis were removed by transforming the corresponding mutants with pCP20, which encodes the FLP recombinase [21]. Gene replacements and deletion of antibiotic-resistance cassettes were confirmed by PCR.

2.3. LPS analysis

LPS samples were prepared as described [22]. Briefly, bacteria obtained from aerobic or anaerobic cultures were adjusted to an $OD_{600} = 2.0$. Next, 1.5 mL of each suspension were centrifuged and bacterial pellets were suspended in 100 μL of lysis buffer containing proteinase K. LPS was separated in 12% (w/v) acrylamide gels using a Tricine-SDS buffer system (Tricine-SDS-PAGE) [23] and visualized by silver staining [22].

2.4. RNA isolation and qRT-PCR assays

RNA isolation was performed as described [24] from at least three independent aerobic or anaerobic cultures grown in E-glucose. Briefly, bacteria were grown to an $OD_{600} = 0.3$ and total RNA was extracted using the "SV Total RNA Isolation" kit (Promega). Samples were treated with RNase-free DNaseI (Qiagen) at 25 °C for 45 min. RNA samples were kept at −20 °C until further use.

For qRT-PCR assays, 1 μg of total RNA was subjected to reverse transcription using SuperScript II (Life Technologies) following manufacturer's instructions. DNA fragments of ~300 bp for each gene were amplified using the "Kapa Sybr Fast" kit (Kapa Biosistems) in an Mx3000P qPCR System with MxPro software (Agilent Technologies, Inc). Data were analyzed using the relative expression software tool REST [25] and *rpoD* was used as housekeeping gene because its expression is not influenced by oxygen availability in *Salmonella* [26].

2.5. Western blot analyses of the 3xFLAG-tagged chain-length regulator Wzz_{fepE}

Total protein samples obtained from bacteria grown under aerobic or anaerobic conditions ($OD_{600} = 0.3$) were separated by SDS-PAGE in a 10% polyacrylamide gel. Gel loading was normalized so that each sample represented the same number of bacterial cells. Western blot analysis was performed as described [27]. Wzz_{fepE}-3xFLAG protein signal was quantified by densitometry analysis using UN-SCAN-IT software (Silk Scientific). Values were

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Source or reference
***Salmonella* Enteritidis**		
NCTC13349	Wild-type strain	Laboratory stock
Δ*fnr*	NCTC13349 Δ*fnr*::FRT	This study
Δ*arcA*	NCTC13349 Δ*arcA*::FRT	This study
Δ*arcA* Δ*fnr*	NCTC13349 Δ*arcA*::FRT Δ*fnr*::Kan, Kan^R	This study
Plasmids		
pGEM-T Easy	TA cloning vector, Amp^R	Promega
pBAD-TOPO	TA cloning vector, Amp^R	Life Technologies
parcA	*arcA* cloned in pBAD-TOPO	This study
pfnr	*fnr* cloned in pBAD-TOPO	This study
pJC142	wzz_{fepE} cloned in pGEM-T Easy	[5]
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS, Amp^R	[33]
pCLF4	*bla* FRT *aph* FRT PS1 PS2 ori R6K, T7 promoter, Amp^R, Kan^R	[19]
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS, Cam^R, Amp^R	[21]
pSUB11	Template plasmid to generate 3xFLAG fusions, ori_{R6K}, Kan^R	[20]

normalized to the total amount of protein in the corresponding lane, as determined by densitometry of a Coomassie-blue stained gel analyzed in parallel.

3. Results

3.1. Oxygen availability modulates the OAg polymerization in S. Enteritidis

To assess the effect of oxygen availability on OAg production by *S.* Enteritidis, the wild-type strain and its isogenic Δ*arcA* and Δ*fnr* mutants were grown under aerobic or anaerobic conditions, and LPS profiles analyzed by Tricine-SDS-PAGE. As described [5], *S.* Enteritidis grown in LB produces OAg with two preferred chain lengths: L-OAg and VL-OAg. However, when bacteria were grown under aerobic conditions in E-glucose medium, VL-OAg was almost absent and a high level of L-OAg was observed (Fig. 1). In contrast, bacteria grown anaerobically produced high levels of VL-OAg with a concomitant slight decrease in L-OAg levels (Fig. 1). Deletion of *arcA* gene resulted in a dramatic reduction of VL-OAg under anaerobic conditions as compared to the wild type. Strikingly, a high increase of VL-OAg in aerobic conditions was observed in the Δ*fnr* and Δ*arcA* Δ*fnr* backgrounds as compared to the wild type (Fig. 1).

