Growth-phase regulation of lipopolysaccharide O-antigen chain length influences serum resistance in serovars of *Salmonella*

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The amount of lipopolysaccharide (LPS) O antigen (OAg) and its chain length distribution are important factors that protect bacteria from serum complement. *Salmonella enterica* serovar Typhi produces LPS with long chain length distribution (L-OAg) controlled by the *wzz* gene, whereas serovar Typhimurium produces LPS with two OAg chain lengths: an L-OAg controlled by Wzz_{ST} and a very long (VL) OAg determined by Wzz_{fepE}. This study shows that serovar Enteritidis also has a bimodal OAg distribution with two preferred OAg chain lengths similar to serovar Typhimurium. It was reported previously that OAg production by *S.* Typhi increases at the late exponential and stationary phases of growth. The results of this study demonstrate that increased amounts of L-OAg produced by *S.* Typhi grown to stationary phase confer higher levels of bacterial resistance to human serum. Production of OAg by serovars Typhimurium and Enteritidis was also under growth-phase-dependent regulation; however, while the total amount of OAg increased during growth, the VL-OAg distribution remained constant. The VL-OAg distribution was primarily responsible for complement resistance, protecting the non-typhoidal serovars from the lytic action of serum irrespective of the growth phase. As a result, the non-typhoidal species were significantly more resistant than *S.* Typhi to human serum. When *S.* Typhi was transformed with a multicopy plasmid containing the *S.* Typhimurium wzz_{fepE} gene, resistance to serum increased to levels comparable to the non-typhoidal serovars. In contrast to the relevant role for high-molecular-mass OAg molecules, the presence of Vi antigen did not contribute to serum resistance of clinical isolates of serovar Typhi.

INTRODUCTION

Lipopolysaccharide (LPS) plays a critical role in the pathogenesis of infections by *Salmonella*. LPS consists of three structural domains: the lipid A region, which is embedded in the outer membrane, the core oligosaccharide and the O-specific polysaccharide chain or O antigen (OAg), which is exposed on the bacterial surface (Raetz & Whitfield, 2002). Lipid A has been extensively investigated as it causes most of the toxic and other biological effects of LPS; for this reason lipid A is also referred to as endotoxin (Raetz & Whitfield, 2002). The OAg plays an important role in colonization of host tissues (Hong & Payne, 1997;

Murray *et al.*, 2003; Nevola *et al.*, 1985; Skurnik *et al.*, 1999) and in resistance to complement-mediated serum killing in several Gram-negative bacteria (Bengoechea *et al.*, 2004; Clas & Loos, 1981; Grossman *et al.*, 1987; Joiner, 1988; Joiner *et al.*, 1986; Liang-Takasaki *et al.*, 1983; Mäkelä *et al.*, 1988; Murray *et al.*, 2003; Saxén *et al.*, 1987; Tomas *et al.*, 1986).

The number of O units that are attached to the lipid A-core is regulated by the chain length determinant Wzz (Rol, Cld) in an as yet unknown manner. *Salmonella enterica* serovar Typhi has a preferred chain length of about 16–35 repeats that is controlled by the *wzz* gene located downstream of the *wba* locus (Parkhill *et al.*, 2001). In contrast, *Salmonella* Typhimurium possesses two functional *wzz* genes, resulting in a bimodal O-antigen length distribution: a long LPS (L-OAg) with 16–35 O-antigen units and a very long LPS (VL-OAg) estimated to contain

more than 100 repeat units. The L-modal OAg length is determined by the Wzz protein encoded by the wzz_{ST} gene located adjacent to the wba locus, and the VL-OAg is controlled by the wzz_{fepE} gene, a homologue of *Escherichia coli fepE* (Bastin *et al.*, 1993; Batchelor *et al.*, 1992; McClelland *et al.*, 2001; Morona *et al.*, 2003; Murray *et al.*, 2003). On the other hand, two preferred lengths have been described in smooth isolates of *Salmonella* Enteritidis: a high-molecular-mass LPS, which has more than 11 OAg units, and a low-molecular-mass LPS that has an average OAg length of 5 units (Guard-Petter, 1998; Rahman *et al.*, 1997).

It has been reported that the proper distribution of OAg chain lengths is required for complement resistance in species of *Salmonella* (Grossman *et al.*, 1987; Hoare *et al.*, 2006; Murray *et al.*, 2003, 2005, 2006). However, not much is known about environmental regulation of OAg modal distribution and its effect on serum resistance. An early study indicated that *Salmonella anatum* OAg size and distribution are affected by the growth temperature (McConnell & Wright, 1979). More recently, Murray *et al.* (2005) reported that incubation of *S.* Typhimurium in heat-inactivated guinea pig serum induced the production of VL-OAg by *S.* Typhimurium, conferring a higher level of survival in serum. The same effect was observed when bacteria were grown under iron-limiting conditions; however, a wzz_{fepE}-*lacZ* transcriptional fusion failed to show a significant change in expression under this growth condition. Thus, these reports did not determine the mechanisms responsible for the observed changes in LPS production. More recently, it has been demonstrated that expression of the *S.* Typhimurium OAg chain length determinant wzz_{ST} is induced in low Mg^{2+} plus Fe^{3+} medium, in a pathway that requires the PhoP/PhoQ and PmrA/PmrB two-component systems (Delgado *et al.*, 2006).

