ABSTRACT

Title of Dissertation: STRUCTRE-FUNCTION RELATIONSHIPS OF PERIPLASMIC MEMBRANE-DERIVED OLIGOSACCHARIDES IN *SALMONELLA* GROWTH AND VIRULENCE

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Membrane-derived oligosaccharides (MDO) consist of branched substituted β-linked sugar chains that are present in the periplasmic space of *Escherichia coli* and other gram-negative bacteria. Their common features are the presence of glucose as a major constituent sugar and their increased levels in low-osmolarity media. In several phytopathogenic bacteria, mutants defective in MDO synthesis failed to incite disease on the host plant. Very little is known about the role of MDO from *Salmonella* in virulence

and osmotolerance. I have studied the structure-function relationship of MDO to understand if they play a role in *Salmonella* growth and virulence.

MDO defective mutants of *Salmonella* Typhimurium were generated using a gene specific mutagenesis protocol and MDO were isolated and their glycosyl composition analyzed. The fractions containing the major peak from Sephadex G-10 gel filtration chromatography were pooled and subjected to DEAE-cellulose anion exchange chromatography to separate charged and neutral MDO. Compositional analysis revealed that MDO of wild-type consist of 94% glucosyl residues (hereafter referred to as glucose) in *Salmonella* Typhimurium FIRN while MDO of the Δ*mdoG* mutant was comprised of only 24% glucose. Rhamnose, mannose, and galactose accounted for the rest. We also found that MDO composition varies in different chromosomal backgrounds. For example, glucose accounted for 41% of sugar residues in MDO of *Salmonella* Typhimurium SL1344. This proportion was further reduced to 24% in the Δ*mdoG* mutant.

Salmonella Typhimurium Δ*mdoG* mutants (in FIRN as well as SL1344 chromosomal backgrounds) displayed reduced virulence in mice. *Salmonella* Typhimurium SL1344 Δ*mdoG* mutant strain was recovered from the intestinal tissues and systemic tissues at a lower frequency than its parental wild-type strain and displayed a reduced ability of intracellular replication in macrophages. This defect in the Δ*mdoG* mutant could be associated with the altered MDO composition. The Δ*mdoG* mutant also invaded macrophages at a reduced efficiency and showed lower respiration rate under conditions mimicking acidic environments, such as stomach and phagosomes (pH 5.0). Correspondingly ATP level in the Δ*mdoG* mutant was significantly reduced compared to

the wild-type. These results support an important role for MDO in the virulence of *S.* Typhimurium.

In competition assays using a mouse host, the Δ*mdoG* mutant had a reduced capacity to colonize the mouse tissues. On the contrary, competitive assays on laboratory media showed that the *mdoG* mutation enhanced the growth of bacteria. A mixed pictured emerged when competition assays were performed by artificially inoculating fresh-cut produce. On tomato and cucumber, the wild-type cells emerged as dominant population after 3 days of growth, while no one strain dominated during the growth on honeydews, cantaloupes, watermelons, as well as acidic fruits such as apples. Together, these data demonstrate that specific wild-type MDO are required for efficient colonization and optimal virulence in mice. For environmental survival under different niches, no evidence was found for a specific need for MDO with particular sugar composition. Enteric pathogens with altered MDO (and reduced virulence) may serve as better “live vaccines”.

Structure-function relationships of periplasmic membrane-derived oligosaccharides in *Salmonella* growth and virulence

By

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DEDICATION

Dedicated to my family

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And most of all, I ascribe to the Lord glory and honor for His grace and peace.

Do not be anxious about anything, but in every situation, by prayer and petition, with thanksgiving, present your requests to God. [7] *And the peace of God, which transcends all understanding, will guard your hearts and your minds in Christ Jesus.* ***(Philippians 4:6-7)***

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LIST OF ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BE	Branching enzyme
CFU	Colony Forming Units
CI	Competitive Index
DEAE	DiEthylAminoEthyl
ECA	Enterobacterial Common Antigen
EPS	ExoPolySaccharide
Gal	Galactose
GC-MS	Gas Chromatography-Mass Spectrometry
Glc	Glucose
Gln	Glutamine
LPS	LipoPolySaccharides
Lys	Lysine
Man	Mannose
MDO	Membrane Derived Oligosaccharides
MOI	Multiplicity Of Infection

NMR	Nuclear Magnetic Resonance
Phe	Phenylalanine
Pro	Proline
Rha	Rhamnose
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	Standard Deviations
Ser	Serine
SPI	*Salmonella* Pathogenicity Islands
SPV	*Salmonella* plasmid virulence
TTSS	Type III Secretion System

CHAPTER 1

INTRODUCTION

Overview

This introduction reviews current knowledge of the topics addressed in this thesis.

Chapter 1 covers most of the basic scientific studies on bacterial MDO focusing on their biosynthesis and the biological functions of MDO. Also discussed are virulence factors in *Salmonella*, one of the leading causes of food poisoning in human beings.

Chapter 2 describes the synthesis of MDO in *Salmonella* Typhimurium. The chapter also presents the composition of MDO and osmotic regulation of MDO.

Details on MDO function in *Salmonella* growth and virulence are described in Chapter 3.

Presented in Chapter 4 are the results of competition assays of *Salmonella* Typhimurium in strains under various environmental conditions in order to further explore the role of MDO in *Salmonella*.

Chapter 5 reviews the results and summarizes the conclusions obtained in this work. Future work is stated.

1.1. History of Bacterial MDO

1.1.1. MDO structures

The first report on periplasmic β-glucan structures is probably the description by McIntire et al. in 1942, of cyclic β-1,2 glucans found in culture filtrates of a crown-gall organism (*Agrobacterium tumefaciens*). In this work, the glucans were essentially thought of as a particular sub-class of exopolysaccharides. The second discovery of MDO came 30 years later during the study of phospholipd turnover in *Escherichia coli* by E.P. Kennedy's group (Golde et al., 1973). In this bacterium, rapid phosphatidylglycerol turnover is associated with the transfer of *sn*-1-phosphoglycerol to a new class of oligosaccharides, MDO (Kennedy 1996). Four families of MDO have been described on the basis of structural features of the glucan backbone.

Family I

In family I, MDO appear to range from 5 to 12 glucosyl residues, with the principal species containing 8 or 9 glucosyl residues. The structure is highly branched, with the backbone consisting of β-1,2-linked glucose to which branches are attached via a β-1,6 linkage. MDO are substituted with phospholipids like phosphoglycerol, phosphoethanolamine, or phosphocholine and also acetate, succinate or methylmalonate (Kennedy, 1996; Lacroix, 1999). These structures are found in the MDO produced by *E. coli* (Kennedy, 1996), enterobacteriaceae *Erwinia chrysanthemi* (Cogez et al., 2001), and *Pseudomonas syringae* (Talaga, 1994). The MDO of *P. syringae* are devoid of any substituents.

Family II

In family II, MDO are composed of a cyclic β-1,2-glucan backbone containing 17 to 25 glucose residues. The backbone may be modified with anionic substituents, such as phosphoglycerol and/or succinate or methylmalonate (Hisamatsu et al., 1992). Among members of the family *Rhizobiaceae*, periplasmic glucans are cyclic and heterogeneous in size (Breedveld et al., 1994). *Agrobacterium, Rhizobium*, *Sinorhizobium* and *Brucella* species synthesize periplasmic glucans with similar structures. Much larger molecules (up to 40 glucose units) were detected within cultures of a strain of *Sinorhizobium meliloti* (Breedveld et al., 1994).

Family III

In family III, MDO are β-1,6- and β-1,3-cyclic glucans containing 10 to 13 glucose units per ring. NMR spectra very similar to those observed for *Bradyrhizobium* (Rolin et al., 1992) were obtained with MDO extracted from *Azaospirillum brasilense*. Three distinct glucans (I, II, and III) were separated. The three glucans consist of a cyclic structure. Glucan I is made of 12 glucose units linked by three β-1,3, eight β-1,6 and one β-1,4 linkages. Glucan II is derived from glucan I by the addition of a glucose linked by an α-1,3 linkage, and glucan III is derived from glucan II by the addition of a 2-*O*-methyl group on the α-linked glucose unit.

Family IV

In family IV, MDO are cyclic and have a unique degree of polymerization (13, 16, and 18, respectively). *Ralstonia solanacearum* (Talaga et al., 1996), *Xanthomonas campestris* (York, 1995), and *Rhodobacter sphaeroides* synthesize MDO of a very similar structure. One linkage is α-1,6, whereas all the other glucose residues are linked by β-1,2 linkages. The presence of this α-1,6 linkage induces structural constraints in this

kind of molecule, which contrast with the very flexible structures of the cyclic β-1,2 MDO of family II.

1.1.2. Biosynthesis of MDO

Three distinct sets of genes were characterized: (i) in bacteria producing MDO of family I (*E. coli*, *P. syringae*, and *E. chrysanthemi*); (ii) in bacteria producing MDO of family II (*A. tumefaciens*, *S. meliloti, Sinorhizobium fredii*, and *Brucella abortus*); (iii) in bacteria producing MDO of family III (*Bradyrhizobium japonicum* and *A. brasilense*).

The *mdo* gene family

In *E. coli*, the two genes forming the *mdoGH* operon are necessary for MDO biosynthesis (Kennedy, 1996). MdoH, the *mdoH* gene product, is a 97-kDa protein spanning the inner membrane. MdoH consists of three large cytoplasmic regions linked by eight transmembrane segments (Debarbieux et al., 1997). This protein is necessary for a glucosyltransferase activity, which allows the *in vitro* production of linear β-1,2 polyglucose chains from the precursor UDP-glucose, if the acyl carrier protein is present (Kennedy, 1996). Actually, the MdoH central domain shows structural features of a family 2-glycosyltransferase. To explain the presence of eight transmembrane segments in MdoH, Debarbieux et al. (1997) have postulated that they could form a channel for MDO translocation to the periplasm during synthesis.

MdoG is a 56-kDa periplasmic protein whose function has not been established but is necessary for the production of MDO. A tempting hypothesis would be that MdoG cooperates with MdoH, perhaps by catalyzing the addition of branches to the linear backbone emerging on the periplasmic side of the inner membrane. Hanoulle et al. (2004)

reported that the structure of MdoG enzyme is organized into two domains in *Escherichia coli*. The *N*-terminal domain is structurally similar to proteins that have a β-sandwich motif, while the *C*-terminal has an Ig-like fold. The *N*-terminal domain architecture is characteristic of proteins involved in carbohydrate interaction and/or enzymatic activities towards carbohydrates. Within this domain, a large cleft has been identified that contains several acidic and aromatic residues. Most of these residues are conserved in related MDO. The charged residues are involved in hydrogen bonding with the substrate, and the aromatic residues participate in stacking with the glycopyrannose rings of the carbohydrate substrates. This observation provides strong support for the hypothesis that MdoG is involved in carbohydrate interaction, and this putative active site may serve as the binding cavity for the linear backbone of MDO during synthesis. In such a model, the *N*-terminal domain would serve as the catalytic core subunit. The *C*-terminal domain of MdoG has architecture comparable to that of protein domains that are involved in the interaction with another protein or with carbohydrates. Interestingly, the *N*-terminal domain of *E. coli* α-1,4-glucan branching enzyme BE is characterized by a related Ig-like β sandwich structure. BE catalyzes the formation of α-1-6 branches in glycogen synthesis. In BE, the linear α-1-4 linked glucose chain is first cleaved and the resulting oligosaccharide is then linked in α-1-6 position to the carbohydrate chain. In the case of BE, the function of the Ig-like domain has been identified and was shown to play a role in the branching patterns of glycogen (Binderup et al., 2002; Devillers et al., 2003). Preiss and co-workers demonstrated that the *N*-terminal domain is implicated in the branching pattern and the length of the glucose chains that are transferred in a α-1,6 position, while the carboxy-terminal domain plays a role in substrate specificity, substrate binding and in

the catalytic efficiency of these branching enzymes (Kuriki et al., 1997). As UDP-glucose is present only in the cytoplasm, the favored hypothesis is that branching may involve a rearrangement of glucose units originally linked through β-1,2 bonds in the linear chains: glucose residues could be cleaved off from β-1,2 linkages and transferred to the same (or a different) polyglucose chain, but now with β-1,6 linkages. Genes closely related to *mdoGH* have been isolated in *Erwinia chrysanthemi* and *Pseudomonas syringae*.

Two cytoplasmic membrane proteins have been implicated in MDO substitutions in *E. coli*. MdoB is a phosphoglycerol transferase with its activity located at the periplasmic face of the cytoplasmic membrane (Bohin et al., 1984), while MdoC is required for the periplasmic succinylation of the MDO (Lacroix et al., 1999).

During the search for genes encoding proteins homologous to MdoG, two paralogous genes were found in *E. coli* and in its close relative *Salmonella typhi*. The *E. coli* gene was named *mdoD* because it influences the size of MDO molecules. When it was inactivated on the chromosome, MDO with a higher degree of polymerization were observed (Lequette et al., 2004).

The *ndv* gene family

In *S. meliloti*, two linked genes are present: *ndvB* and *ndvA* (nodule development) (Breedveld, 1994). NdvB is a very large cytoplamic membrane protein with a molecular mass of 310 kDa. The NdvB enzymatic activity can be assayed efficiently *in vitro*. Unpurified membrane preparations from the $ndvB^+$ strain were able to catalyze the formation of cyclic β-1,2 glucans from UDP-glucose, and a high molecular-mass membrane protein is labeled when radioactive UDP-glucose is present. NdvA is a 67-kDa cytoplasmic protein which shares amino acid sequence similarity with several ATP-

binding cassette transporters. The greatest similarity is found with the type I secretion protein HlyB. For this reason it was proposed that NdvA functions in the translocation of cyclic molecules toward the periplasm, and eventually extracellularly, since several strains secrete MDO in their environment. Consistent with this hypothesis, is that *ndvA* mutants are impaired in MDO secretion and in some MDO modifications which would occur in the periplasmic space (Wang, 1999). Very similar genes were found in *A. tumefaciens* (*chvB* and *chvA*), in *S. fredii* (*ndvB*), *B. abortus* (*cgs*) (Inon de Iannino, 1998) and *R. fredii* (*ndvB*) (Bhagwat, 1992). These genes are strictly homologous and can complement MDO biosynthetic deficiency of *ndvB* mutants of *S. meliloti*.

A third gene family

Bhagwat et al. (1996, 1999) identified *ndvB* and *ndvC* from *Bradyrhizobium japonicum* which are required for cyclic β(1,3),(1,6)-D-glucan synthesis and successful symbiotic interaction with soybean. A new gene *ndvD* was identified (Chen, 2002). A mutation in the *ndvB* locus results in a hypo-osmotically sensitive strain that is unable to synthesize β-glucans and is symbiotically ineffective (Bhagwat et al., 1996).

Mutation of *ndvC* results in the synthesis of structurally altered β-glucans, composed almost entirely of β-(1,3)-glucosyl linkages. Strains with a mutation in *ndvC* are only slightly sensitive to hypo-osmotic growth conditions but are severely impaired in nodule development (Bhagwat et al., 1996). NdvB could be responsible for the primary polymerization of β-1,3-glucan molecules, and NdvC could catalyze the formation of β-1,6 linkages by addition of new glucose units or by a rearrangement of previously synthesized molecules.