3.2. Oxygen availability regulates the expression of wzz_{SE} and wzz_{fepE} in S. Enteritidis

The above results lead us to hypothesize that the expression of genes encoding the OAg chain-length regulators Wzz_{SE} and Wzz_{fepE} are regulated by ArcA or Fnr in response to oxygen availability. In support of this notion, our bioinformatics analyses showed that the promoter regions of wzz_{SE} and wzz_{fepE} include putative binding sites for ArcA and Fnr (Fig. S1). To determine whether the expression of wzz_{SE} and wzz_{fepE} is affected by oxygen availability, we measured the relative abundance of transcripts for both genes in bacteria grown anaerobically and aerobically. We observed that expression of wzz_{SE} in the wild-type strain was 4 times lower after anaerobic growth than after aerobic growth (Fig. 2A). In contrast, expression of wzz_{fepE} showed a 2-fold induction when subjected to the same comparison (Fig. 2A). Of note, the oxygen-dependent regulation of wzz_{SE} and wzz_{fepE} was lost in the Δ*fnr* mutant (Fig. 2B). These observations are supported by the oxygen-dependent regulation of control genes *hybO* and *cyoA* [28,29] (Fig. 2A), that was lost in the Δ*fnr* mutant (Fig. 2B). Unfortunately, in the case of the Δ*arcA* mutant we were not able to find a housekeeping gene to validate eventual differences in the relative expression of genes wzz_{SE} and wzz_{fepE} under anaerobic and aerobic conditions.

Next, we evaluated if the dramatic effect of oxygen availability on VL-OAg production and wzz_{fepE} expression correlates with variations in Wzz_{fepE} protein levels. To this end, we evaluated the abundance of Wzz_{fepE} under aerobic and anaerobic conditions using translational fusions to the 3xFLAG epitope (Fig. 3). The relative amounts of Wzz_{fepE} detected by Western blot were determined by densitometry analysis and values were normalized to the total amount of protein loaded in the corresponding lane of a Coomassie-blue stained gel analyzed in parallel. In agreement with the LPS profile of the wild-type strain, the amount of Wzz_{fepE} produced under anaerobiosis was higher (70% increase) than that produced under aerobiosis (Fig. 3). In addition, the amount of Wzz_{fepE} produced by the Δ*arcA* mutant was similar to the level exhibited by the wild-type strain grown under aerobiosis. Of note, no oxygen-dependent regulation of Wzz_{fepE} production was observed in this mutant. The same was observed in the Δ*fnr* mutant; however, in this case the amount of Wzz_{fepE} produced was similar to that of the wild-type strain under anaerobiosis (Fig. 3).

3.3. Overexpression of wzz_{fepE} restores bi-modal distribution of OAg in aerobiosis and in the ΔarcA mutant grown in anaerobiosis

As shown, oxygen availability dramatically affects the amount of VL-OAg produced by *S.* Enteritidis. In addition, deletion of *arcA* results in the production of minimal amounts of VL-OAg. Furthermore, these phenotypes correlate with the expression levels of wzz_{fepE} and the production of the encoded protein. According to this idea, one could expect that overexpression of wzz_{fepE} would bypass the effect of ArcA and oxygen availability on VL-OAg production. To test this, the wild type and Δ*arcA* mutant were transformed with a plasmid including wzz_{fepE}. Production of VL-OAg under aerobiosis was increased when the wild-type strain overexpressed wzz_{fepE} (Fig. 4A). Also, the Δ*arcA* mutant overexpressing wzz_{fepE} produced similar levels of VL-OAg under aerobic and anaerobic conditions (Fig. 4B). These results indicate that reduced production of VL-OAg results directly from reduced levels of Wzz_{fepE}. In addition, a reduction in L-OAg chain lengths was observed when bacteria overproduced Wzz_{fepE} (Fig. 4).

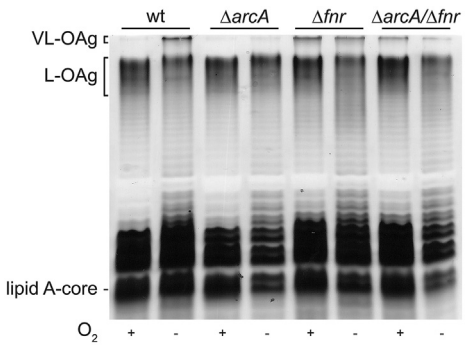

Fig. 1. Effect of oxygen availability and *arcA* and *fnr* deletion on OAg modal distribution. LPS samples obtained from bacteria grown in the presence (+O_2) or absence (- O_2) of oxygen were analyzed by Tricine-SDS-PAGE.