Previous work from our laboratory demonstrated that production of OAg by *S.* Typhi Ty2 is regulated during bacterial growth, increasing at the late exponential and stationary phases. Increased amounts of OAg produced during stationary phase correlated with an increase in expression of the transcription elongation factor RfaH, which allows complete transcription of the *wba* operon (Bittner *et al.*, 2002; Rojas *et al.*, 2001). In this study, we show that OAg production by *S.* Typhimurium and *S.* Enteritidis is also growth-regulated; however, while the L-OAg modal distribution increases during growth, the amount of VL-OAg molecules remains relatively constant. Growth-regulation of L-OAg influences serum resistance of *S.* Typhi, conferring higher levels of protection to bacteria grown to stationary phase. The presence of VL-OAg in the non-typhoidal serovars protects bacteria from the lytic action of serum complement, irrespective of the growth phase. Expression of VL-OAg by *S.* Typhi increases serum resistance to levels comparable to those of the non-typhoidal serovars. In contrast, the Vi capsule does not contribute further to resistance to serum complement.

METHODS

Bacterial strains, plasmids, media and growth conditions. Table 1 summarizes the properties of the bacterial strains and plasmids used in this study. Bacteria were grown aerobically at 37 °C in Luria–Bertani medium (LB) to exponential (OD_{600} 0.1) or stationary (OD_{600} 1.8–2) phase. Growth curves and details of growth conditions are shown in Supplementary Fig. S1, available with the online version of this paper. Where appropriate, media were supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}).

Mutagenesis of *wzz* and *tviC* genes. Mutagenesis was performed by the method described by Datsenko & Wanner (2000) to construct chromosomal deletions by homologous recombination using PCR products. To disrupt *tviC*, which encodes UDP-GlcN-AcA 4-epimerase that is involved in Vi capsule synthesis, from *S.* Typhi and the chain-length determinant genes from *S.* Typhi Ty2 (*wzz*), *S.* Typhimurium LT2 (wzz_{ST} and wzz_{fepE}) and *S.* Enteritidis PT4 (wzz_{SE} and wzz_{fepE}), cells were first transformed with the thermosensitive plasmid pKD46, which expresses the λ Red recombinase system, and subsequently transformed with PCR products that were generated using pKD4 , which contains an FRT-flanked kanamycin resistance gene (*aph*), as a template. Each primer pair also carried 30 bases that were homologous to the edge of the gene targeted for disruption. The sequences of the oligonucleotide primers used in this study are shown in Supplementary Table S1. In the presence of the Red recombinase system, the integration of the amplicons resulted in the targeted replacement of the wild-type gene by the antibiotic resistance cassette. The Kan^R transformants were replica plated in the absence of selective antibiotics at 42 °C, and finally assayed for ampicillin sensitivity to confirm the loss of pKD46. To obtain non-polar deletion mutants, the antibiotic resistance gene was removed by transforming the gene replacement mutants with pCP20, which encodes the FLP recombinase (Cherepanov & Wackernagel, 1995). Transformants were plated on LB agar plates containing ampicillin and kanamycin at 30 °C. Individual colonies were replica plated on LB agar plates, on LB agar plates containing ampicillin and on LB agar plates containing kanamycin and incubated at 42 °C. Transformants that had lost the resistance gene (*aph*) and plasmid pCP20 were selected as those colonies that were able to grow only on LB agar plates. Correct insertional gene replacements and the deletion of the antibiotic gene cassette were confirmed by PCR analysis.

LPS analysis. Culture samples obtained at different times during growth were adjusted to an OD_{600} 2.0 in a final volume of 1.5 ml LB. Cells were centrifuged and the pellets were suspended in 150 μl of lysis buffer containing proteinase K, followed by hot phenol extraction and a subsequent extraction of the aqueous phase with diethylether. LPS was separated on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system and visualized by silver staining (Marolda *et al.*, 2006). Densitometry analysis was performed using the UN-SCANT-IT gel software (Silk Scientific). To perform this analysis, LPS samples were separated on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system (Marolda *et al.*, 2006) to visualize the unsubstituted lipid A-core region. The ratio of the relative intensity of the lipid A-core band to the average intensity of the bands corresponding to total OAg and VL-OAg was calculated by quantifying the pixels in a narrow window across the centre of each lane. The densitometric analysis was calibrated by determining the ratio of the relative intensity of the lipid A-core region relative to the average intensity of the OAg bands using a range of loading volumes of LPS samples. As shown in Supplementary Fig. S2, the total pixels increased linearly with respect to the volume loaded, indicating that there is no saturation of bands in the range of loading volumes (from 1–4 μl) used. Representative densitograms are shown in Supplementary Fig. S3.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Source or reference
S. enterica serovar Typhi		
Ty2	Wild-type	ISP†
M985	Ty2 Δwzz	Hoare *et al.* (2006)
Ty2/pJC142	Ty2/pGEMwzz_{fepE}, Amp^R	This study
M985/pJC142	Ty2 Δwzz/pGEMwzz_{fepE}, Amp^R	This study
CC761	Clinical isolate, Vi$^+$	Hospital de San Bernardo, Chile
CC737	Clinical isolate, Vi$^-$	Hospital de San Bernardo, Chile
CC761$\Delta tviC$	CC761$\Delta tviC$::aph, Kan^R, Vi$^-$	This study
S. enterica serovar Typhimurium		
LT2	Wild-type	ISP†
MTM4	LT2 Δwzz_{ST}	This study
MTM113	LT2 Δwzz_{fepE}	This study
SL1344	Wild-type, virulent strain	Dr G. Mora
S. enterica serovar Enteritidis		
PT4 NCTC 13349	Wild-type	Sanger Center
MSE4	PT4 Δwzz_{ST}	This study
MSE113	PT4 Δwzz_{fepE}	This study
Di62	Clinical isolate	Dr C. Toro
Plasmids		
pKD46	*bla* P$_{BAD}$ *gam bet exo* pSC101 oriTS	Datsenko & Wanner (2000)
pKD4	*bla* FRT *aph* FRT PS1 PS2 oriR6K	Datsenko & Wanner (2000)
pCP20	*bla cat cI*857 λP$_R$ *flp* pSC101 oriTS	Cherepanov & Wackernagel (1995)
pGEM-T Easy	TA cloning vector	Promega
pJC142	*fepE* cloned into pGEM-T Easy	This study