1.1.3. MDO substitution

In several bacterial species, MDO are substituted by one or several of a series of different residues, originating from either membrane phospholipids (phosphoglycerol, phosphoethanolamine, and phosphocholine) or from metabolic intermediates (acetyl, succinyl, and methylmalonyl). Thus, depending on the bacterial strain and growth conditions, MDO can be found unsubstituted, neutral or anionic. MDO originating from *Escherichia coli* are linear glucans known to be substituted with three different substituents, phosphoglycerol, phosphoethanolamine, and succinyl residues (Kennedy, 1996). A majority of the MDO molecules have a high anionic character (up to five negative charges), while a minority of them are neutral. Wang et al. (1999) have identified a novel cyclic β-(1,2)-glucan mutant of *S. meliloti* (also known as *Rhizobium meliloti*) which we refer to as mutant S9. Mutant S9, created by Tn*5* insertional mutagenesis, is specifically impaired in its ability to transfer *sn*-1 phosphoglycerol substituents to the cyclic β-(1,2)-glucan backbone. Although the cyclic β-(1,2)-glucans of mutant S9 lack phosphoglycerol substituents, high levels of succinyl substituents are present on these molecules. Indeed, the overall anionic charge on the cyclic β-(1,2)-glucans of this mutant is similar to that found in WT cells. Interestingly, this mutant is able to effectively nodulate alfalfa and can grow as well as WT cells in hypo-osmotic media. These results reveal that the phosphoglycerol substituent is not required for either process and suggest that it is the overall anionic charge on the cyclic β-glucans that may be important for nodulation and/or hypo-osmotic adaptation. The MDO of *S. meliloti* and *A. tumefaciens* are cyclic structures of family II that may be modified with anionic substituents such as phosphoglycerol and/or succinyl moieties (Breedveld et al., 1994),

the MDO of *B. japonicum* are cyclic structures of family III that may be modified by substitution with phosphocholine (Rolin et al., 1992), while the MDO of *P. syringae* are linear and highly branched and devoid of any substituents (Talaga et al., 1994). Also, cyclic glucans of *Ralstonia solanacearum* (Talaga et al., 1996) and *Xanthomonas campestris* (York, 1995) are devoid of substituents. *E. Chrysanthemi* MDO can be substituted by acetyl and succinyl resides, and the presence of succinyl depends on the growth conditions (Cogez et al., 2001). Cyclic glucans of *Azospirillum brasilense* (Altabe et al, 1998) are substituted only with succinyl residues and the cyclic glucans of *Rhodobacter sphaeroides* (Talaga et al., 2002) are substituted with succinyl and acetate residues. In *B. abortus*, cyclic ß-1,2-glucan is substituted with O-succinyl residues (Roset et al., 2006). Although other mutants of glucan substituent transferases have been described for *E. coli*, *S. meliloti*, and *R. sphaeroides*, those mutants contain increased levels of other substituents, such as phosphoethanolamine, succinyl, and acetyl substituents, respectively (Breedveld et al., 1994; Cogez et al., 2001; Fiedler et al., 1988). *B. abortus cgm* mutants accumulated neutral cyclic ß-1,2-glucans; the lack of succinyl residues did not promote replacement or addition with other nonglycosidic substituents.

1.1.4. Biological function of the MDO

MDO provide two major functions regarding vegetative properties and interaction with hosts.

Vegetative properties

Mutants defective in MDO synthesis are highly impaired in vegetative properties such as osmotolerance, motility, antibiotic sensitivity, and biofilm formation.

Osmoprotection

MDO biosynthesis appears to be controlled by osmoregulation in the periplasmic space. This was first discovered for the MDO of *E. coli* by Kennedy, who proposed that the glucans functioned during osmotic adaptation (Kennedy, 1982). MDO are synthesized in abundance when the osmolarity of the medium is very low. The studies by Kennedy and coworkers (Geiger, 1992) have indicated that the accumulation of MDO within the periplasm may be advantageous during growth at low osmolarity for the following reasons: (i) the accumulation of these molecules provides a mechanism for the cell to regulate the relative volumes of periplasmic and cytoplasmic compartments; (ii) anionic MDO contribute to the ionic strength of the periplasm, which appears to be important for porin regulation (Delcour, 1992) and possibly other processes; (iii) high concentrations of MDO within the periplasm should lead to a reduction in turgor pressure across the cytoplasmic membrane ; and (iv) the accumulation of anionic MDO within the periplasm should lead to the development of a Donnan potential across the outer membrane. Additionally, anionic forms of the cyclic β-(1,2)-glucans [which represent 50% or more of the total cyclic β-(1,2)-glucans synthesized by *A. tumefaciens* and *R. meliloti* strains] would be expected to be the most effective form of periplasmic solute because the counterions of these glucans also contribute to periplasmic osmolarity. This osmoreglulation was observed, more or less, in every bacterial species studied, including *Rhizobium*, *Agrobacterium*, and *Bradyrhizobium* strains, including *R. sphaeroides*, *R. solanacerarum*, and *E. chrysanthemi*, (Gogez, 2001) with the exception of *Brucella* strains (Briones, 1997) and *S. meliloti* strain GR4. It has been reported that MDO synthesis is not osmoregulated in *Brucella* and *S. meliloti* strain GR4. In the case of *R leguminosarum*,

cells excrete large amounts of cyclic β-(1,2)-glucans (e.g., 1,600 mg/liter) during growth at elevated osmolarity, indicating, perhaps, that the integrity of the outer membrane of these bacteria is modified under these conditions (Breedveld et al., 1994). It is important to note, however, that these *R. leguminosarum* strains synthesize only neutral, unsubstituted cyclic β-(1,2)-glucans. Thus, it appears that they lack the ability to synthesize anionic cyclic β-(1,2)-glucans. Because anionic forms of the periplasmic cyclic β-(1,2)-glucans may be critical for hypoosmotic adaptation, *R. leguminosarum* strains may not utilize periplasmic cyclic β-(1,2)-glucans for this purpose.

Studies with *ndv* and *chv* mutants of *R. meliloti* and *A. tumefaciens* have provided the most direct evidence that periplasmic cyclic β-(1,2)-glucans do, indeed, function during hypoosmotic adaptation. These mutants have been shown to be specifically impaired for growth in hypoosmotic media, with growth restored to WT levels on the addition of solutes to the growth medium (Cangelosi et al., 1990; Dylan et al., 1990). Curiously, similar experiments performed with *mdoGH* mutants of *E. coli* have failed to reveal impaired growth in low osmolarity media (Kennedy, 1982).

Motility and sensitivity to antibiotics

The synthesis of cyclic β-(1,2) glucan, nodule invasion, and virulence in *R. meliloti* and *A. tumefaciens* were associated with the recovery of motility (Inon de Iannino et al., 1998). A pleotropic nonmotility effect was observed for *ndvB* assembly (Douglas, 1985). Whatever the species considered, motility is altered and this phenotype was reported to be the consequence of a reduced synthesis of flagellin. *ndv, chv* and *mdo* mutants have several altered cell surface properties (Fiedler et al, 1988; Geiger et al, 1992; Holtje et al, 1988), including (i) a loss of motility with reduced numbers of flagella

(Cangelosi et al., 1990; Dylan et al., 1990), (ii) a greater resistance to certain bacteriophages (Cangelosi et al., 1990; Dylan et al., 1990), (iii) increased sensitivity to certain antibiotics (Dylan et al., 1990; Dylan et al., 1986; Mah et al., 2003), (iv) bile salt hypersensitivity (Page et al., 2001), and (v) modified cell surface protein composition (Breedveld, 1994). These characterization studies of MDO defective mutants suggest that MDO within the periplasmic compartment greatly influences overall structure of the cell envelope. From the above, it is possible that the cell envelope alterations of MDO defective mutants are responsible for the failure of these bacteria to properly osmoregulate or infect hosts. How the periplasmic MDO could influence the structure of the cell envelope is unclear; however, one possible mechanism is through interactions with other cell envelope components such as membrane proteins. Evidence to support this possibility is derived from studies with *E. coli* that have indicated an influence of MDO on outer membrane porin synthesis and activity with an increased amount of OmpC (Delcour et al, 1992; Geiger et al, 1992).

Biofilm formation

Several reports point to a role for MDO in regulation of an exopolysaccharide (EPS) required for development of biofilm architecture (Parsek et al, 2003). Microorganisms organized into biofilms are resistant to host defense mechanisms and antibiotic therapy. For instance, biofilms of uropathogenic *E. coli* persist in the urinary tract and catheter surfaces and may cause resistance in urinary tract infections, one of the most common bacterial infections of any organism (Hanna et al, 2003). Although numerous regulators of EPS have been described, osmotic shift is one of the first environmental signals shown to strongly and rapidly induce EPS synthesis (Sledjeski et al, 1996). In

E. coli, colanic acid synthesis is induced through the response of a two-component regulatory system, RcsB and RcsC (Ferrieres et al., 2003). Using *mdoH* inactivation as a model system, it has been suggested that the low levels of periplasmic MDO act to signal RcsC to activate EPS synthesis via RcsB (Ebel et al., 1997). Inactivation of the *mdoH* gene leads to increased expression of colanic acid required for normal biofilm formation (Danese et al., 2000). However, premature colanic expression inhibits adhesion of planktonic cells (Hanna et al., 2003). These data illustrate the important role of periplasmic glucans and of the *mdoGH* operon in bacterial pathogenesis. The preponderance of biofilm communities in nature, as well as their medical impact, provide an incentive for researching information about the molecular structure of enzymes involved in MDO biosynthesis.

MDO in bacterium-host interactions

The MDO of pathogenic and symbiotic bacteria perform various functions during the infection process. Mutations at the loci *ndvA* and *ndvB* in *Sinorhizobium meliloti* (Geremia, 1987), *ndvB* and *ndvC* in *Bradyrhizobium japonicum* (Bhagwat, 1999), *chvA* and *chvB* in *Agrobacterium tumefaciens* (Puvanesarajah, 1985), and *hrpM* in *Pseudomonas syringae* (Loubens, 1993) impair MDO biosynthesis, and these mutants fail to interact properly with a host plant as a symbiont or a pathogen. Mutations of *Rhizobia* prevent the formation of normal nodules on the roots of specific plant hosts, an essential step in the biologically and agriculturally important process of symbiotic nitrogen fixation. Failure to produce cyclic glucan leads to the delayed production of small, white nodules that are not invaded by the mutant bacteria and hence do not fix nitrogen (Dylan et al., 1986). Cyclic glucans therefore play a part in the complex interchange of signals

between bacteria and specific plant hosts. In *B. japonicum*, mutations of the *ndvB* gene, which result in total absence of MDO, led to the formation of ineffective nodules on soybean plants. However, mutations in the *ndvC* gene, which result in the formation of structurally distinct MDO (predominantly β-1,3), do not affect nodulation (Bhagwat et al., 1993; Bhagwat et al., 1996). In *E. chrysanthemi*, mutations in both the *mdoG* and the *mdoH* homologues result in a complete loss of virulence (Page et al., 2001). One of three highly attenuated mutants of *Salmonella* Typhimurium possesses a MduJ insertion in a gene homologous to *mdoB* (Valentine, 1998). The absence of cyclic β-1,2-glucan in *B. abortus* is associated with a reduction of virulence in mice (Briones, 2001). Also, a mutant of *Pseudomonas aeruginosa* PA14, which has a transposon insertion in mdo*H*, had dramatically reduced virulence against *Caenorhabditis elegans* and was not able to induce mortality in mice (Mahajan-Miklos et al, 1999).

MDO can also affect vegetative properties. *Chv* mutants of *A. tumefaciens* and *hrpM* mutants of *P. syringae* are unable to elicit the hypersensitive reaction (necrosis of plant cells resulting in the containment and death of invading bacteria) in non-host plants. Similarly, *ndv* mutants of *S. meliloti* form defective nodules on alfalfa.

MDO as suppressors of host defense responses

The bradyrhizobial β(1,3),(1,6)-glucans suppressed plant responses at concentrations reported as physiological levels in the legume-symbiont interaction (Bhagwat et al.,1992). Cyclic β-(1,2)-glucans mediate the attachment of *Rhizobium* and *Agrobacterium* species to specific sites on the plant cell surface. Interestingly, the cyclic β(1,6),(1,3)-glucans of *B. japonicum* have been shown to be elicitors of isoflavonoid production by the soybean as suppressors of plant defense responses (Miller et al., 1994).

Increasing concentrations of the bacterial β(1,3),(1,6) glucans progressively inhibit stimulation of phytoalexin synthesis in a bioassay induced by the fungal β(1,3),(1,6) glucans (Mithofer et al., 2001). β-glucan, derived from soybean suspension cultures of *Bradyrhizobium japonicum,* can induce a transient cytosolic Ca^{2+} increase as a prerequisite for phytoalexin synthesis. Mutants deficient in *ndv* were unable to induce nodules typical of *Bradyrhizobium japonicum* infection. However, with a delay of nearly 2 weeks, *ndv* mutants did form very small pseudonodules which did not contain viable bacteria. These pseudonodules contained higher concentrations of glyceollins. The *P. sojae* β-glucan-induced transient cytosolic Ca^{2+} increase is not necessary for the elicitation of H_2O_2 production, but it is very likely required for phytoalexin synthesis (Mithofer, 2001).

The role of MDO during attachment to plant cells

A role for the cyclic β-(1,2)-glucans was first revealed with *A. tumefaciens chvA* and *chvB* mutants. These mutants were found to be defective in their ability to attach to isolated Zinnia leaf mesophyll cells and tobacco suspension culture cells (Douglas et al., 1985). Subsequently, it has been shown that *R meliloti ndv* mutants are severely reduced in their ability to attach to the roots of alfalfa seedlings (Dylan et al, 1990). From the above results, it is possible that cyclic β-(1,2)-glucans mediate the attachment of *Rhizobium* and *Agrobacterium* species to specific sites on the plant cell surface.

1.2. Pathology of *Salmonella* infection

The genus *Salmonella* is a member of the family of *Enterobacteriaceae* which also includes the genera *Escherichia* and *Yersinia* (Woese et al., 1990).

Salmonella species infect both animal and human hosts and are the causative agents of diseases including enteric (typhoid) fever, gastroenteritis, bacteremia, and a variety of localized systemic infections. In the United States an estimated 1.41 million cases and more than 500 human deaths occur annually (Mead et al., 1999). Approximately 95% of the human *Salmonella* infections are foodborne, corresponding to approximately 30% of deaths caused by foodborne infections in the United States (Mead et al., 1999). *Salmonella* infection is even more serious in the developing world. Much research has been conducted on *Salmonella* Typhimurium, a serotype of particular interest since it is one of the leading causes of food poisoning in human beings and also induces a typhoid-like disease in susceptible mice (Xu et al., 1992). Murine typhoid pathogenesis has served as a model of human typhoid infection and has enabled a detailed analysis of the organism's spread beyond the bowel to deeper tissues and the systemic circulation. The disease (non-typhoidal fever) is usually self-limiting and recovery follows within days but, occasionally, systemic infection may occur in vulnerable human patients such as infants and the elderly, leading to serious syndromes (Xu et al., 1992). Following ingestion, bacteria pass through the acidic environment of the stomach. Thus, bacterial resistance against these acidic conditions plays an important role in infection (Foster, 1995). Prior to the invasion of any cell type, bacteria must encounter and attach to one or more cell types found in intestinal tissue. Bacteria attach to target cells via firm interaction of bacterial surface components with distinct host receptors. Many bacterial pathogens use subcellular surface appendages that radiate from the bacterial surface for initial adherence. Typical examples are the bacterial pili (fimbriae) and flagella.

Flagella also play a major role in *Salmonella* motility. In many studies, bacterial motility was found to be essential for adherence or invasion (Jones et al., 1992; Khoramian-Falsafi et al., 1990). It can be imagined that in many systems, flagella provide the driving force that enable the bacteria to penetrate the host mucus layer more rapidly and reach the host cell surface. Information about attractants and repellents is conveyed to the flagellar motors through a signal transduction network, allowing chemotactic movement in response to external chemical stimuli (Harshey et al., 1994).

Salmonella are thought to next colonize the intestine and penetrate the intestinal barrier (Frost et al., 1997). Bacterial penetration of the intestinal mucosa is often an important step in establishment of an infection as it enables microorganisms to pass the epithelial barrier. In mice, *S*. Typhimurium invades the mucosal tissue rapidly. Both enterocytes and M cells can be invaded, but the subsequent destruction of M cells is thought to contribute to spreading of the infection and the occurrence of systemic disease (Jones et al., 1994).