4. Discussion

In this study, we describe that ArcA and Fnr are involved in the oxygen-dependent control of OAg chain-length distribution in *S.* Enteritidis. A simultaneous and opposing change in the production levels of L-OAg and VL-OAg was observed in response to oxygen availability. Of note, LPS profiles showed that *S.* Enteritidis cultured aerobically in minimal medium was almost devoid of VL-OAg. To our knowledge, this phenotype has not been described for any bimodal serovar of *Salmonella enterica*. Interestingly, our unpublished data show that the effect of oxygen is not observed in bacteria grown in LB, indicating a direct influence of culture medium on the LPS modal distribution in *S.* Enteritidis.

Our data also indicate that the described OAg chain-length modulation occurs by controlling the expression of genes encoding the Wzz regulators in response to oxygen availability. Thus, the increased production of VL-OAg and concomitant decrease in L-OAg production observed during anaerobic vs. aerobic growth (Fig. 1) correlate with the expression levels of wzz_{fepE} and wzz_{SE} under both conditions (Fig. 2). In addition, the lack of oxygen-dependent control of OAg modal distribution in the Δ*fnr* and Δ*arcA* mutants (Fig. 1) correlates with a deregulated expression of wzz_{fepE} and wzz_{SE} in response to oxygen availability (Fig. 2). Therefore, Fnr and ArcA participate in the oxygen-mediated control

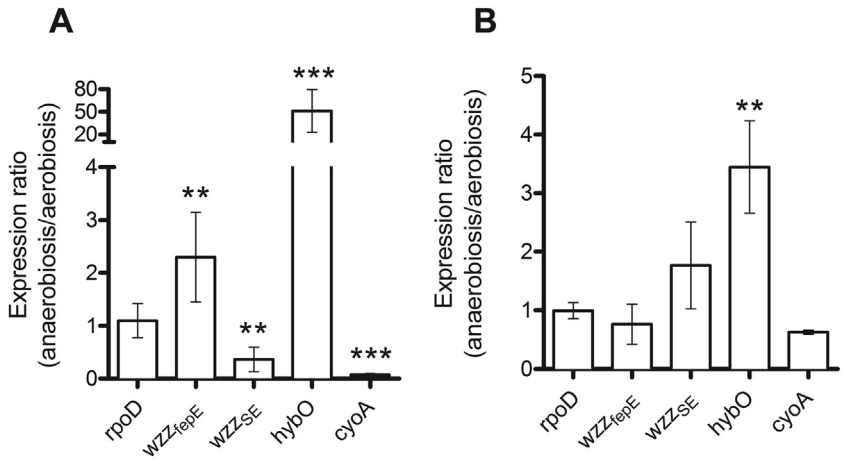

Fig. 2. Effect of oxygen availability and *fnr* deletion on the expression of genes wzz_{SE} and wzz_{fepE}. Total RNA samples were obtained from aerobic and anaerobic cultures of wild type (A) and Δ*fnr* (B) strains, and the anaerobic/aerobic ratio of mRNA levels was obtained for each gene. Oxygen-regulated genes *cyoA* and *hybO* were used as controls. Bars represent mean values from three independent replicates. Error bars denote standard errors. Statistical significance of observed differences was determined using tools implemented in REST software (**, P < 0.01; ***, P < 0.001).

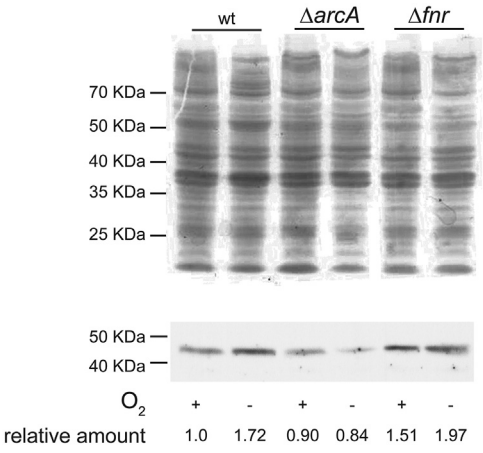

Fig. 3. Effect of oxygen availability and *arcA* and *fnr* deletion on the Wzz_{fepE} level. Total protein samples were obtained from bacteria grown in the presence (+O_2) or absence (- O_2) of oxygen. Samples were resolved by SDS-PAGE for Coomassie blue staining and detection of Wzz_{fepE}-3xFLAG by Western blot. The signal intensity of the fusion protein under each condition was determine by densitometry, to calculate its relative abundance with respect to the wild type grown aerobically. These values were normalized to total protein loaded as determined by densitometry of each whole lane.