*Amp^R, ampicillin resistant; Kan^R, kanamycin resistant.
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Western blot analyses were performed as described by Marolda *et al.* (2006). Briefly, LPS was transferred to a PVDF membrane for 75 min at 250 mA and blocked for 90 min in 5 % (w/v) skim milk at room temperature. The membrane was incubated overnight with a polyclonal rabbit antiserum against *Salmonella* group D1 or group B (Probac do Brasil, Produtos Bacteriológicos) as a primary antibody, and horseradish peroxidase-conjugated, goat anti-rabbit (Pierce) as a secondary antibody. Detection was performed using the SuperSignal West Pico chemiluminiscent substrate (Pierce).

Serum resistance. Human serum samples obtained from healthy donors (provided by the blood bank of the Clinical Hospital from the University of Chile) devoid of antibodies against *Salmonella* group D1 and *Salmonella* group B, were pooled, divided into small aliquots and stored at -70 °C until use. Bacteria were grown in LB medium to exponential or stationary phase (OD$_{600}$ 0.1 and 1.8–2, respectively). Culture samples were diluted in LB medium to 10^3 c.f.u. ml^{-1} and 20 µl was mixed with 20 µl serum, diluted in LB to 0, 2.5, 5, 10, 20 and 40 % final concentration. After 20 min incubation at 37 °C, complement function was stopped by the addition of 60 µl cold LB medium. Tubes were kept on ice until the full contents of each tube were plated onto LB agar plates to determine viable bacterial numbers. Resistance to serum was calculated as percentage survival, taking as 100 % the bacterial counts obtained after incubation in LB (0 % serum). Each experiment was carried out in duplicate in at least three independent assays. Data are expressed as means $\pm$ SE.

RESULTS AND DISCUSSION

Growth-phase-dependent regulation of O-antigen synthesis in *Salmonella* serovars