Many of the *Salmonella* virulence genes are clustered in certain areas of the chromosome known as "*Salmonella* pathogenicity islands" (SPI). To date, five SPI have been described. *Salmonella* invasion of nonphagocytic cells has been an important virulence trait conferred by the type-III-secretion system (TTSS) located on SPI-1 (Galan, 2001). The function of SPI-1 is required for the invasion of nonphagocytic cells, an important virulence trait of *Salmonella*. The SPI-1-encoded TTSS form needle-like surface appendages (Kubori et al., 1998) that can mediate the delivery of proteins by extracellular *Salmonella* organisms into host cells. These effector proteins encoded by SPI-1 modify signal transduction pathways resulting in the temporal reorganization of the

actin cytoskeleton of the host cell leading to cell membrane "ruffling". The localized modification of the cytoskeleton is followed by dramatic changes in the host cell surface that appear as the membrane ruffles. As a consequence, nonphagocytic cells, in this case epithelial cells, internalize larger particles such as bacteria in a process termed macropinocytosis.

Following the invasion process, the bacteria can reach the Peyer's patches (Penheiter et al, 1997). Here, the bacteria can be taken up by macrophages that are believed to carry these engulfed bacteria to systemic sites through the lymphatic system. In the mouse liver, *Salmonella* can invade and survive within macrophages (Richter-Dahlfors et al, 1997). To survive within macrophages, pathogens have developed defense mechanisms to counter antibacterial attack such as toxic oxygen derivatives, reactive nitrogen intermediates, and defensins. Organisms that remain within phagosome membranes must also combat nutrient limitation, fusion with lysosomes, and phagosome acidification. Phagosome acidification is probably not directly toxic to most bacteria but has been suggested to facilitate the microbial killing process by inducing spontaneous generation of hydrogen peroxide from superoxide (Fridovich, 1978), influencing fusion of lysosomes with phagosomes (Horwits et al, 1984), and providing an optimal environment for the activity of hydrolytic enzymes (Coffey et al., 1968).

The *Salmonella* pathogenicity island 2 (SPI-2) was identified as a gene cluster required for survival of Typhimurium inside host cells. This virulence phenotype is linked to the ability of *S.* Typhimurium to survive in phagocytic cells and to replicate within the *Salmonella*-containing vesicle in a variety of eukaryotic cells.

SPI-3 and SPI-4 also play a role in survival of *Salmonella* in macrophages (Marcus et al., 2000). For intracellular replication, *Salmonella* must adapt to the microbicidal and nutrient-poor environment of the phagosome, which is limiting for purines, pyrimidines, particular amino acids, and Mg^{2+}. A large number of metabolic pathways and transport systems are required for adaptation to this environment (Garcia-del et al, 1992). SPI-3 encoded proteins allow *Salmonella* to acquire magnesium under low magnesium conditions. These proteins were shown to be important for survival of the bacterium in macrophages and for virulence in mice (Blanc-Potard et al., 1997). SPI-4 is involved in secretion of a cytotoxin (Wong et al., 1998). The survival of *Salmonella* within macrophages is generally considered to be essential for translocation of bacteria from the lymphoid tissue to the mesenteric lymph nodes, liver, and spleen.

1.3. Objectives

Infections with *Salmonella* spp. are among the major cause of foodborne illness in humans worldwide. *Salmonella* spp. are able to infect a wide variety of animal species, such as chicken, cattle and mice, and survive in diverse environments, including most environmental ecosystems, food production and processing systems, and intestinal tracts of host animals (Bean and Griffin, 1990). The study of this organism provides important knowledge about processes involved in bacterial pathogenesis and may lead to the development of more effective methods for controlling *Salmonella*.

Membrane-derived oligosaccharides (MDO) consist of branched substituted β-linked sugar chains that are present in the periplasmic space of *Escherichia coli* and other gram-negative bacteria. Their common features are the presence of glucose as a major constituent sugar and their increased levels in low osmolarity media. In several

phytopathogenic bacteria, mutants defective in MDO synthesis fail to incite disease on the host plant. However, MDO from *Salmonella* species have not been studied for their role in virulence or osmotolerance even though extensive studies of their role in host-pathogen interactions are recognized. Therefore, the aim of this research was to gain insight into the structure-function relationship of MDO to better understand if and how they play a role in *Salmonella* growth and virulence.

At the beginning of our studies, MDO of *Salmonella* Typhimurium strains were isolated and their sugar composition analyzed. However, MDO in *Salmonella*, supposedly producing glucose polymers as in other gram negative bacteria, resulted in the production of mixed sugar-oligosaccharides with components, such as rhamnose, mannose, glucose, and galactose. Subsequently, to study the function of MDO in *Salmonella* Typhimurium, the *mdoG* gene encoding the glycosyltransferase enzyme, one of the enzymes responsible for MDO synthesis, was cloned through a genetic approach. Using a gene specific mutagenesis protocol, an *mdoG* gene was deleted and a kanamycin resistance gene was replaced to obtain a MDO defective mutant. Glycosyl composition of *Salmonella* Typhimurium WT and the MDO defective mutant were compared. MDO composition was altered in the mutant compared to the WT, revealing that the *mdoG* deletion affected MDO composition. We also detected various MDO compositions under different chromosomal backgrounds.

We characterized the *mdoG* deletion mutant to examine the function of MDO in *Salmonella* growth and virulence. Survival assays including virulence-related characteristics were performed. Mouse virulence experiments besides survival assays were also tested with macrophage survival assays to determine the role of MDO in

Salmonella pathogenicity. These studies suggest that MDO play an important role in the virulence of *S.* Typhimurium. Additionally, we investigated the role of MDO production in competition between *Salmonella* Typhimurium SL1344 and the Δ*mdoG* mutant in mice *in vivo*, a laboratory medium, and fresh-cut fruit slices.

CHAPTER 2

The synthesis of periplasmic MDO in *Salmonella* spp.

2.1. Abstract

Membrane-derived oligosaccharides (MDO) consist of branched substituted β-linked sugar chains that are present in the periplasmic space of *Escherichia coli* and other gram-negative bacteria. Their common features are the presence of glucose as a major constituent sugar and their increased levels in low-osmolarity media. In several phytopathogenic bacteria, the chemical structure and biosynthesis of MDO have been studied extensively, whereas little is known about MDO in *Salmonella.*

MDO defective mutants of *Salmonella* Typhimurium were generated using a gene specific mutagenesis protocol and MDO were isolated and their glycosyl composition analyzed. The fractions containing the major peak from Sephadex G-10 gel filtration chromatography were pooled and subjected to DEAE-cellulose anion exchange chromatography to separate charged and neutral MDO. Compositional analysis revealed that MDO of the wild-type consist of 94% glucosyl residues (hereafter referred to as glucose) in *Salmonella* Typhimurium FIRN while MDO of the Δ*mdoG* mutant was comprised of only 24% glucose. Rhamnose, mannose, and galactose accounted for the rest. We also found that MDO composition varies in different chromosomal backgrounds. For example, MDO of *Salmonella* Typhimurium SL1344 was composed of only 41% glucose, and the glucose amount was further reduced to 24% in the Δ*mdoG* mutant.

The synthesis of MDO appeared to be osmoregulated, since lower amounts of MDO were produced when bacteria were grown in media of higher osmolarities.

Here I suggest that (i) Δ*mdoG* mutants differ in MDO composition and (ii) elevated osmotic pressure inhibits MDO biosynthesis in *Salmonella*.

2.2. Introduction

Membrane derived oligosaccharides (MDO) are a family of oligosaccharides found in the periplasm of gram-negative bacteria. As described in the literature, their common features are the presence of glucose as the sole constituent sugar and their increased levels in low-osmolarity media. Theses MDO are cyclic, branched cyclic, or branched linear, and they may be substituted by various glycosyl residues in different species (Bohin, 2000).

Four families of MDO have been described on the basis of structural features of the glucosyl backbone. In family I, MDO appear to range from 5 to 12 glucose residues, with the principal species containing 8 or 9 glucose residues. Their structure is highly branched, the backbone consisting of β-1,2-linked glucose residues to which branches are attached via β-1,6 linkages. In family II, MDO are composed of a cyclic β-1,2-glucan backbone containing 17 to 25 glucose resides. In family III, MDO are β-1,6 and β-1,3 cyclic glucans containing 10 to 13 glucose units per ring. In family IV, MDO are cyclic and have a unique degree of polymerization (DP, = 13, 16, or 18). One linkage is α-1,6 whereas all other glucose residues are β-1,2 linked. Depending on the species considered, MDO can be modified to various extents by a variety of substituents (Cogez et al., 2001). In general, neutral or anionic functional groups are found in MDO, such as phosphoglycerol, methylmalonic acid, succinic acid, or phosphocholine (Miller et al., 1988; Cogez et al., 2001; Rolin et al., 1992). These anionic and neutral oligosaccharides

within the periplasmic place are directly involved in the osmotic adaptation of microorganisms.

MDO structure and its biosynthesis have not been studied in *Salmonella* Typhimurium. In this report we present the results of our studies on the periplasmic MDO from *Salmonella* Typhimurium. The effect of osmotic strength of the growth medium on MDO biosynthesis is also described. In chapter 2, the structure-function relationship of MDO is explored. The role of MDO in S*almonella* growth and virulence will be discussed in chapter 3.

2.3. Materials and methods

Bacterial strains and media

All of the bacterial strains used are listed in Table 1. *Salmonella enterica serovar* Typhimurium strains were grown at 37^{o}C with shaking at 200 rpm in Lauria-Bartani (LB) medium without NaCl for 24 h to enhance MDO synthesis. To obtain the high-osmolarity medium, NaCl was added to LB medium up to 0.5 M.

Genomic library construction of *S*. Typhimurium LT2

Genomic DNA from strain LT2 was PCR amplified using the forward primer 5′ - ACACCAACTCCGACAACCT - 3′ and the reverse primer 5′ - CCCTTCGCCAATGA ATC - 3′ of the *mdoG* gene. The amplified DNA fragment of *mdoG* was cloned in the pGEM-T Easy vector (Promega, Madison, WI) using the manufacturer's protocol, and subsequently transformed into *E. coli* JM109 competent cells (Promega, Madison, WI). Appropriate transformants were selected after growth on LB/ampicillin/IPTG/X-Gal

plates. Through propagation of the transformants, plasmid (pMDOG1) was obtained which was digested with HpaI and NdeI to determine orientation of the cloned *mdoG*.

Sequence analysis of *mdoG*

The plasmid pMDOG1, *mdoG* clone, was isolated from *E. coli* transformants with the QIAGEN plasmid mini kit (QIAGEN, Valencia, CA). DNA of *mdoG* was sequenced by the Iowa State University DNA Facility. Resulting sequences were analyzed by the BLAST program at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) web site.

Construction of the *mdoG* deletion strains

The plasmid pMDOG1 was digested with Bsu36I and HpaI to remove sequences encoding residues 13 to 306 of the *mdoG* gene and the gene for resistance to kanamycin of a PstI restriction fragment from pUC4K was introduced into the deleted part of the gene. The resulting fragment was blunt-ended using T4 DNA polymerase, and inserted into pMDOG1 to yield pMDOG11. The EcoRI restriction fragment of pMDOG11 was ligated into pKNG101, and the ligation mixture was transformed into $CaCl_2$-competent *E. coli* S17-1 λ pir cells, yielding pMDOG111. Transformants were selected on LB agar containing kanamycin and streptomycin.

The plasmid pMDOG111 was introduced into the WT *S.* Typhimurium FIRN from *E. coli* S17-1 λ pir by conjugal transfer. Exconjugants with the deletion allele integrated into the corresponding chromosomal gene locus were selected by growth on LB agar plates supplemented with kanamycin. Growth on LB agar supplemented with 5% sucrose was then used to counterselect for bacteria in which the vector sequences had been excised by a second recombination event. Correct construction of deletion mutants

was confirmed by PCR amplification of the gene of interest and further screened by Southern blot analysis and DNA sequencing. The *mdoG* deletion mutation was introduced into the chromosome of *S.* Typhimurium SL1344 by P22-mediated transduction to yield *S.* Typhimurium strain SG111. The deletion of DNA in different background strains was confirmed by Southern blot analysis

Complementation studies

To investigate the possibility of the functionally interchangeable complementation of *mdoG* gene, a DNA fragment harboring a single copy of mdoG1 was subcloned into pACYC184 and mobilized into exconjugants FG111 and SG111 to examine functional complementation.

Purification of MDO for structural analysis

Cultures (12 L) were harvested and centrifuged at 4 ^{o}C for 10 min at 20,000 x g. After cell pellets were washed by centrifugation two times with 0.9% saline, 50% hot EtOH was added to the pellet and the mixture was incubated at 55 ^{o}C for 10 min. The EtOH extract was condensed 10-fold by rotary evaporation (Schneider et al., 1979; Bhagwat et al., 1999). The crude extract was freeze-dried and dissolved in 60 mL of distilled water at 50 ^{o}C for 10 min. After centrifugation for 10 min at 20,000 x g, the supernatant was resuspended in 10X EtOH at -20 ^{o}C for 1 h and the residue then precipitated by centrifugation for 10 min at 20,000 x g. The precipitate, which contained MDO, was suspended in 7% (v/v) 1-propanol and purified by chromatography on a column (1.5 cm by 100 cm) of Sephadex G-10. The column was eluted at room temperature with 7% (v/v) 1-propanol and fractions (3 ml) were collected. Sephadex G-10 fractions containing MDO were pooled and concentrated. The concentrated sample

was further fractionated on a column (1.5 by 5 cm ; diameter × height) of DEAE-cellulose (Whatman DE53). Columns were eluted with 20 ml water (neutral MDO), 20 ml 0.25 M NaCl (anionic MDO), and 20 ml 0.5 M NaCl (anionic MDO). MDO were then desalted by Sephadex G-10 chromatography as described above. Carbohydrates were measured by the anthrone-sulfuric acid method (Mokrasch, 1954).

Protein determination

The amount of total cellular protein was determined according to the BCA protein assay (Pierce Biotechnology, Inc**.,** Milwaukee, WI) using bovine serum albumin as a standard.

Composition and methylation analyses

Glycosyl composition was determined by acid hydrolysis of the polysaccharides with 2N TFA for 1 h at 120°C, preparation of the alditol acetate derivatives, followed by gas chromatography analysis (Henry et al., 1983). A part of each sample was methylated, hydrolysed with 2 M TFA, reduced with $NaBD_4$, peracetylated and then partially methylated alditol acetates were analyzed by GC-MS for glycosyl linkage analysis as described by Priem et al. (1993).

2.4. Results

Construction of the *mdoG* deletion mutant *S*. Typhimurium Δ*mdoG::kan*

Based on the known sequence of the *S.* Typhimurium LT2 *mdoG* gene, a 294 amino acid deletion of the open reading frame of the *mdoG* gene and insertion of *kan* was introduced into the chromosome of *S.* Typhimurium FIRN to yield *S.* Typhimurium strain FG111 (Fig. 1A). These mutations were introduced into the chromosome of *S.*

Typhimurium SL1344 by P22-mediated transduction to yield *S.* Typhimurium strain SG111. Southern hybridization analysis using the *mdoG* DNA probe was performed to confirm the structure of the Δ*mdoG::kan* mutants. A single hybridizing band was revealed in each wild-type strain when the genomic DNA was cut with *Hind*III or *Hind*III-*Sma*I because the *mdoG* probe hybridized to identical bases of genomic DNA and the presence of the *kan* insertion in each of the mutants revealed two hybridizing bands because kan had HindIII and SmaI cut sites (Fig. 1B).

Purification of MDO

Cultures (12 liter) of S. Typhimurium strains were grown at 37^{o}C in LB medium without NaCl. The cells were harvested by centrifugation and the MDO extracted with 50% (v/v) EtOH from stationary-phase cultures. Then, MDO were precipitated with 10 volumes of EtOH and subjected to Sephadex G-10 chromatography. Comparison of Sephadex G-10 chromatography profiles of WT and the *mdoG* deletion mutant is shown in Figure 2. The peak of the *mdoG* deletion mutant was shifted slightly to the right, indicating that the molecular size of the carbohydrate was slightly smaller than that of the WT MDO.