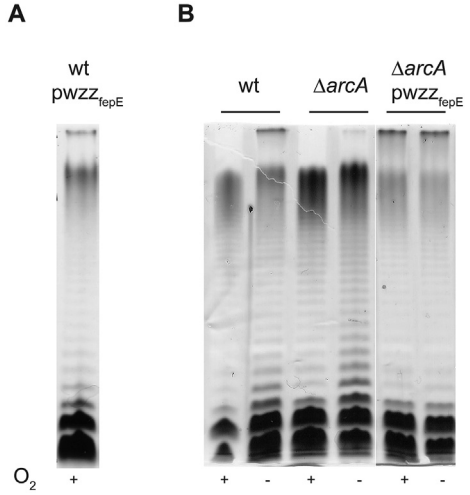

Fig. 4. Effect of wzz_{fepE} overexpression on OAg modal distribution. Wild type (A) and Δ*arcA* (B) strains were transformed with a multicopy plasmid carrying the wzz_{fepE} gene. LPS samples obtained from bacteria grown in the presence or absence of oxygen were analyzed by Tricine-SDS-PAGE.

of VL-OAg production, probably by controlling the transcription of wzz_{fepE} and wzz_{SE}. In support of this hypothesis, our bioinformatics analyses revealed the presence of a putative binding sites for Fnr at position −262, and three putative binding sites for ArcA at positions −13, −85 and −107 with respect to the potential transcription initiation site of wzz_{fepE} (Fig. S1). In the case of wzz_{SE}, we also identified a putative binding site for Fnr at position −14, and three putative binding sites for ArcA at positions −117, −208 and −314, with respect to its assumed transcription initiation site (Fig. S1). Several genes regulated by oxygen availability in *Escherichia coli*, such as *cydAB* [30], *cyoABCDE*, *fixABCX* [28] and *dpiBA* [31], also possess similarly-positioned Fnr and ArcA biding sites. Moreover, both Fnr and ArcA are able to bind *in vitro* to the promoter regions of wzz_{fepE} and wzz_{SE} in *S.* Enteritidis (Fig. S2), suggesting a direct role for these regulators in the control of wzz_{fepE} and wzz_{SE} transcription.

On the other hand, the Δ*fnr* Δ*arcA* and the Δ*fnr* mutants produced similar levels of VL-OAg under aerobic conditions. This indicates that the LPS phenotype associated to the Δ*fnr* allele does not depend on the presence of a functional ArcA. In contrast, the LPS phenotype associated to the Δ*arcA* allele requires the Fnr function. Therefore, these observations reveal a functional interplay between ArcA and Fnr to control the OAg modal distribution in *S.* Enteritidis in response to oxygen availability. In agreement with our results, both ArcA and Fnr regulate several genes in *E. coli* [13,16,32]. In some cases, this regulation cannot be explained only by simple additive effects of each regulator, suggesting complex functional interactions between ArcA and Fnr [16]. Thus, how ArcA and Fnr influence the expression of wzz_{fepE} and wzz_{SE} remains unknown. We are currently performing site-directed mutagenesis of putative binding sites and protein binding experiments to gain insight into the mechanism of the oxygen-dependent transcriptional regulation of these genes.

Previously, we demonstrated that the relative levels of chain-length regulators WzzB and Wzz_{pHS-2} are critical to determine the OAg modal distribution in *Shigella flexneri* 2a [27]. We also showed that both regulators compete to control the degree of OAg

polymerization. Thus, increase of one modal length results in the concomitant decrease of the other [27]. Here, we showed that the relative amounts of L-OAg and VL-OAg in *S.* Enteritidis also changed in an opposite way in response to oxygen availability, so that the decrease in L-OAg levels during anaerobiosis may be the result of an increase in VL-OAg levels. In support of this, when *S.* Enteritidis overproduced Wzz_{fepE}, not only an increment in VL-OAg, but also a reduction in L-OAg production was observed (Fig. 4).

At present, we can only speculate on the physiological role of the described oxygen-dependent control of OAg modal distribution and its association to virulence in *Salmonella*. For instance, given that VL-OAg protects *Salmonella* serovars from lytic action of serum complement [5] and egg albumen [4], increased amounts of these molecules under oxygen limitation could contribute to survival of *S.* Enteritidis in blood, eggs and during systemic spread in different hosts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.06.074.

Transparency document

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