Previous work from our laboratory demonstrated that the production of OAg by *S.* Typhi increases at the onset of stationary phase and correlates with a growth-regulated expression of the transcription elongation factor RfaH (Bittner *et al.*, 2002; Rojas *et al.*, 2001). To investigate whether LPS production is subject to growth-phase regulation in the non-typhoid serovars Typhimurium and Enteritidis, we analysed the LPS profiles in silver-stained polyacrylamide gels of bacterial cells collected at different stages of growth. Gel loadings were normalized by the bacterial density at the starting point of the LPS preparations, as described in Methods. In accordance with our previous results (Bittner *et al.*, 2002; Rojas *et al.*, 2001), the amount of OAg produced by serovar Typhi increased throughout the bacterial growth cycle (Fig. 1a, left panel, lanes 1–4). The densitometric quantification of the lanes in the gel (Fig. 1a, right panel) showed a significant increase in the ratio of OAg to lipid A-core region in the sample grown to stationary phase (OD$_{600}$ 1.8) compared to the sample grown to exponential phase (OD$_{600}$ 0.1). In *S.*
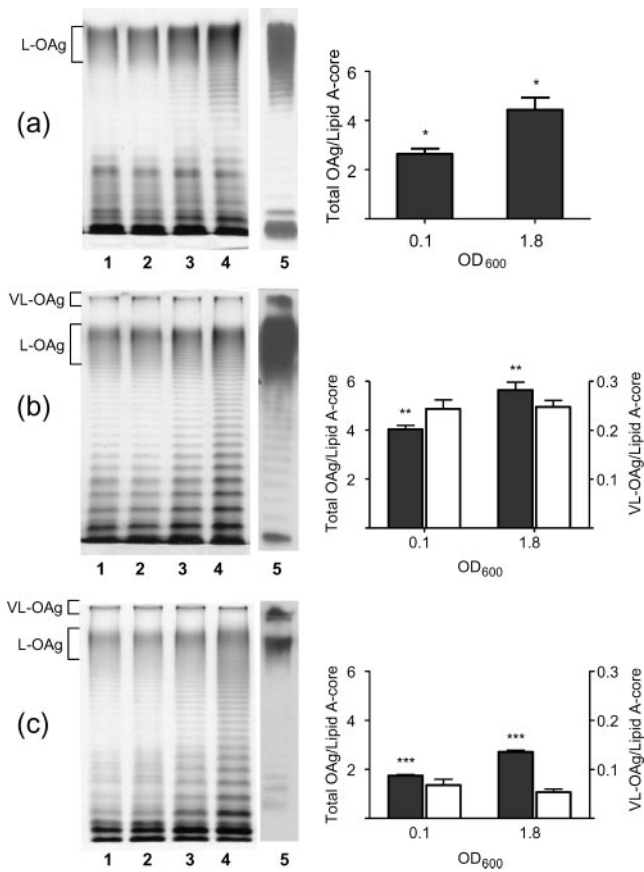
Fig. 1. Production of LPS by *Salmonella* serovars (a) Typhi, (b) Typhimurium and (c) Enteritidis during growth. Left panels show the LPS profiles of bacterial cells grown in LB to the following OD_{600} values: lane 1, 0.1; lane 2, 0.4; lane 3, 0.7; lane 4, 1.8. Samples from equal numbers of bacterial cells were loaded in each lane and analysed on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system. Brackets indicate the long (L) and very long (VL) chain length distributions. Lane 5 shows Western blot analysis of LPS production by bacteria grown to stationary phase. Right panels show densitometry analysis of the levels of total-OAg (black bars) or VL-OAg (white bars) relative to the lipid A-core region at exponential (OD_{600} 0.1) and stationary phase (OD_{600} 1.8). Densitometry analysis was performed in four independent gels. Means ± SE are shown. Asterisks, significant differences determined by the one-way ANOVA and Tukey post test (*, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$).

confirmed that these bands contained LPS OAg by Western blotting using an anti-group D1-specific antibody (Fig. 1c, left panel, lane 5). Western blots were also performed using anti-group D1-specific and anti-group B-specific antibodies for detecting LPS OAg from serovar Typhi (Fig. 1a, left panel, lane 5) and serovar Typhimurium (Fig. 1b, left panel, lane 5), respectively. A differential growth-phase-regulation of OAg synthesis was observed in *S.* Enteritidis, similar to serovar Typhimurium. This was confirmed by densitometric quantification of the lanes in the gels. In both serovars, the amount of total OAg relative to the lipid A-core region increased significantly during growth, yet no significant differences in the ratio of VL-OAg to lipid A-core were observed (Fig. 1b, c, right panels).

At the moment, we can only speculate how environmental conditions could differentially modulate the OAg modal distribution; however, several reports have begun to unravel a variety of mechanisms involved in OAg chain length regulation. It has been shown in *S.* Typhimurium that while wzz_{ST} expression is regulated in response to conditions of low Mg^{2+} plus Fe^{3+} through the PmrA/PmrB and RcsC/YojN/RcsB systems (Delgado *et al.*, 2006), wzz_{fepE} expression is regulated by conditions that stimulate swarming motility (Wang *et al.*, 2004). Additional control of wzz_{fepE} expression by the flagellar master regulator FlhDC has been reported in *E. coli* (Stafford *et al.*, 2005). In addition, genome-wide analysis of the DNA adenine methyltransferase (Dam) regulon in *E. coli* described *wzzB* as one of many genes found to be repressed by the presence of this regulator (Robbins-Manke *et al.*, 2005). Since Dam levels are downregulated in stationary phase (Seshasayee, 2007), the increase of *wzzB* transcription could be a result of diminished levels of Dam.

In a previous study, we demonstrated a differential growth-phase regulation of OAg production in *Shigella flexneri* 2a (Carter *et al.*, 2007). We showed that, while VL-OAg increased significantly during growth, the short LPS (S-OAg) distribution remained relatively constant. VL-OAg production correlated with growth-dependent regulation of the *rfaH* gene. Our results also indicated that RfaH is essential for expression of the *wzy* polymerase gene, but not for *wzzB* or wzz_{pHS-2}, suggesting that the cellular levels of Wzy are critical for VL-OAg production and normal OAg chain-length distribution in *Shigella flexneri* 2a. In *Salmonella*, the *wzy* gene is a single transcriptional unit and thus it is probably not regulated by RfaH. Therefore, at present we do not have any insights about the mechanisms of differential growth-phase regulation of OAg modal distribution in serovars of *Salmonella*.