Effect of osmolarity on cellular periplasmic MDO accumulation

Production of MDO from both *S.* Typhimurium wild-type and *mdoG* deletion mutant in a high-osmotic pressure medium was examined. When cells of *S.* Typhimurium SL1344 wild-type were grown in LB medium supplemented with 0.5M NaCl, the amount of MDO recovered in the Sephadex G-10 peak was reduced 3-fold compared to MDO obtained from cells grown without NaCl (Table 2). When cells of the *mdoG* deletion mutant were grown without NaCl, there was a slight reduction of MDO (1.7-fold less in

high osmolarity) accumulation compared to cells of the wild-type grown without NaCl. This result indicated that MDO synthesis by the *mdoG* deletion mutant was not as highly regulated as in the WT.

The *mdoG* deletion mutants synthesize a structurally altered MDO

The MDO that eluted in a single peak on Sephadex G-10 were recovered, pooled, concentrated, and further fractionated by DEAE-cellulose column chromatography, which allowed for the separation of MDO fractions by their anionic charge. In Figure 3, MDO in WT *S*. Typhimurium FIRN, which bound to DEAE-cellulose, contained the greatest amount of cellular MDO modified with anionic constituents. The Δ*mdoG* mutant produced 54% of charged MDO compared to the WT, indicating that *mdoG* deletion affected MDO substitution. In addition, 74% of charged MDO in *S*. Typhimurium SL1344 was synthesized while 63% of charged MDO in the Δ*mdoG* mutant was synthesized, showing that the anionic substitution of MDO was inhibited by *mdoG* deletion. Thus, the amounts of anionic oligosaccharides were increased in both WT *S*. Typhimurium strains and the anionic substitution of MDO in both *mdoG* deletion mutant strains was decreased.

Subsequently, glycosyl composition analysis was performed to test the effect of the deletion of the *mdoG* gene in two different chromosomal backgrounds on monosaccharide composition of the respective MDO (Table 3). WT *S*. Typhimurium LT2 MDO was comprised of 80% glucose and the WT *S*. Typhimurium FIRN was 94% glucose. In contrast, only about 24% of *mdoG* was glucose with about 25% of the MDO being comprised of rhamnose, mannose, and galactose. This demonstrated that the *mdoG* deletion mutant synthesized a significantly structurally altered MDO. The glucose

content synthesized by the WT *S*. Typhimurium SL1344 was 41% which is lower than the WT *S*. Typhimurium FIRN while the glucose content was 50% reduced in the *mdoG* deletion mutant strain, demonstrating that lack of expression of *mdoG* affected MDO structure quite dramatically. When the pWG21 plasmid carrying the WT copy of the *mdoG* gene was introduced into strain SG111 (the *mdoG* deletion mutant), the composition of MDO was restored to that of the wild-type strain, SL1344. The results of glycosyl linkage methylation analysis showed that the MDO were branched linear structures (Table 4). Wild-type MDO were linear chains composed of (1→4)-rhamnose, (1→2)-glucose, (1→3)-galactose, (2→3)-mannose, and (2→6)-glucose residues. In contrast, (1→2)-glucose and (2→6)-glucose residues were absent in MDO from the Δ*mdoG* mutant. This indicated that *mdoG* deletion affected MDO structure and that a glucose chain "defect" in the MDO of SG111 may be associated with its function.

2.5. Discussion

This is the first report on the role of *S*. Typhimurium MDO. In this study, we compared MDO structure of *S*. Typhimurium wild-type and an isogenic Δ*mdoG* mutant in order to investigate structure-function relationships.

We cloned and sequenced the *S*. Typhimurium genes involved in MDO biosynthesis (*mdoGH*). They exhibit high homology to *E. coli mdoGH* (84% identity) (Lequette et al., 2004) and *Erwinia chrysanthemi OpgGH* (83% identity) (Page et al., 2001). The fact that highly similar genes control the biosynthesis of MDO belonging to the same structural family may seem obvious in *E. coli* and *E. chrysanthemi*. In these bacteria and other Gram-negative organisms (Golde et al., 1973; Schulman et al., 1979)

glucose is the only monosaccharide present in the periplasm that was detected in the MDO by GC analysis. However, in addition to glucose, in our study, rhamnose, mannose, and galactose were detected in the MDO of *Salmonella*. This is unique in that these additional sugars, not found in other gram-negative bacteria, may have significance in the role of MDO in *Salmonella*. Thus, structural analysis of MDO will be necessary even though the gene sequences are known.

Compositional analysis of the MDO from the Δ*mdoG* mutant showed that MDO structure was affected by the mutation. The amounts of rhamnose, mannose, glucose, and galactose were present in similar proportions in FG111. Glucose content was slightly higher in SG111. Both 2-glucosyl and 2,6-glucosyl residues were absent from the MDO of both FG111 and SG111, showing that these mutants synthesize MDO with different glucosidic linkages. MdoG is thus a very interesting candidate for structure/function studies aimed at identifying amino acid residues responsible for glucosidic bond specificity. These results may also suggest that the function of the MdoG protein, which is involved in glucan elongation in *E. coli* (Lacroix, 1991), depends on the structure of MDO molecules. Structural analysis showed that OpgG is an OPG branching enzyme in *E. coli* presenting a two domain structure: an N-terminal catalytic domain and a C-terminal substrate binding domain (Hanoulle et al., 2004). Lequette et al. (2004) have characterized the new gene *mdoD,* a paralog of *mdoG* in *E. coli*. They found that the *mdoD* mutant produced a highly polymerized MDO. Even though *mdoG* deletion mutants affect MDO backbone synthesis, there is still an accumulation of periplasmic MDO because MDO are synthesized by a multiprotein complex (Bohin, 2000; Lequette et al., 2004). Thus, it is possible that *mdoD* might replace the role of *mdoG* in *Salmonella*

strains containing mutations in the *mdoG* gene. When *mdoG* is absent, it can be assumed that other MDO biosynthesis genes can still catalyze MDO formation.

S. Typhimurium MDO content increased when the osmolarity of the growth medium was decreased (3-fold more in low osmolarity). This result suggested that in *S*. Typhimurium MDO biosynthesis is controlled by osmoregulation. The effect of external osmolarity on MDO production has been examined in many bacteria (Miller et al., 1986; Dylan et al., 1990). Consensus suggests that suppression of biosynthesis of the periplasmic MDO during growth in high-osmolarity media indicates a role of MDO in adaptation of the bacteria to low osmolartiy conditions (Miller et al., 1986).

The anionic characteristic of MDO was confirmed by DEAE-cellulose ion exchange chromatography (Fig. 3). This investigation revealed that most MDO in *S*. Typhimurium are highly anionically charged, and only small portions are uncharged. In *E. coli*, the glucose backbone is substituted with *sn*-1-phosphoglycerol and phosphor-ethanolamine, and with succinic acid O-ester residues from the cytoplasmic pool (Kennedy, 1996; Lacroix et al., 1999). Ninety-two percent of MDO in FIRN are anionic while only 74% of MDO in SL1344 are anionic. This reveals differences in the pattern of MDO substitution depending on the strains being studied. Thus, we must consider that MDO can be modified to various extents and that further analysis of MDO structural variations in *S*. Typhimurium must be pursued.

MDO extracted from Δ*mdoG* mutants showed reduced degrees of substitution with negatively charged residues and increased neutral residues compared to MDO of the wild type. This indicated that *mdoG* might be involved in residue substitution of MDO in *S*. Typhimurium. It was also observed that the accumulation of MDO changes with the

osmolarity of the media (Breedveld et al., 1994). This change due to media osmolarity needs to be studied since MDO biosynthesis is controlled by osmoregulation. Further studies are needed to identify the structure of MDO in *Salmonella* Typhimurium and to elucidate the structure-function relationship of MDO in relation to virulence.

Table 1. Bacterial strains.

Strain	Description	Reference
S. Typhimurium		
S. Typhimurium LT2	Wild type	SGSC
S. Typhimurium FIRN	Wild type Nal^r	SGSC
FG111	FIRN Δ*mdoG::kan*	This study
S. Typhimurium SL1344	Wild type *his* Sm^r	Hoiseth et al. (1981)
SG111	SL1344 Δ*mdoG::kan*; Kan^r	This study
SG112	SG111 containing pWG21; Kan^r Cm^r	This study
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ (*lac-proAB*) *mcrA* (F' *traD36 proAB*$^+$ *lacI*^{q}ZΔM15)	Promega
S17-1 (λ pir)	Tp^r *recA thi pro hsdR*$^-$*M*$^+$ *RP4:2-Tc:MuKm* Tn*7*, λ pir	Kim et al. (2002)
Plasmids		
pGEM-T	Vector for cloning of PCR products	Promega
pUC4K	Source of the kanamycin cassette	Stratagene
pKNG101	Sm^r, oriR6K, mobRK2, *sacB*, suicide vector	Kim et al. (2002)
pACYC184	Low-copy-number cloning vector; Cm^r Tc^r	New England Biolabs
pMDOG1	pGEM-T derivative harboring 1,924-bp *mdoG* PCR fragment	This study
pMDOG11	882-bp HpaI/Bsu36I fragment containing *mdoG* coding region from pMDOG1 was removed and km cassette was inserted into the deleted part (Δ*mdoG::km*); Kan^r	This study
pMDOG111	2,100-bp EcoRI fragment from pMDOG11 cloned in pKNG101 at SmaI site; Kan^r	This study
pWG1	pGEM-T derivative harboring 2,030-bp *mdoG* PCR fragment	This study
pWG21	2,086-bp NotI fragment containing *mdoG* from pWG1 cloned in pACYC184 at EagI site; Cm^r	This study

SGSC; *Salmonella* Genetic Stock Center, Alberta, Canada

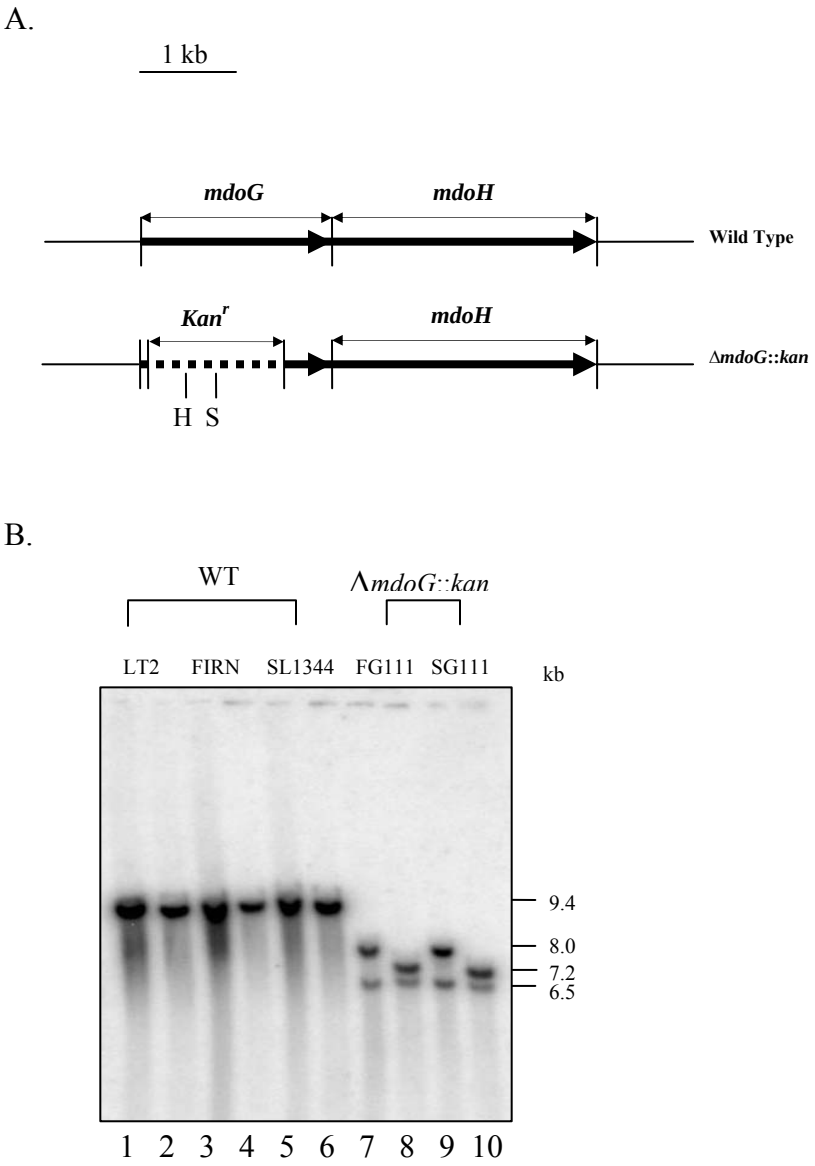


Fig. 1. Construction (A) and characterization of the *mdoG* deletion mutant *S.* Typhimurium Δ*mdoG*::*kan* by southern blot analysis (B). A. Dotted lines represent where *mdoG* was deleted and the kanamycin resistant gene was inserted. H, *HindIII*; S, *SmaI*. B. Southern blot hybridization analysis with both *Hind*III and *Hind*III-*Sma*I digested chromosomal DNA from the WT *S.* Typhimurium and the Δ*mdoG* mutant using a *mdoG*

DNA probe. Lanes 1, 3, 5, 7, and 9 show *Hind*III digested chromosomal DNA and lanes 2, 4, 6, 8, and 10 show *Hind*III-*Sma*I digested chromosomal DNA.

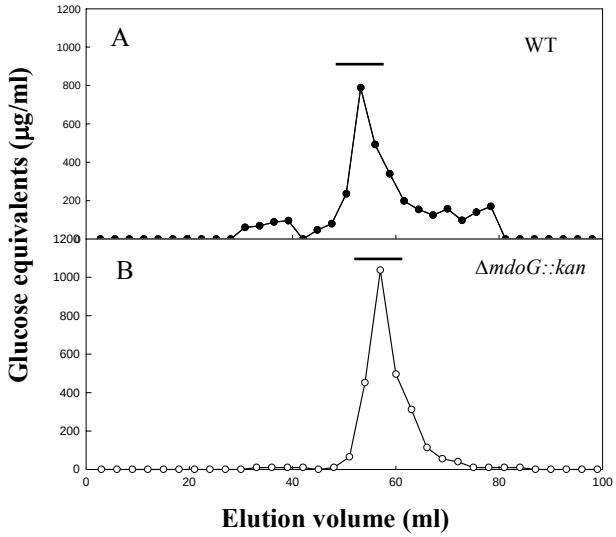


Fig. 2. Sephadex G-10 chromatography of MDO from different strains grown in LB medium with or without NaCl. The column (1.5 by 100 cm) was eluted with 7% propanol at 22 to 25°C and aliquots were analyzed for total carbohydrate by the anthrone-sulfuric acid method. Results are expressed as glucose equivalents per milliliter of eluant. Fractions indicated by the horizontal bar were pooled, lyophilized, and further fractionated on a DEAE-cellulose anion exchange column. A, *S*. Typhimurium SL1344 (Wild type); B, SG111 (Δ*mdoG::kan*).

Table 2. MDO biosynthesis in different *S.* Typhimurium strains growing in high-osmotic pressure medium.

NaCl	Bacterial protein (mg/ml culture)		MDO (μg glucose equivalents /mg bacterial protein)	
	WT	SG111	WT	SG111
None (control)	0.44	0.33	23.8	15.6
0.5 M NaCl	0.33	0.32	8.4	9.1

S. Typhimurium strains were grown in 1940 ml of LB medium with or without 0.5M NaCl. MDO content indicates the total amount of glucan eluting in a single peak on Sephadex G-10.

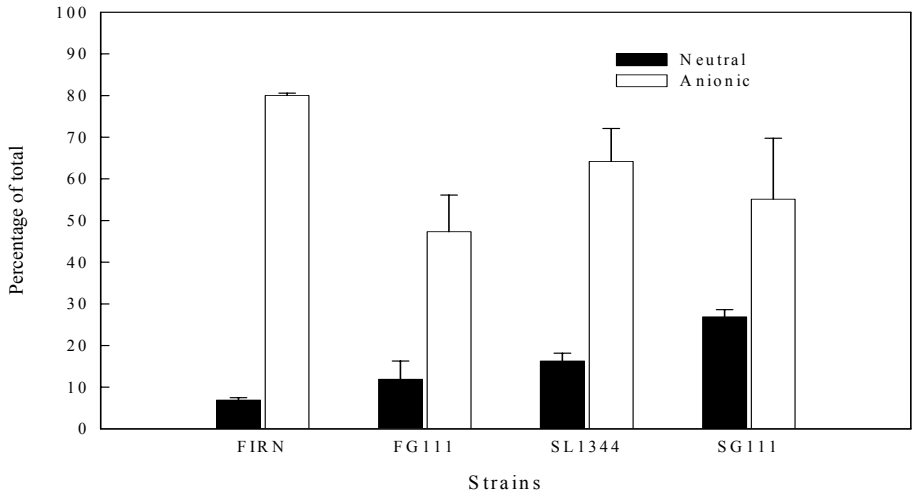


Fig. 3. DEAE-cellulose anion exchange chromatography profiles of MDO from different strains. MDO recovered from Sephadex G-10 columns were subjected to DEAE-cellulose chromatography. Columns (1.5 by 5 cm ; diameter × height) were eluted with 20 ml water (neutral MDO) and 20 ml 0.25 M NaCl (anionic MDO). Fractions of 20 ml were collected and total hexose measured by the anthrone-sulfuric acid method (Mokrasch, 1954).

Table. 3. Comparison of the glucosyl composition of MDO isolated from different strains.

	% of MDO			
	Rha	Man	Glc	Gal
S. Typhimurium LT2	10.5 ± 1.0	6 ± 0.1	78 ± 1.2	5.2 ± 0.1
S. Typhimurium FIRN	0	6 ± 0.2	94 ± 0.2	0
FG111	25 ± 0.4	25 ± 0.6	24 ± 0.6	26 ± 0.3
S. Typhimurium SL1344	20 ± 0.9	18 ± 1.3	41 ± 2.4	21 ± 2.1
SG111	26 ± 1.6	23 ± 0.2	24 ± 1.4	27 ± 0.0
SG112	23 ± 1.2	13 ± 1.2	48 ± 1.6	16 ± 1.6

Rha, rhamnose; Man, mannose; Glc, glucose; Gal, galactose. MDO recovered from Sephadex G-10 columns were purified on a DEAE-cellulose anion exchange column and anionic MDO were analyzed for glycosyl composition by GC

Table. 4. Glycosyl linkage methylation analysis of the MDO produced by *Salmonella* Typhimurium strains.

Linkage type[a]	SL1344	SG111	SG112
	(% of total)		
4 Rha	26 ± 0.03	26 ± 0.02	16 ± 0.01
T Glc	11 ± 0.04	37 ± 0.00	38 ± 0.01
2 Glc	13 ± 0.00	NT[b]	12 ± 0.03
3 Gal	19 ± 0.00	8 ± 0.01	5 ± 0.02
2,3 Man	18 ± 0.01	16 ± 0.02	14 ± 0.02
2,6 Glc	13 ± 0.00	NT[b]	14 ± 0.01
3,6 Glc	NT[b]	13 ± 0.03	14 ± 0.00

[a]Separated as partially methylated alditol acetates and characterized by retention times and fragmentation patterns by GC-MS.

[b]Not detected

CHAPTER 3

The ability of *Salmonella* Typhimurium to synthesize MDO is involved in mouse virulence

3.1. Abstract

MDO defective mutants of *Salmonella enterica serovar* Typhimurium were generated using a gene specific mutagenesis protocol as described in Chapter 2. Mutants showed an impaired motility only when in the *Salmonella* Typhimurium SL1344 chromosomal background. Mutants in all chromosomal backgrounds displayed reduced virulence in mice. Challenging mice with the Δ*mdoG* mutant resulted in less colonization (100- to 1000-fold) of the intestine, liver, and spleen than the parental strain SL1344. The *Salmonella* Typhimurium SL1344 Δ*mdoG* mutant displayed defective intracellular replication in RAW262.7 cells. Under growth conditions of reduced pH (observed in stomach and phagosomes of macrophages), the Δ*mdoG* mutant respired at a reduced rate and correspondingly had reduced levels of ATP. These results suggest that MDO play an important role in the virulence of *S.* Typhimurium. However, stress tolerance studies (such as desiccation and detergent shock) showed that the *mdoG* deletion mutant of *S.* Typhimurium strains showed similar survival rates to the parental wild-type strain. It appears that the ability of *Salmonella* Typhimurium to synthesize MDO influences its virulence in mouse but not *in vitro* stress-tolerance.

3.2. Introduction

Salmonellae are enteric Gram-negative bacteria that cause gastroenteritis and enteric (typhoid) fever after oral ingestion (Alpuche-Aranda et al., 1995). *Salmonella* serotypes exhibit either a narrow or broad host range. *Salmonella typh*i infects only humans and causes typhoid fever. After oral ingestion, bacteria spread to the liver, spleen, and bone marrow, where they persist in the phagosomal compartment of macrophages. In contrast, *Salmonella* Typhimurium is a broad host range serotype that causes host-specific clinical manifestations. Non-typhoidal *Salmonella* serovars are one of the leading causes of food-transmitted infections in developed countries. Inbred mice develop a systemic illness similar to human typhoid fever after *S*. Typhimurium infection. Other animals, including humans and cattle, develop gastroenteritis, a disease characterized by diarrhea and intestinal neutrophil infiltration. *S*. Typhimurium gastroenteritis is usually self-limiting. However, in young, old or immunocompromised individuals, it can be lethal. Despite the varying host ranges and diseases caused by *Salmonella* serotypes, many virulence determinants are conserved among most *Salmonellae*, many of which are clustered within *Salmonella* pathogenicity islands (SPIs) (Marcus et al., 2000; Hensel, 2004). SPI1 and SPI2 each encode a structurally and functionally different type III secretion system, which is responsible for the delivery of effector proteins into the host cell cytosol. SPI3 is necessary for survival within macrophages and growth in low Mg^{2+} environments during the systemic phase of the disease. SPI4 is suspected to be required for intramacrophage survival and may also contribute to toxin secretion. Like SPI1, SPI5 appears mainly involved in enteropatho-genesis, being associated with inflammation and chloride secretion. Some of the genes not residing in SPIs that also play a role in disease are slyA, phoP/Q, agfA and stn,

encoding a transcriptional regulator of virulence genes required for survival within macrophages (initially identified as salmolysin) (Watson et al., 1999), a two-component global transcriptional regulator of *Salmonella* virulence (Miller, 1991), the thin aggregative fimbriae and an enterotoxin (Collinson et al., 1996a, b), respectively. Furthermore, many *Salmonella* serovars harbor virulence (V) plasmids with variable size, depending on the serovar (Rotger & Casadesus, 1999; Marcus et al., 2000). All V plasmids share a highly conserved 7.8 kb region with *spv* operon - *spvRABCD* (*Salmonella* plasmid virulence). The *spv* operon positively regulated by *rpoS* appears to promote growth and survival of *Salmonella* within the host cells which is important for systemic infection in experimental animals.

Bacterial membrane-derived oligosaccharides (MDO) play a role in host-pathogen interaction. Since we found a different composition of MDO in the Δ*mdoG* mutant, as described in Chapter 2, we hypothesize that the change in composition of the MDO may affect survival and virulence of *Salmonella*. For example, a *ndvB* mutant of *Bradyrhizobium japonicum* resulted in the total absence of MDO and led to the formation of ineffective nodules on soybean plants (Bhagwat et al., 1996). Mutations in the *ndvC* gene, which result in the formation of structurally distinct MDO, were severely impaired in symbiotically in their ability to interact (Bhagwat et al., 1996). In *E. chrysanthemi*, mutations in both the *mdoG* and the *mdoH* homologues involved in MDO biosynthesis resulted in a complete loss of virulence (Page et al., 2001). *Chv* in *Agrobacterium tumefaciens* and *ndv* in *Sinorhizobium meliloti* are the genes coding for cyclic β-1,2-glucan synthetase. Mutations at these loci impaired host-pathogen interaction (Zorreguieta et al., 1986). These results suggest that MDO play a critical role in bacteria

pathogenicity and the presence of MDO in many bacteria determines interactions with other organisms.

Despite the fact that MDO are important molecules in many bacterial organisms, to our knowledge the structure and function of MDO in *Salmonella* have not been reported or studied.

To investigate the potential role of MDO in the host-bacterium interaction, we examined the function of MDO in *Salmonella* Typhimurium strains relative to phenotypic differences, particularly in relation to virulence factors. We found that the *Salmonella* Typhimurium Δ*mdoG* mutant has impaired motility, reduced virulence in mice, and defective intracellular replication in RAW262.7 cells. The mutant also showed reduced survival in BALB/c mouse intestine, liver, and spleen tissues. These data indicate that MDO are important molecules in *Salmonella* pathogenesis. The respiration rate by evaluating ATP levels for the wild-type strain SL1344 and for Δ*mdoG* mutant SG111 at pH 5 was also evaluated. Furthermore, the modulation of SPI1 and SPI2 gene expression in *S*. Typhimurium Δ*mdoG* mutant was analyzed.

3.3. Materials and methods.

Bacterial growth conditions

Strains used in this study are listed in Table 5. Bacteria were grown overnight in LB (Luria-Bertani) medium with aeration. For SPI1 expression assays, late logarithmic phase bacteria (overnight bacteria back diluted 1:100 and grown for 4 h) in LB medium with aeration were used. For SPI2 expression, bacteria were grown in N minimal medium overnight with aeration (Deiwick et al., 1999).

DNA manipulations and analysis

DNA manipulations and analysis are described in Chapter 2.

Growth assay

A comparison of the general growth patterns for wild type and mutant strains was performed. Strains were grown in 500 ml flasks containing 20 ml of either LB or EG minimal medium (Lin et al., 1996) supplemented with 1% casamino acids. One percent (v/v) of a culture grown overnight was inoculated in the appropriate flask and incubated at 37°C. Growth was monitored by viable cell counts over a 24 hour period. This experiment was carried out in duplicate.

Motility assay

Motility assays were performed in 0.3% LB soft agar medium. Five µl of stationary phase cells grown in LB medium were inoculated onto motility plates. Motility was examined after 6 h of incubation at 37°C. At least three independent motility assays were carried out for each strain.

Virulence in mice

Six-week-old female BALB/c mice were infected orally with 0.2 ml of a suspension containing the appropriate number of viable *Salmonella* Typhimurium strains in collaboration with Dr. Allen Smith (USDA/ARS, Beltsville) and mortality rates over time were determined. Strains were grown statistically overnight at 37°C in LB medium, suspended in sterile saline, and adjusted to the appropriate concentration.

Analysis of *Salmonella* Typhimurium loads in the intestine, spleen, and liver

To analyze colonization, 6-week-old female BALB/c mice were infected orally with a 0.2 ml suspension containing either 1.5×10^7 CFU SL1344 organisms or 1.5×10^7

CFU Δ*mdoG* mutant organisms (five mice per strain). Mice were sacrificed 6 days later. Organs (ileum, livers, and spleens) were isolated and homogenized, and the number of bacteria present determined by viable counting on Brilliant green agar plates (Difco, Franklin Lakes, NJ) containing 50 μg of streptomycin per ml for wild-type and 25 μg of kanamycin per ml for mutants. Statistical significance was analyzed by ANOVA.

Macrophage survival assays

For macrophage survival assays, RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (FCS) at 37°C. Bacterial strains were grown without shaking overnight in LB broth at 37°C. Stationary phase bacteria were diluted in DMEM–10% FCS to an OD600 of 1.0. Bacteria were centrifuged onto macrophages seeded in 24-well tissue culture plates at a multiplicity of infection (MOI) of 5:1 and 1:1, and incubated for 25 min. Infected monolayers were washed three times with PBS to remove extracellular bacteria and incubated for 90 min in DMEM–10% FCS containing 50 mg/ml gentamicin. Thereafter, the gentamicin concentration was reduced to 10 mg per ml in DMEM–10% FCS. At either 2 or 24 h p.i., cells were lysed in 1% (v/v) Triton X- 100 with 0.1% (w/v) SDS and plated on LB agar to enumerate CFU (Knodler et al., 2002).

Measurement of luminescence and ATP expression at pH 5

Both the Δ*mdoG* mutant and the wild-type strain were tested in a luminescence assay to determine levels of ATP. Cells from frozen stock were streaked onto EGC minimal medium agar and were grown overnight at 37°C. Cells were then re-streaked on EGC agar and again grown overnight at 37°C to deplete nutrient reserves so that subsequent growth in microplates was entirely dependent on the nutritional supplements

provided in the medium. Colonies were collected from the surface of EGC plates using a cotton swab. These cells were suspended in GN/GP-IF (inoculating fluid) at a density corresponding to 85% transmittance (optical density, 0.05) in the Biolog trubidimeter using a 20 mm diameter tube. The suspensions were then inoculated into the triethanol-amine/glutaric acid (30 mM, pH 5) reagent and a volume of 100 µl/ well was placed into 96-well microplates (Bochner et al., 2001). The microplates were incubated at 37°C for 12 h, 15 h, 18 h, and 24 h, at which time cultures were diluted properly and plated on LB for viable cell counting. ATP was quantified by mixing 100 µl luciferase reagents with bacterial cells in medium (100 µl) taken from a 96-well microplate at different times using a bactiter-glo microbial cell viability assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the mixture of a 100 µl sample and 100 µl luciferin/luciferase reagent was place in a sample tube, and the luminescent signal was measured after 5 min with a Berthold LB9501 luminometer (Berthold Technologies GmbH & Co KG., Bad Wildbad, German). For maximum sensitivity, the light signal was integrated for 1 s.

Generation of reporter gene fusions

pEM67 (kindly provided by Dr. Ferric Fang) listed in Table 5 was introduced into the *Salmonella* Typhimurium SL1344 wild-type and the Δ*mdoG* mutant by conjugation, and single chromosomal integration events that create gene duplications were confirmed by Southern blotting. EE656 (kindly provided by Dr. Ferric Fang) listed in Table 5 was transduced into the Δ*mdoG* mutant to yield *S.* Typhimurium strain SG170 and screened for Tet^R and Kan^R. The targeted integration into the chromosomes of *S*. Typhimurium was confirmed by Southern blot analysis.

β-Galactosidase assays

β-Galactosidase activity was assayed using a bacterial cell density of about 7 x 10^7 CFU as described by Miller (1992).

Luciferase assays

Bacteria with a cell density of about 7 x 10^7 CFU were suspended in 500 µl of luciferase buffer (Gunn et al., 1996), frozen, thawed and lysed by sonication for 20 s. A 20 µl aliquot of this lysate was assayed for luciferase activity using the Dual Luciferase Reporter Assay System from Promega. Firefly and renilla luciferase units were recorded sequentially for 10 s or 30 s and 10 s (*r-luc*) respectively, in a Berthold LB9501 luminometer (100 µl manual injection for *f-luc* readings, 100 µl manual injection for *r-luc* readings). Firefly relative luciferase light units were divided by relative renilla luciferase light units to normalize for cell lysis and number. Assays were performed in triplicate and repeated at least twice.

Survival assays

Acid tolerance. Stationary-phase cells grown overnight in LB were diluted in EG (pH 3.0) to a cell density of about 1 x 10^7 per ml and incubated for different time points at 37°C. Cultures were diluted and plated on LB for viable cell counting.

Heat shock survival. WT and mutant of *Salmonella* Typhimurium strains were grown overnight in LB. Stationary-phase cells were diluted in EG to a cell density of about 1 x 10^7 per ml. Samples (3 ml) in plastic snap cap tubes were pre-warmed to 58°C; proper dilutions were plated directly on LB for viable cell numbers.

SDS resistance. SDS was added at a final concentration of 5% (w/v) to cultures in stationary phase, and the cultures were monitored for cell growth or lysis 3 h after inoculation.

Nutrient starvation. Five µl containing10^8 cells of *Salmonella* serovar Typhimurium were placed on individual polyethersulfone membranes (0.22 µm thick) (Durapore membrane; Millipore, Inc.) in a petri dish and allowed to incubate at room temperature for different times. At day 13, membranes were transferred to EGC plates (EG plates supplemented with 1 % casamino acid). Viable counts of cells from polyethersulfone membranes were measured by extracting with saline and plating on LB agar at various time intervals.