Effect of growth-dependent regulation of O-antigen on serum resistance

As described above, the wild-type LT2 strain grown to stationary phase expressed higher amounts of low-molecular-mass OAg molecules and of L-OAg than bacteria grown to exponential phase, whereas VL-OAg levels remained

Typhimurium LT2 (Fig. 1b, left panel, lanes 1–4) growth-phase-dependent regulation of OAg synthesis was also observed; however, while the amount of low-molecular-mass OAg molecules and of the L-OAg modal distribution increased during growth, the amount of VL-OAg chains remained relatively constant. Examination of LPS production by *S.* Enteritidis PT4 (Fig. 1c, left panel, lanes 1–4) revealed the presence of high-molecular-mass OAg molecules, similar to the VL-OAg distribution of serovar Typhimurium. To our knowledge, this modal length has not been described previously in serovar Enteritidis, so we

relatively constant (Fig. 2a, lanes 1 and 2). Similar growth-phase regulation of LPS production was observed in the virulent *S.* Typhimurium SL1344 strain (Fig. 2a, lanes 3 and 4), suggesting that differential regulation of OAg is not restricted to a particular strain, but rather is most likely a property of this serovar. Additionally, we studied the effect of growth phase on LPS production by deletion mutants in the wzz_{ST} and wzz_{fepE} genes. The LT2 Δwzz_{ST} mutant (strain MTM4) did not produce the L-OAg distribution but expressed higher amounts of random-length OAg when grown to stationary phase, compared to exponential phase. This mutant produced VL-OAg chains similar to the wild-type (Fig. 2a, lanes 5 and 6). On the other hand, the LT2 Δwzz_{fepE} mutant (strain MTM113) was devoid of VL-OAg while L-OAg distribution and the production of low-molecular-mass molecules was

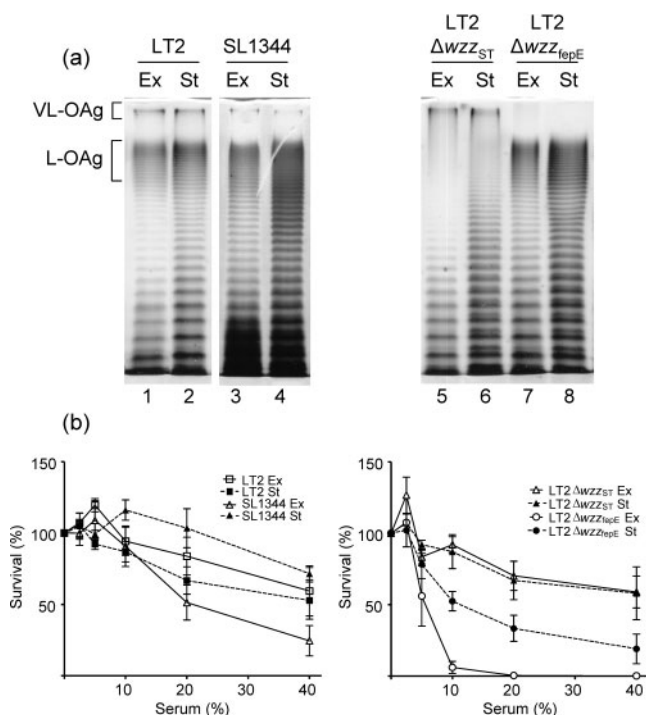growth-phase-regulated, as in the wild-type LT2 strain (Fig. 2a, lanes 7 and 8). The *S.* Enteritidis wild-type PT4 and Di62 strains and the PT4 isogenic Δwzz_{SE} (MSE4) and Δwzz_{fepE} (strain MSE113) mutants showed LPS patterns (Fig. 3a) that were similar to those of *S.* Typhimurium. Thus, differential regulation of OAg modal distribution appears to be a property common to both these *Salmonella* serovars.

To ascertain whether growth-phase regulation of LPS production influences resistance to the killing effect of human serum, bacteria grown to exponential or stationary phase were added to different concentrations of serum and assayed for serum resistance as described in Methods. The results showed that the wild-type *S.* Typhimurium LT2 and its isogenic Δwzz_{ST} mutant (strain MTM4) were quite