LPS isolation and analysis

Bacterial cultures for analysis of LPS were grown overnight in LB at 37°C, diluted 1:100 in the same media, and grown at 37°C for 3 h to the late-log-phase. A 10 ml sample was collected and centrifuged. The cell pellet was resuspended in PBS to an OD of 600nm of 0.5 to 0.6. The cell suspension (1.5 ml) for LPS extraction was centrifuged and resuspended in 50 µl of SDS-PAGE lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue in 1M Tris-HCl buffer, pH 6.8). Samples were incubated at 100°C for 10 min. Samples were mixed with 25 µg Proteinase K and incubated for 1 h at 60°C and then mixed with 50 µl of 90% phenol for 15 min at 70°C. Samples were centrifuged for 10 min and the aqueous phase was transferred to a new tube and extracted once with 500 µl of ethyl ether to remove traces of phenol. The upper ether phase was aspirated and the lower phase then was mixed with an equal volume of SDS-PAGE lysis buffer. Ten µl of each sample was incubated at 100°C for 5 min and

was submitted to electrophoresis on a 15% SDS-PAGE gel containing 4 M urea. LPS was visualized by silver staining (Tsai et al., 1982).

3.4. Results

Growth study

Growth of the Δ*mdoG* mutant was compared to the parent strain in both LB and EGC media; there was no difference in general growth (Fig. 4), indicating that the reduced virulence of this mutant was probably not due to a mutation in a gene required for growth since growth was unaffected by this mutation.

The *mdoG* deletion mutant is defective in motility

A comparison of the motility of different *S.* Typhimurium strains was performed in motility agar at 37°C. The *S.* Typhimurium FIRN *mdoG* mutant was as motile as the WT, demonstrating that the lack of expression of *mdoG* did not influence the motility of *S.* Typhimurium FIRN (data not shown). However, the *S.* Typhimurium SL1344 *mdoG* mutant had reduced motility while *S.* Typhimurium SL1344 WT was highly motile (Fig.5), suggesting that *mdoG* deletion may affect flagellin synthesis or stability. In addition, the introduction of the pWG21 plasmid carrying the WT copy of the *mdoG* gene restored motility to the *mdoG* mutant. Therefore, the reduction in motility of the mutant must have been the consequence of the *mdoG* deletion.

S. Typhimurium *mdoG* deletion mutant has reduced virulence in mice

To determine the functional role of *mdoG*, we orally challenged mice with *Salmonella* Typhimurium WT and *mdoG* deletion mutant strains at three different doses (10^5, 10^7, and 10^9 CFU/mouse for the *Salmonella* Typhimurium FIRN strain and 10^3, 10^5,

and 10^7 CFU/mouse for the *Salmonella* Typhimurium SL1344 strains (a lower infectious dose was necessary for SL1344, indicating that this strain is more virulent than FIRN) by oral route. Mortality rates were recorded over time (Fig. 6). The deletion of *mdoG* in *S.* Typhimurium strains had no discernible effect on mortality at a 10^3 dose challenge. However, at 10^5 and 10^7 doses, mice infected with *S.* Typhimurium FIRN died slowly compared to *S.* Typhimurium SL1344; only a marginal loss in virulence of the *mdoG* deletion mutant was observed (Fig. 6A). On the other hand (Fig. 6B), all mice infected with a 10^7 dose of *S.* Typhimurium SL1344 died 9 days after infection while the *mdoG* deletion mutant displayed a slower rate of death. At the lower dose of 10^5, one mouse died on day 6, and one mouse died on each of days 7 and 10 after infection. All the rest of the mice survived this infection, showing that the *mdoG* deletion caused significantly less mortality than the WT *S.* Typhimurium SL1344. It seems clear that MDO are important virulence factors since a mutation in the *mdoG* gene showed reduced virulence in mice. This result confirms that altered MDO structure leads to virulence attenuation.

Bacterial growth *in vivo* was performed (Fig. 7). Two groups of five BALB/c mice were infected orally with 1.5 x 10^7 CFU of the mutant SG111 or the WT strain SL1344. Animals were sacrificed 6 days after inoculation and colonization of the intestine, spleen, and liver were analyzed. Levels of colonization of *S.* Typhimurium WT were >100-fold higher than mutants in mice tissues, and the differences between WT and mutants for all tissues were significant. The colonization defect of mutant SG111 in mice tissues indicated that *mdoG* of *Salmonella* Typhimurium is required for intestinal and systemic infection in mice. These data are consistent with results described above and suggested that MDO are required for optimal *Salmonella* virulence.

mdoG is required for intracellular survival

The capacity to survive within macrophages is an absolute requirement for *Salmonella* virulence. Therefore, the ability of a Δ*mdoG* mutant to survive and replicate within RAW264.7 cells was assayed using the gentamicin protection assay. Monolayers were lysed and plated to enumerate CFU at 2, 6, and 22 h post-infection. The Δ*mdoG* mutant was impaired in intracellular growth in phagocytic cells compared with WT bacteria 2, 6 and 22 h post-infection (Fig. 8). At both high (figure 8A) and low doses (figure 8B), the survival ability of wild type versus mutant bacteria remains comparatively similar. Based on these results, it was concluded that *mdoG* is required, but not sufficient, for normal *S.* Typhimurium intracellular replication in phagocytic cells.

The *S*. Typhimurium *mdoG* deletion mutant has a low ATP level at pH 5

To understand the mechanism involved in MDO defective mutant-mediated reduction of *Salmonella* virulence and colonization in mice, we examined ATP levels during growth of *Salmonella* Typhimurium. *Salmonella serovar* Typhimurium is a facultative anaerobe (Macvanin et al., 2004). It grows faster aerobically because a proton motive force is generated across the bacterial membrane with oxygen as a terminal electron acceptor (Neidhardt et al., 1990). This process drives the efficient synthesis of ATP which is an essential part of the respiratory process (Gennis et al., 1996). The basis of the assay is the measurement of intracellular ATP, the universal energy molecule in all living cells. When a cell dies, the intracellular ATP is rapidly degraded by ATPases and ATP levels decline rapidly. In the presence of luciferase and the substrate luciferin, extracted from the firefly *Photinus pyralis*, ATP-dependent oxidation of the luciferin produces oxyluciferin, carbon dioxide, AMP, inorganic phosphate, and light. The amount

of light emitted is directly correlated with the amount of ATP present. We considered the possibility that the Δ*mdoG* mutant, showing the same viability as the WT, might be defective in aerobic respiration since Biolog data showed that the Δ*mdoG* mutant displayed a lower respiration rate than the WT at pH 5 (data not shown). We measured respiration by determining ATP level for the WT strain SL1344 and for the Δ*mdoG* mutant SG111 in Biolog medium (pH 5). The mutant had a significantly lower ATP level than the wild-type (Fig. 9). The ATP level for the mutant was only 20% that of the WT at 12 h. However, both strains had the same level of ATP at 24 h. It was concluded that the Δ*mdoG* mutant has a significantly reduced rate of respiration at pH 5.

Regulation of SPI and SPI2 gene expression

We studied the expression of genes for the SPI1 and SPI2 strains under conditions that induce SPI1 genes and SPI2 genes in the Δ*mdoG* mutant background (Fig. 10). A reduction in the expression of the *prgH*::*lacZ* fusion in the Δ*mdoG* mutant background was observed, indicating that the expression of SPI1 is affected by the function of *mdoG*. This suggests that MdoG is required for the expression of *prgH*. The effect of the mutation in *mdoG* on the expression of the SPI2 gene was tested. Under conditions inducing SPI2 gene expression, inactivation of *mdoG* had no effect on the expression of the *sseI*::*luc* fusion.

Characterization of the *S*. Typhimurium *mdoG* deletion mutant

Survival assays were performed in *S*. Typhimurium strains to determine if MDO are required for virulence related characteristics. These included acid and base resistance, heat resistance, SDS resistance, polymyxin B resistance, oxidative stress resistance, and bile resistance and it was observed that MDO had no effect on these stresses (Fig. 11). It

is shown in Fig. 12 that the *mdoG* deletion mutation of *S.* Typhimurium strains showed similar survival rates as the parental WT strain against nutrient starvation and SDS stresses. Analysis of the nutrient starvation experiment revealed that differences between the WT and mutant for the recovery of bacteria at 13 days after the shift to EGC agar plates were not significant. These results indicate that MDO do not affect stress resistance. The only phenotypic differences that occurred were related to motility and mouse virulence, thus supporting the hypothesis that MDO play a role during *Salmonella* host interaction.

LPS

In order to determine if the *mdoG* deletion is a factor involved in virulence reduction, we examined whether the outermost portion of the cell wall was affected by the *mdoG* deletion mutation. The bacterial outer membrane represents a platform for many surface structures involved in virulence. Lipopolysaccharide (LPS) makes up a portion of the outermost region of the bacterial cell wall. LPS is widely considered to protect bacteria against host defense mechanisms such as gastric acidity, bile salts and the bactericidal activity of complement and phagocytes (Doerrler and Raetz, 2002).

To determine if LPS influenced virulence in the Δ*mdoG* mutant, the LPS from *Salmonella* strains were extracted and run on an SDS-PAGE gel. Electrophoresis of LPS revealed no significant differences between the WT and the Δ*mdoG* mutant (Fig. 13). SG112 had the same LPS content as the WT and mutant suggesting that *mdoG* mutation does not affect LPS.

3.5. Discussion

In this study, we showed that the MDO defective mutant in *S*. Typhimurium is less virulent than its WT parent in mice and is impaired in intracellular replication in RAW 264.7 cells. The fact that the reduced virulence of the *mdoG* mutant strain SG111 was consistent with defective motility, but not in FG111 (since FG111 was as motile as the WT), suggests a role for the stability of the flagella. Although the data support an involvement for *mdoG* in motility phenotype, it may be that some strains of *Salmonella* Typhimurium have compensatory mechanisms for *mdoG* mutations. *Rhizobium* and *Agrobacterium* MDO defective mutants are nonmotile due to a defective stability of the flagella, a process that is known to occur in the periplasmic space (Ugalde, 1999). Swart et al. (1993) suggested that the presence of MDO in the bacterial periplasm may stabilize membrane proteins against improper assembly or disassembly. Recently, Arellano-Reynoso et al. (2005) showed that glucan-defective mutants in *Brucella* lose the ability for phagosome-lysosome fusion and are not able to replicate in macrophages. So, this virulence defect in the *mdoG* mutant of *Salmonella* might be explained to some extent by a decrease in MDO synthesis, or partly by an alteration in its function resulting from cell envelope disorganization.

The complementation of the defect with an intact copy of the gene confirmed *mdoG* as an important component responsible for the mutant phenotype motility. However, the complementation strain did not restore the virulence level in mice due to the plasmid loss in the absence of antibiotics.

The role of MDO in bacterium-host interaction was extensively studied in *Agrobacterium, Rhizobium*, and other gram-negative bacteria. Synthesis and transport

mutants have some associated pleiotropic phenotypes. For example, defective flagellum assembly (Breedveld et al., 1994), defective plant attachment (Puvanesarajah et al., 1985), decreased temperature stability of the virB10 protein (Banta et al., 1998), reduced virulence of *B. abortus* in mice, impeded normal intracellular multiplication in HeLa cells (Briones et al., 2001), a complete loss of virulence of *E. chrysanthemi* in plants (Page et al., 2001), and the formation of ineffective nodules on soybean plants in *B. japonicum* (Bhagwat et al., 1996), are all traits that may affect bacterium-host interaction.

The mutant colonized much less than its WT parent in mouse organs suggesting that the Δ*mdoG* mutant was not able to interact with epithelial surfaces and invade the tissues. This result was relevant to SPI1 gene expression study with the Δ*mdoG* mutant using a reporter gene fusion assay. MDO affected the SPI1 type III secretion system, whose function is important for bacterial translocation across the gut epithelium. Although only *prgH* for SPI1 expression was studied, additional work is required to address other regulatory mechanisms of SPI1 gene expression in *Salmonella* Typhimurium.

During systemic infection, *Salmonella* cells are thought to reside and replicate mainly inside phagocytic cells (Hensel et al., 1995; Jantsch et al, 2003). The present study indicated that the Δ*mdoG* mutant was impaired in intracellular growth in phagocytic cells. This may indicate that MDO may be required during intracellular stages of the *Salmonella* life cycle. SPI1 gene expression studies also implied that SPI1 may be important in mediating epithelial cell death and could possibly also affect macrophages immediately adjacent to the epithelium.

To understand the mechanism involved in MDO defective mutant-mediated reduction of *Salmonella* virulence and colonization in mice, we examined ATP levels during growth of *Salmonella* Typhimurium. The acidic environment of the stomach (pH 4.5 to 5.0) forms the first line of defense. Organisms that survive in the acidic stomach must combat nutrient limitation, fusion with lysosomes, and phagosome acidification to remain within macrophages. Thus, changes in acidity may affect survival and virulence of *Salmonella*.

In this study, the viability of the *Salmonella* Typhimurium Δ*mdoG* mutant was not affected at pH 5, but the ATP level was significantly affected, indicating that the reduced virulence in mice, the impaired intracellular replication in macrophage cells, and the decreased colonization in mouse organs by the mutant can be explained by an affect on the ability of the mutant to respire at low pH in the stomach or phagocytic cells. These results provide the first direct evidence of a virulence role for MDO in mice.

Salmonella responds to various stresses in a host, including acid, oxidants, heat, high salt level, nutrient deprivations, SDS, and polymyxin. *Brucella abortus, E. coli, Rhizobium* glucan defective mutants showed sensitivity to surfactants such as SDS, DOC, and Zwittergent or certain antibiotics (Briones et al., 2001,; Rajagopal, 2003; Breedveld, 1994), but this is not the case with *Salmonella* Typhimurium. Responses to stresses mentioned above in *Salmonella* Typhimurium strains were examined in order to determine the function of MDO. The *mdoG* mutants grew as well as the WT in LB broth. Even though growth of the *mdoG* mutants was attenuated in mice, no correlation was observed between sensitivity to stressful agents and virulence.

Both Δ*mdoH* and Δ*mdoB* mutant generation was attempted using a gene specific

mutagenesis protocol similar to Δ*mdoG*. However, we were unable to make a Δ*mdoH* mutant construct, and the Δ*mdoB* mutant had the same composition of MDO as the WT (data not shown). We tested the Δ*mdoB* mutant for mice virulence since Bowe et al. (1998) reported that the disruption of *mdoB*, an osmoregulated transferase which adds phosphoglycerol to periplasmic oligosaccharides, leads to attenuation. However, our findings contrast with those of Bowe et al. (1998); the Δ*mdoB* mutant did not reduce virulence in mice (data not shown). It is indicated that different strains of *S.* Typhimurium and two different mutagenesis methods were used in the two experimental systems.

All of the above observations suggest that the proper MDO synthesis is important for virulence. Our results have a number of important implications for other gram-negative pathogens. Similar genetic approaches could be used to investigate the extent of the contribution of MDO to bacterial disease. Enteric pathogens with altered MDO (and reduced virulence) may serve as better “live vaccines”.

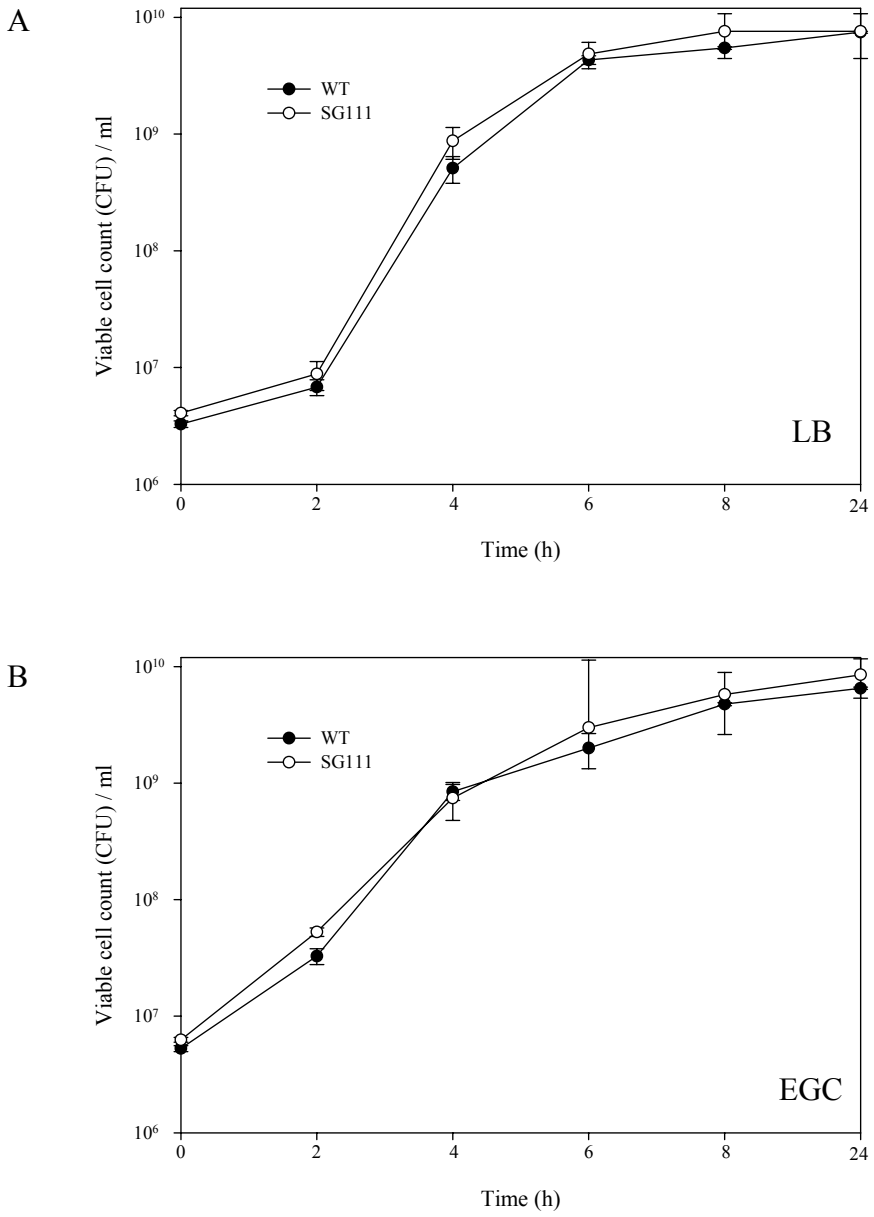


Fig. 4. Growth study in WT strain and Δ*mdoG* mutant SG111 in LB (A) and EGC (B) medium. Viability is expressed as colony forming units (CFU) and determined at the indicated times. Data are expressed as means ± SE (not shown when smaller than the symbol), n = 3.

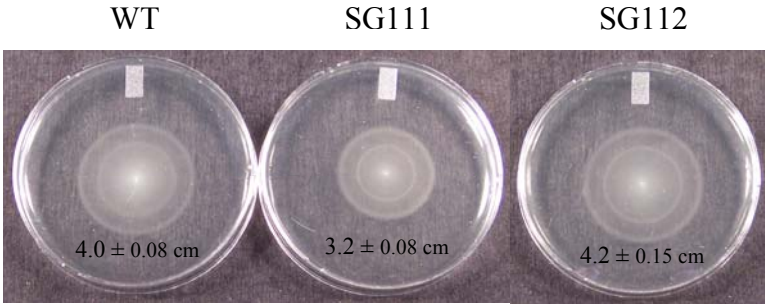


Fig. 5. Motility of *S.* Typhimurium SL1344 (WT), SG111 (Δ*mdoG::kan*), and SG112 (SG111 complemented by a wild type copy of the *mdoG* gene located on the pACYC184 plasmid) in semisolid LB medium containing 0.35% agar. The plates were incubated at 37°C, and the migration distance was measured after 6 h of spot inoculation (5 µl). The data represent the averages of three independent experiments with standard deviation. The representative plates were photographed.

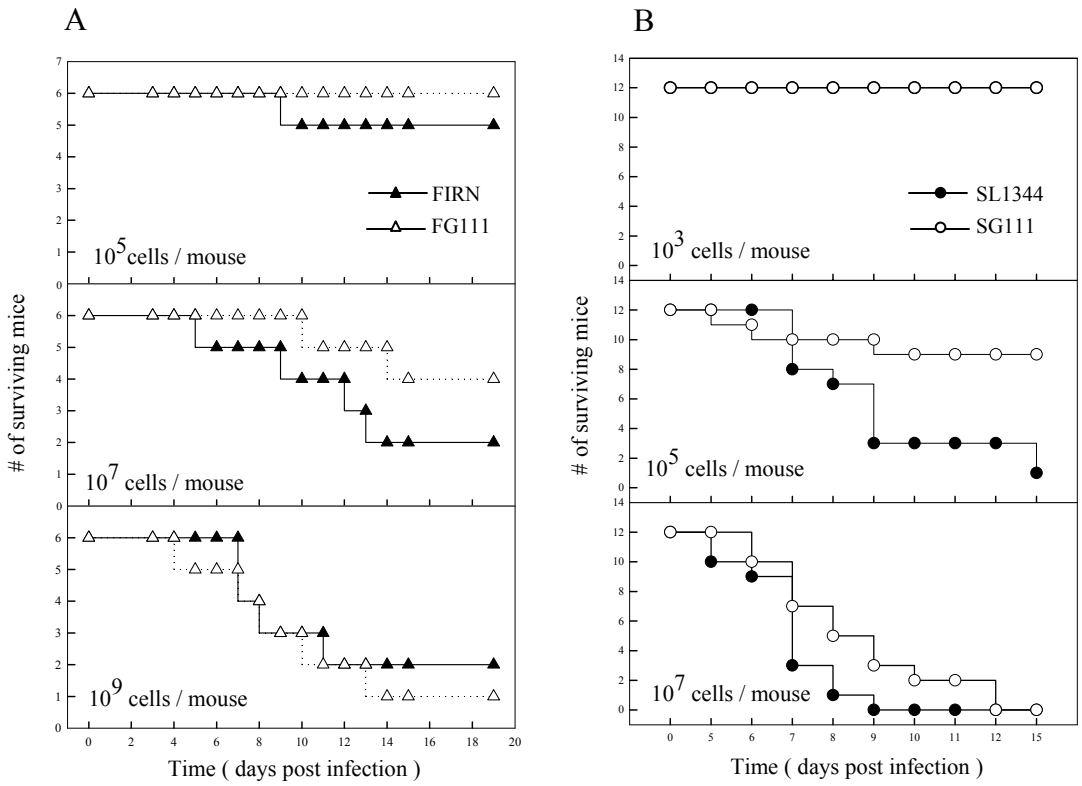


Fig. 6. Competitive virulence study of *S.* Typhimurium strains in mice. In panel A, BALB/c mice were orally infected with 10^5, 10^7, and 10^9 CFU of *S.* Thyphimurium FIRN wild-type (wt) or FG111 strains in 0.2 ml of saline on day 0 and mortality was determined. Lines represent the number of animals surviving in each experimental group over time (n = 6 per group). In panel B, BALB/c mice were orally infected with 10^3, 10^5, and 10^7 CFU of *S.* Thyphimurium SL1344 wild-type (wt) or SG111 strains in 0.2 ml of saline on day 0 and mortality determined. Lines represent the number of mice surviving in each experimental group over time (n = 12 per group). Survival curves from a representative experiment are shown.

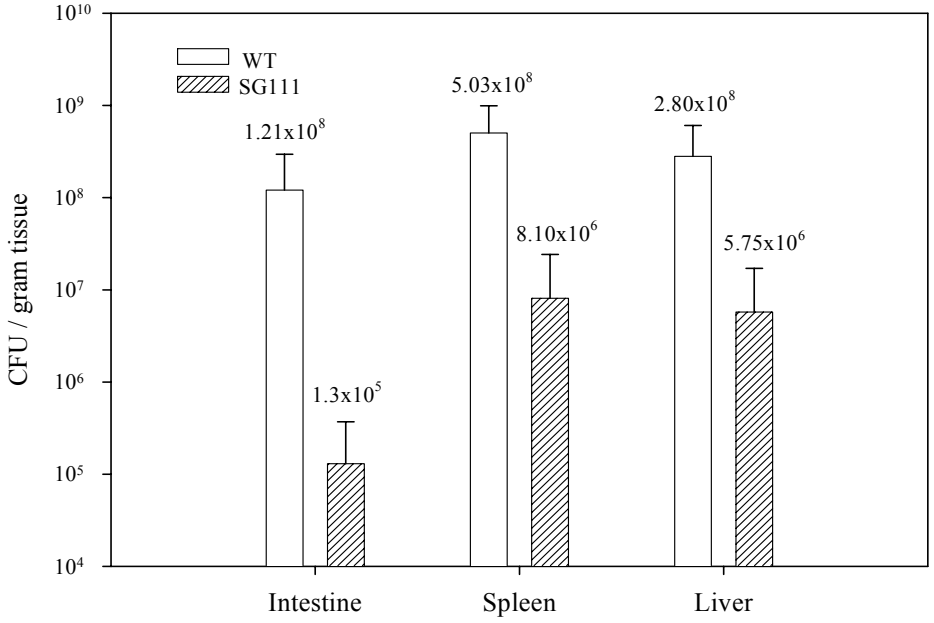


Fig. 7. Effect of the *mdoG* mutation on the ability of *Salmonella* Typhimurium to colonize mouse organs. Groups of five mice were infected orally with either the WT strain SL1344 or the Δ*mdoG* mutant SG111 (1.5×10^7 organisms per mouse) and the number of bacteria present in different organs determined 6 days after infection. Each bar represents the mean for five mice; error bars are the SE. Data shown are representative of three independent experiments. $P < 0.0001$ for all tissues.

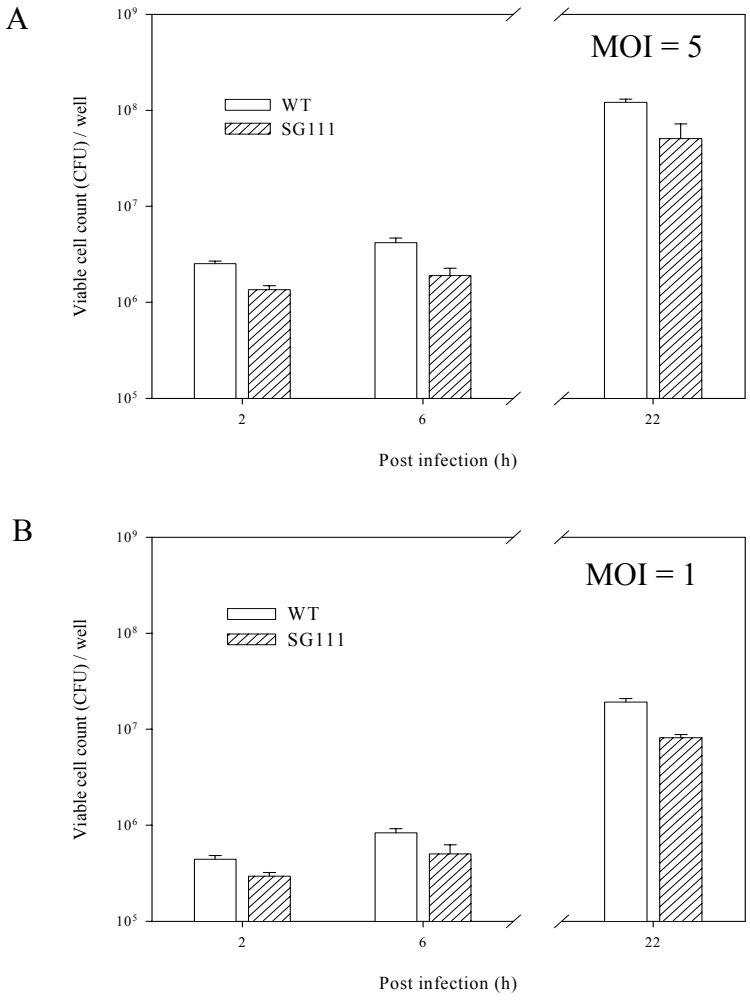


Fig. 8. Intracellular survival and replication of *S.* Typhimurium SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) within macrophages after phagocytosis. RAW262.7 cells were challenged with *Salmonella* Typhimurium strains (graph A: MOI= 5 bacteria/cell; graph B: MOI= 1 bacteria/cell). The ability of bacteria to survive within RAW264.7 cells was examined at 37 °C. The numbers of CFU were determined at the indicated times. The data are the means of triplicate determinations, and the error bars indicate the SE of the means. The statistical difference in the mean CFU with WT and SG111 was determined with the Student *t* test. The differences in mean CFU were significant at all time intervals examined ($P < 0.01$).

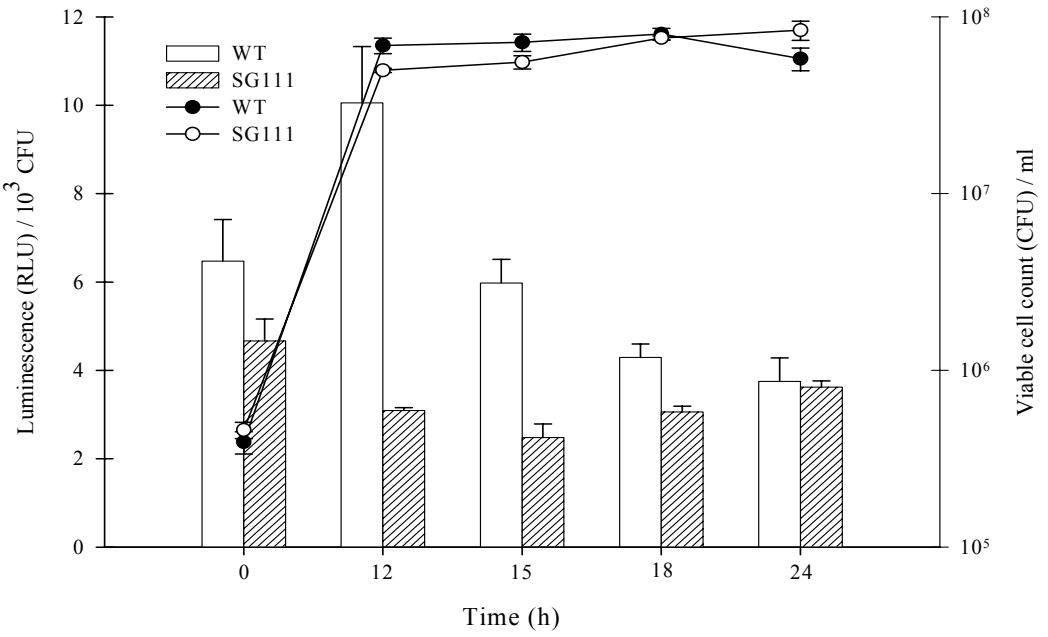


Fig. 9. Respiration (ATP level) in the WT strain and the Δ*mdoG* mutant SG111. ATP levels were monitored by enzyme assay as described in Experimental procedures. RLU, relative light units. Values (luminescence) were normalized for viable cell count (CFU) and represent the mean ± SE (not shown when smaller than the symbol) from three separate experiments. The graph depicts results from one experiment performed in triplicate and is representative of three independent experiments.