Fig. 2. Effect of growth phase on LPS production and serum resistance of wild-type *S.* Typhimurium strains and isogenic wzz_{ST} and wzz_{fepE} mutants. (a) LPS profiles of *S.* Typhimurium strains SL1344 and LT2 (left panel) and LT2 isogenic Δwzz_{ST} (MTM4) and Δwzz_{fepE} (MTM113) mutants (right panel). Samples from equal numbers of bacterial cells grown to exponential (Ex) or stationary (St) phase were loaded in each lane and analysed on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system. Brackets indicate the long (L) and very long (VL) chain length distributions. (b) Serum resistance of wild-type *S.* Typhimurium strains SL1344 and LT2 (left panel) and LT2 isogenic Δwzz_{ST} (MTM4) and Δwzz_{fepE} (MTM113) mutants (right panel). Bacteria grown to exponential or stationary phase were incubated for 20 min in 2.5, 5, 10, 20 or 40 % human serum as described in Methods. Means ± SE are shown.
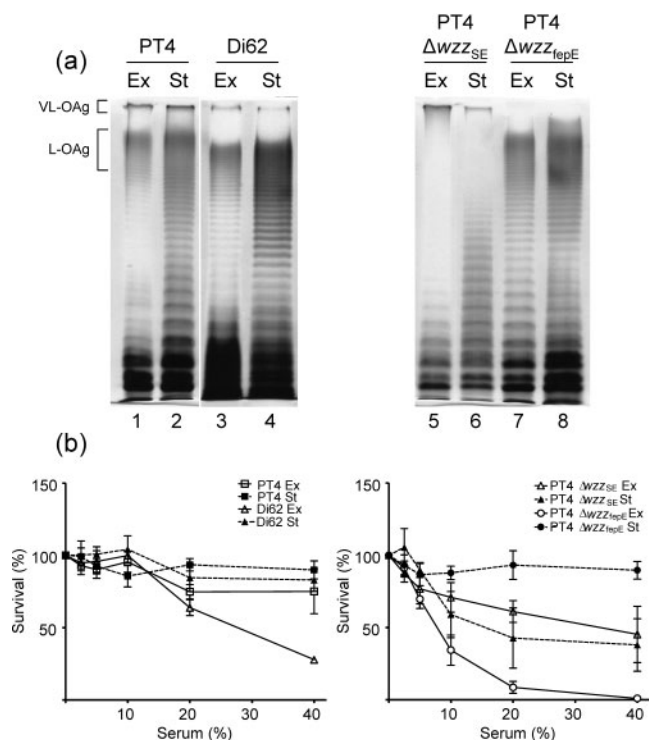
Fig. 3. Effect of growth phase on LPS production and serum resistance of wild-type *S.* Enteritidis strains and isogenic wzz_{SE} and wzz_{fepE} mutants. (a) LPS profiles of *S.* Enteritidis strains Di62 and PT4 (left panel) and PT4 isogenic Δwzz_{SE} (MSE4) and Δwzz_{fepE} (MSE113) mutants (right panel). Samples from equal numbers of bacterial cells grown to exponential (Ex) or stationary (St) phase were loaded in each lane and analysed on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system. Brackets indicate the long (L) and very long (VL) chain length distributions. (b) Serum resistance of wild-type *S.* Enteritidis strains Di62 and PT4 (left panel) and PT4 isogenic Δwzz_{SE} (MSE4) and Δwzz_{fepE} (MSE113) mutants (right panel). Bacteria grown to exponential or stationary phase were incubated for 20 min in 2.5, 5, 10, 20 or 40 % human serum as described in Methods. Means ± SE are shown.

resistant to the lytic action of complement, showing over 70 % survival after 20 min of incubation in 20 % serum, irrespective of the growth phase (Fig. 2b). In contrast, *S.* Typhimurium MTM113 (Δwzz_{fepE}) was very sensitive to serum killing, particularly when grown to exponential phase; no survivors were recovered after 20 min of incubation in 20 % serum compared to approximately 30 % survival of bacteria grown to stationary phase (Fig. 2b). The virulent strain SL1344 was highly resistant to the lytic action of complement when grown to stationary phase, similar to LT2 (Fig. 2b, left panel). Bacteria grown to exponential phase were significantly more sensitive; nevertheless, over 50 % survival after 20 min incubation in 20 % serum was obtained (Fig. 2b, left panel). These results indicate that, although small differences may occur among different strains, serovar Typhimurium is quite resistant to human serum irrespective of differences in virulence.

Our results demonstrate the importance of VL-OAg molecules in protection of *S.* Typhimurium from the bactericidal action of human complement. They also indicate that the growth-regulated amount of L-OAg is relevant to serum resistance when VL-OAg distribution is absent. Our data are in contrast to findings by Murray *et al.* (2005) indicating that the wzz_{ST} gene is primarily responsible for serum resistance of serovar Typhimurium strain C5, whereas wzz_{fepE} provides little serum resistance alone. These authors reported an important role for the VL-OAg distribution either when it was induced by environmental conditions such as incubation in serum or under iron limitation, or when the wzz_{fepE} was over-expressed. In this study we clearly show that the VL-OAg distribution produced by aerobically grown bacteria in LB confers serum resistance to serovar Typhimurium at both exponential and stationary phases of growth. The amount of L-OAg further contributes to resistance to human serum. The differences between our results and those of Murray *et al.* (2005) could be due to differences in growth conditions or strains used in the two studies and/or to the use of guinea pig serum instead of human serum in the study by Murray *et al.* (2005).

Similar to *S.* Typhimurium, the wild-type PT4 and Di62 strains of *S.* Enteritidis were highly resistant to complement; approximately 70 % survival was obtained after 20 min incubation in 20 % serum, irrespective of the growth phase (Fig. 3b, left panel). However, the relative impact of the two *wzz* genes of serovar Enteritidis on serum resistance was different from that in *S.* Typhimurium LT2. Deletion of the PT4 wzz_{SE} gene caused a considerable reduction in the ability to resist the lytic action of serum at both exponential and stationary phases (Fig. 3b, right panel). On the other hand, the Δwzz_{fepE} mutant (strain MSE113) was as resistant as the wild-type when grown to stationary phase, but it was highly sensitive when grown to exponential phase (Fig. 3b, right panel). These results indicate that the amount of L-OAg is crucial for survival of *S.* Enteritidis while the VL-OAg modal distribution

contributes to serum resistance when a small amount of L-OAg is produced.

Expression of VL-OAg increases serum resistance of *S.* Typhi

To confirm the significance of high-molecular mass OAg in resistance of *Salmonella* to complement-mediated killing, we transformed *S.* Typhi Ty2 and its isogenic Δwzz mutant with the *S.* Typhimurium wzz_{fepE} gene cloned in pGEM-T (pJC142). In *S.* Typhi wzz_{fepE} is a pseudogene (Deng *et al.*, 2003) and thus this serovar cannot synthesize VL-OAg (Fig. 4a, lanes 1 and 2). The complemented Ty2/pJC142 strain produced VL-OAg as in *S.* Typhimurium, whereas expression of L-OAg was similar to that of the wild-type Ty2 strain (Fig. 4a, lanes 3 and 4). The Δwzz mutant (M985) showed a random distribution of OAg lengths (Fig. 4a, lanes 5 and 6), while the complemented M985/pJC142 strain showed a similar LPS profile but expressed
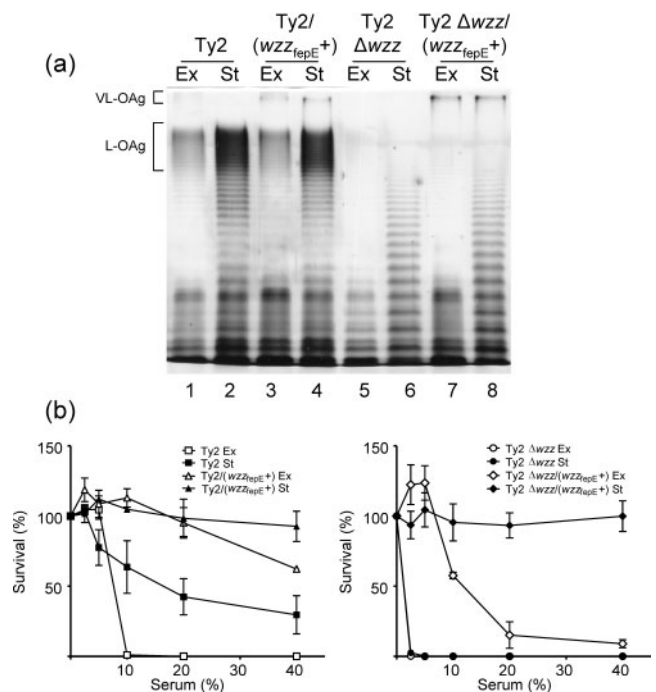

Fig. 4. Effect of expression of VL-OAg on serum resistance of *S.* Typhi Ty2 and its isogenic *wzz* mutant during growth. (a) LPS samples from equal numbers of bacterial cells grown to exponential (Ex) or to stationary (St) phase were loaded in each lane and were analysed on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system. Brackets indicate the long (L) and very long (VL) chain length distributions. (b) Serum resistance of wild-type *S.* Typhi strains Ty2 and Ty2/(wzz_{fepE}^+) (left panel) and Ty2 Δwzz and Ty2 Δwzz/(Δwzz_{fepE}^+) mutants (right panel). Samples of bacteria grown to exponential or stationary phase were incubated for 20 min in 2.5, 5, 10, 20 or 40 % human serum as described in Methods. Means $\pm$ SE are shown.

VL-OAg (Fig. 4a, lanes 7 and 8). Higher amounts of OAg were produced at the stationary phase of growth.

The effect of growth-phase regulation of OAg on serum resistance is shown in Fig. 4(b). *S.* Typhi Ty2 grown to exponential phase was highly susceptible to the bactericidal action of serum; no survivors were recovered after 20 min incubation in 10 % serum. In contrast, stationary-phase-grown bacteria showed approximately 60 % survival under the same conditions, indicating that a higher density of OAg molecules confers a better level of protection against lysis by complement. The *S.* Typhi Δ*wzz* mutant was completely killed by serum irrespective of the growth stage (Fig. 4b, right panel), indicating that OAg with a proper distribution of chain lengths is required for serum resistance. As a result of this, in *S.* Typhi which is naturally devoid of VL-OAg, the presence and the amount of L-OAg are crucial for survival in serum. Expression of VL-OAg by Ty2/pJC142 increased serum resistance to levels comparable to those of the non-typhoidal serovars under both growth conditions (Fig. 4b, compare with Figs 2b and 3b). A different result was obtained in M985/pJC142. As shown in Fig. 4(c), the presence of VL-OAg protected the bacteria from complement, but a growth-phase-dependent behaviour was still observed: bacteria grown to stationary phase were completely resistant in up to 40 % serum, while bacteria grown to exponential phase showed only 10 % survival under this condition. This provides stronger evidence for the importance of the L-OAg distribution for serovar Typhi resistance to lysis by complement.

In summary, our data demonstrate that growth-regulation of OAg production and chain length distribution are central for complement resistance of *Salmonella* serovars, and underline the importance of controlling the bacterial stage of growth to assay serum sensitivity. Although other growth effects cannot be ruled out, our results strongly suggest that the regulation of OAg modal distribution is central for serum resistance. This notion is supported mainly by two observations: (i) serum resistance is growth-phase independent in non-typhoidal serovars expressing VL-OAg (Figs 2b and 3b), and (ii) serum resistance of *S.* Typhi Ty2 becomes growth-phase independent when complemented to produce VL-OAg (Fig. 4).

The *S.* Typhi Vi antigen does not contribute to serum resistance

Our results showing that serovar Typhi is more sensitive to the lytic action of human serum than the non-typhoidal serovars were unexpected considering that *S.* Typhi causes a systemic disease in its human host. Strain Ty2 does not express the Vi capsule due to the spontaneous loss of a portion of the pathogenicity island 7 (SPI7) that contains the *viaB* genes encoding the proteins for the biosynthesis and export of the Vi capsular antigen (Bueno *et al.*, 2004). Considering previous reports indicating that the Vi capsule may enhance survival of *S.* Typhi in serum (Hashimoto *et al.*, 1993; Looney & Steigbigel, 1986), we asked whether

the presence of Vi antigen confers additional protection from complement-mediated lysis. To this end, we tested the sensitivity of Vi⁻ and Vi⁺ clinical isolates of *S.* Typhi to serum (CC737 and CC761, respectively). Both strains were characterized by an agglutination assay with Vi-specific antisera. The clinical isolates showed LPS profiles (Fig. 5a) and abilities to resist the lytic action of serum (Fig. 5b) similar to those of the Ty2 strain at both exponential and stationary phases of growth. These results suggested that the Vi antigen does not contribute to survival of *S.* Typhi in human serum. To confirm this notion, we constructed a Vi⁻ mutant (CC761Δ*tviC*) from the clinical isolate CC761 by deletion of the *tviC* gene encoding UDP-GlcN-AcA 4-epimerase, involved in the synthesis of the Vi antigen (Hashimoto *et al.*, 1993). Both strains showed growth phase regulation of LPS production and serum sensitivity similar to the other serovar Typhi strains (Fig. 5a, b).

Thus, our results do not agree with previous studies indicating that capsulated strains of *S.* Typhi exhibit increased serum resistance (Hashimoto *et al.*, 1993; Looney & Steigbigel, 1986). Rather, they are in accordance with studies conducted in uropathogenic *E. coli* O75 : K5, which
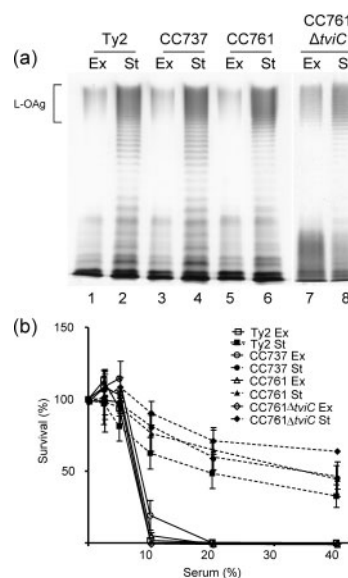

Fig. 5. Effect of Vi antigen production on serum resistance of *S.* Typhi. (a) LPS profiles of wild-type Ty2 (Vi⁻) and clinical isolates CC737 (Vi⁻), CC761 (Vi⁺), and its isogenic Vi⁻ mutant CC761Δ*tviC*. Samples from equal numbers of bacterial cells grown to exponential (Ex) or to stationary (St) phase were loaded in each lane and were analysed on 12 % (w/v) acrylamide gels using a Tricine-SDS buffer system. Brackets indicate the long (L) chain length distribution. (b) Serum resistance of wild-type Ty2 (Vi⁻) and clinical isolates CC737 (Vi⁻), CC761 (Vi⁺) and its isogenic Vi⁻ mutant CC761Δ*tviC*. Bacteria grown to exponential or stationary phase were incubated for 20 min in 2.5, 5, 10, 20 or 40 % human serum as described in Methods. Means ± SE are shown.

suggest that the capsular antigen plays a minor role in protecting bacteria from the bactericidal action of human serum (Burns & Hull, 1998).

The Vi capsular polysaccharide is thought to be expressed during infection, as indicated by protection against human typhoid fever following vaccination with the Vi antigen (Klugman et al., 1987) and by the observation that isolates from blood of patients almost always express the Vi antigen (Robbins & Robbins, 1984). Studies conducted recently to understand the pathogenesis of infection by S. Typhi have suggested new roles for the Vi antigen as a virulence factor. It has been demonstrated that expression of the viaB genes is induced during infection of human macrophages in vitro (Raffatellu et al., 2005), indicating a role of the Vi antigen during interaction of bacteria with host phagocytes. In addition, it has been suggested that the Vi capsule allows S. Typhi to downregulate a Toll-like-receptor-mediated host response in the intestinal mucosa that leads to neutrophil infiltration and consequently to evasion of innate immune recognition (Raffatellu et al., 2006). Thus, the Vi capsule may contribute to survival of serovar Typhi in human tissue or blood by a mechanism different from serum resistance.

Concluding remarks

Collectively, our results demonstrate that the LPS OAg is more important for survival in serum than the Vi antigen, and that regulation of OAg production and chain length distribution is crucial for protection of Salmonella from complement-mediated lysis. In addition, our data showing that non-typhoidal serovars are more resistant than serovar Typhi to human serum suggest that resistance to human serum does not contribute to host specificity or to pathogenesis of systemic disease by S. Typhi.

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