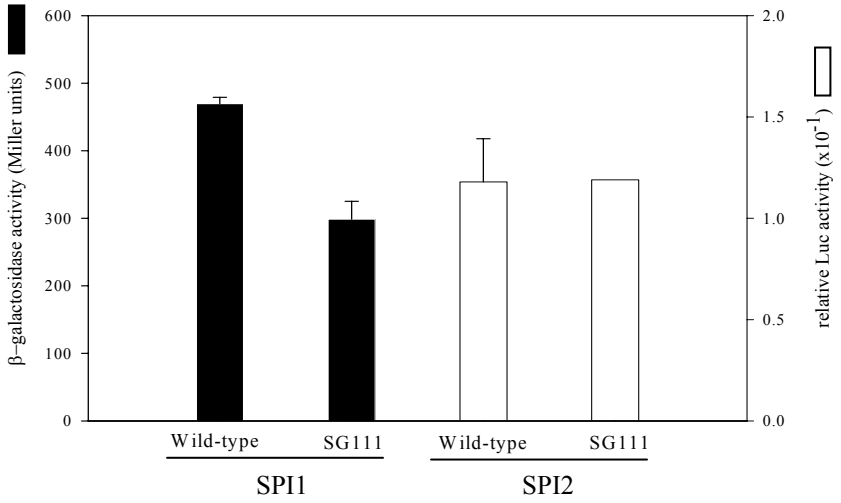


Fig. 10. Expression of genes in SPI1 and SPI2. The expression of reporter gene fusion in SPI1 (*prgH*::*lacZ*, solid bars) and SPI2 (*sseI*::*luc*, open bars) was analyzed in the background of the *Salmonella* Typhimurium wild-type strain and the Δ*mdoG* mutant strain. Strains were grown under conditions as highly inducing for SPI1 gene expression (Bajaj et al., 1996) or as inducing for SPI2 gene expression (Deiwick et al., 1999). Cultures were grown for about 16 h in LB containing 1% NaCl for the induction of SPI1, or in N salts minimal medium for the induction of SPI2. The Luc activities of lysates of strains harbouring the *sseI*::*luc* fusion (open columns) and β-galactosidase activities of strains harbouring the *prgH*::*lacZ* (solid columns) were determined. Each bar represents the mean of three replications; error bars, SE. Data shown are representative of three independent experiments. The statistical difference in the mean Miller units with WT and SG111 was determined with the Student *t* test. The differences in mean Miller units were significant ($P < 0.05$), but not in Luc activity.

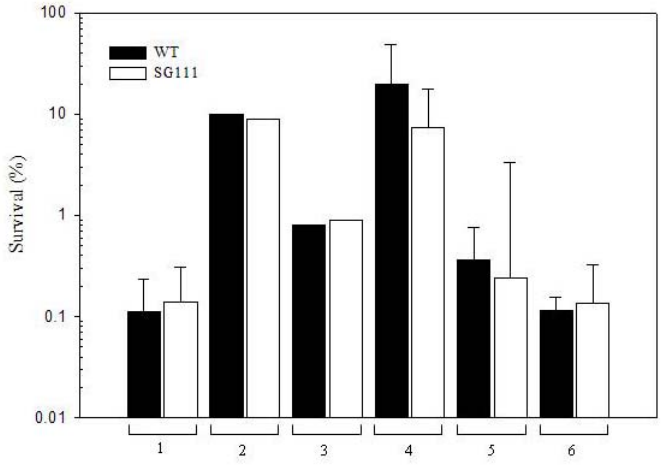


Fig. 11. Survival assays of *S.* Typhimurium strains: Filled bars, *Salmonella* Typhimurium strain SL1344 wild-type; open bars, SG111 (Δ*mdoG::kan*). 1. Effect of MDO on acid tolerance. Survival of stationary phase cells after 2 h of EG (pH 3) acid challenge; 2. Base tolerance. Survival of stationary phase cells after 2 h of EG (pH 9.8) base challenge; 3. Heat tolerance. Survival of stationary phase cells after 5 min at 58 °C; 4. Bile tolerance. Survival of stationary phase cells after 24 h of 15 % bile challenge; 5. Polymyxin B resistance. Exponentially growing *Salmonella* strains were treated with 1 μg per ml polymyxin for 1 h.; 6. H_2O_2 resistance. Stationary phase cells were exposed to 20 mM H_2O_2 for 2 h. Percent survival was determined as the viable cell number at each time point divided by the viable cell number before exposure to stress. Error bars indicate SE (not shown when smaller than the symbol).

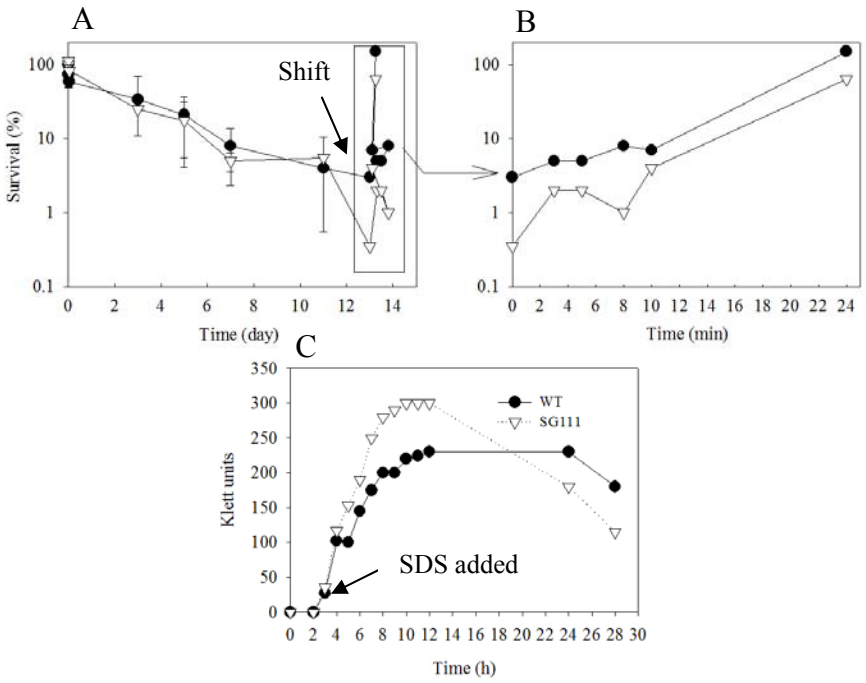


Fig. 12. Survival assays of *S.* Typhimurium strains. Nutrient starvation (A) and recovery of *Salmonella* strains (B). Cells were placed on polyethersulfone membranes and allowed to starve for 13 days after which they were transferred to EGC media for re-growth. Error bars indicate standard error. (C) Effect of detergent (5% SDS) on growth in LB media. SDS was added at 3 h after inoculation (5%, v/v).

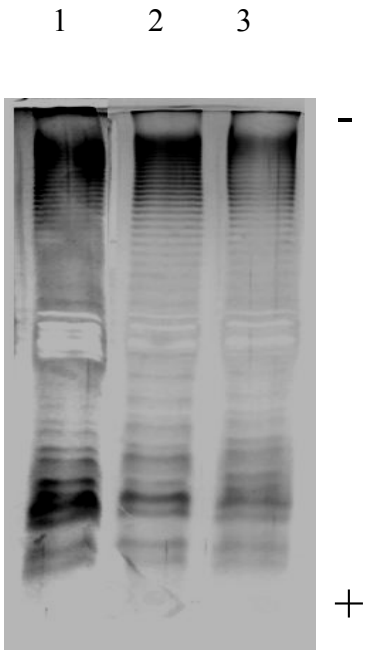


Fig. 13. Gel electrophoresis of *Salmonella* Typhimurium LPSs. The gel (15% acrylamide) was run and LPS was detected by silver stain (Tsai et al., 1982). Samples of 10 μg were analyzed. 1, *Salmonella* Typhimurium SL1344 (WT); 2, SG111; 3, SG112.

Table 5. Bacterial strains.

Strain	Description	Reference
S. Typhimurium		
S. Typhimurium FIRN	Wild type Nal^r	SGSC
FG111	FIRN Δ*mdoG::kan*	This study
S. Typhimurium SL1344	Wild type *his* Sm^r	Hoiseth et al. (1981)
SG111	SL1344 Δ*mdoG::kan*; Kan^r	This study
SG112	SG111 containing pWG21; Kan^r Cm^r	This study
EE656	*prgH*::*lacZY*; Tc^r	Ferric Fang
SG170	SG111 containing *prgH*::*lacZY*; Kan^r Tc^r	This study
SL167	*sseI*::*f-luc*; Amp^r Kan^r	This study
SG167	SG111 containing *sseI*::*f-luc*; Amp^r Kan^r	This study
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ (*lac-proAB*) *mcrA* (F' *traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15)	Promega
S17-1 (λ pir)	Tp^r *recA thi pro hsdR*$^-$*M*$^+$ *RP4:2-Tc:MuKm* Tn*7*, λ pir	Kim et al. (2002)
Plasmids		
pGEM-T	Vector for cloning of PCR products	Promega
pUC4K	Source of the kanamycin cassette	Stratagene
pKNG101	Sm^r, oriR6K, mobRK2, *sacB*, suicide vector	Kim et al. (2002)
pACYC184	Low-copy-number cloning vector; Cm^r Tc^r	New England Biolabs
pMDOG1	pGEM-T derivative harboring 1,924-bp *mdoG* PCR fragment	This study
pMDOG11	882-bp HpaI/Bsu36I fragment containing the *mdoG* coding region from pMDOG1 was removed and km cassette was inserted into the deleted part (Δ*mdoG::km*); Kan^r	This study
pMDOG111	2,100-bp EcoRI fragment from pMDOG11 cloned in pKNG101 at SmaI site; Kan^r	This study
pWG1	pGEM-T derivative harboring 2,030-bp *mdoG* PCR fragment	This study
pWG21	2,086-bp NotI fragment containing *mdoG* from pWG1 cloned in pACYC184 at EagI site; Cm^r	This study
pEM67	pGPLFR03 containing *sseI*::*f-luc*; Amp^r Kan^r	Ferric Fang

SGSC; *Salmonella* Genetic Stock Center, Alberta, Canada

CHAPTER 4

Synthesis of MDO in *Salmonella* Typhimurium confers a bacterial growth advantage in mice but not on media *in vitro*

4.1. Abstract

Foodborne illness caused by *Salmonella* is associated increasingly with fresh fruits and vegetables. The role of MDO as related to survival of *Salmonella* Typhimurium SL1344 and the Δ*mdoG* mutant was determined by competitive growth experiments in mouse organs, on laboratory media, and fresh-cut fruit slices. Wild type bacterium, *S.* Typhimurium SL1344 colonized very efficiently in mouse organs, while the Δ*mdoG* mutant exhibited slow growth in mouse organs and was outcompeted by the WT SL1344 strain. These results demonstrate the importance of the *mdoG* gene for increasing the fitness of *S.* Typhimurium for colonization.

The effects on competition and survival of these bacteria on both LB rich medium and EGC minimal medium were examined next. The changing proportion of each bacterium in these media was determined by plate counting at regular intervals. When competition experiments with WT and Δ*mdoG* mutant strains were performed using LB medium, *S.* Typhimurium SL1344 Δ*mdoG* mutant outcompeted the WT. Similar results were found using EGC medium. Mutation in *mdoG* resulted in a great advantage in minimal medium where casamino acids were the sole source of carbon and energy. To investigate this result further, we checked the doubling time for individual bacterium, but the generation time between the WT and mutant was similar. Thus, they might have different survival strategies.

A fresh-cut produce challenge was performed to examine the competition between bacteria in different nutrient composition environments. Competition between the bacteria was evident on tomato and cucumber slices. The WT had a slight advantage, with the difference increasing during a longer incubation time. Also, the WT grew more effectively in a mixed culture.

4.2. Introduction

Salmonella enterica serovar Typhimurium is a major cause of gastroenteritis or food poisoning in humans (Miller et al., 1995). During infection, *Salmonella* spp. must sense and respond to harsh environments within the host, such as the small intestine and spleen and have evolved a number of distinct strategies to survive and propagate in a wide variety of cell types in the host.

In a rich medium, such as LB broth, during the exponential growth phase, which follows an initial lag phase, the population of bacteria grows rapidly, doubling every 20 to 30 min. During this period of rapid growth, carbohydrates initially serve as the primary carbon and energy source but are quickly depleted, and other nutrients, including peptides, amino acids, nucleic acids, nucleotides,
and fatty acids, are utilized to sustain growth (Zinser et al, 1999; Farrell et al., 2003). Acetate is released at this time and the medium becomes acidic (Luli et al., 1990). As these remaining nutrients are depleted, the population enters a stationary phase, during which time little change is observed in the number of viable cells in the culture for 2 to 3 days and ammonia released into the medium causes it to become basic. As the culture enters the death phase, cell viability abruptly declines and approximately 99% of the cells

die. Finally, during the long-term stationary phase, there is a lengthy period of slow decline in the remaining viable cell population (Farrell et al., 2003).

Indeed, in their natural environment bacteria spend only a small fraction of their existence in exponential growth due to a limited availability of nutrients. The vast majority of their existence must, by necessity, be in a non-growth state. Yet most of our understanding of bacterial physiology has been obtained from cells undergoing exponential growth.

The rapid, stressful changes that occur in the hosts or in the medium place a heavy burden on the *S.* Typhimurium population. How rapidly and to what extent bacteria can genetically adapt to novel environments has been examined mostly in evolution experiments under apparently homogenous and constant laboratory conditions. These studies show that considerable potential to increase niche-specific fitness often exists (Lenski et al., 1994; Rosenzweig et al., 1994; Rainey et al., 1998; Rainey et al., 2000; Elena et al., 2003). Such experimental adaptation was associated with a substantial fitness increase, changed cell size, and an altered pattern of gene expression (Lenski et al., 1994; Lenski et al., 1998; Lenski et al., 2003).

The gram-negative pathogen *Salmonella* Typhimurium can adhere to and invade gut epithelial cells, disseminate to systemic sites (*e.g.*, liver and spleen) via blood and lymph glands, and survive and replicate within a variety of host cells, including macrophages (Richter-Dahlfors et al., 1997; Salcedo et al., 2001; Santos et al., 2003). To successfully adapt to these different niches the bacterium regulates the expression of an extensive repertoire of virulence factors that confer an appropriate physiological response (Clements et al., 2001). In addition, during growth within a host there might be selection

for bacterial mutants, which, due to specific regulatory/physiological changes, are better adapted to one or more of the specific host niches (Eriksson et al., 2000).

Here we investigated the role of the production of MDO in the ability of the competition between *Salmonella* Typhimurium SL1344 and the Δ*mdoG* mutant in various environmental conditions, such as mouse organs, a laboratory medium, and fresh-cut fruit slices. We examined how the Δ*mdoG* mutant adapts to the complex and variable natural environment of a host during competition against its parental strain. We found that a laboratory medium influenced the outcome of such competition in contrast to the mice virulence study. To learn more about the function of MDO *in vivo*, we tested the competition between a Δ*mdoG* mutant and a WT strain while growing on various fresh-cut fruit slices. We had previously observed that MDO do not contribute significantly to the ability of *S.* Typhimurium to survive *in vivo*, but that it is required for the rapid bacterial growth that occurs in the intestine, spleen, and liver. These competitive population studies were complemented by carbon utilization profiling of the Δ*mdoG* mutant and its parental strain.

4.3. Materials and Methods

Bacterial stains and culture conditions

A *S.* Typhimurium SL1344 derivative (Nal^r) was obtained by plating aliquots of overnight cultures on LB plates containing nalidixic acid (50 μg/ml) to distinguish strains between *Salmonella* Typhimurium SL1344 (Nal^r) and the Δ*mdoG* mutant (Kan^r) in the competition study. This marker has no effect on competitive advantage under growth conditions. Cultures were routinely started from frozen stocks for growth on LB agar and

after incubation overnight at 37°C, a single colony was selected and inoculated into either 10 ml LB broth or minimal EG medium (Lin et al., 1996) supplemented with 1% casamino acids at pH 7.0 (EGC medium) to facilitate growth in a 125-ml Erlenmeyer flask. The cells were grown for 17 to 19 h at 37°C with shaking at 200 rpm to obtain stationary-phase cultures.

Competition assays

Competitive index assay. Wild-type and mutant bacteria were grown without shaking for 24 hr at 37°C in 10 ml of LB until they reached the stationary phase. Wild-type and mutant bacteria were diluted in saline and mixed in equal proportions. BALB/c mice were infected orally with a total of 3 x 10^7 mixed inoculum in a volume of 0.2 ml containing the Δ*mdoG* mutant strain resistant to kanamycin and a WT competing strain. At 6 days after infection, mice were sacrificed. The intestine, liver, and spleen were homogenized in LB, diluted, and plated on Brilliant green agar medium (Difco, Franklin Lakes, NJ.) containing streptomycin for determination of total *Salmonella* CFU. Colonies were plated under kanamycin selection for enumeration of SL1344 Δ*mdoG* mutant (Δ*mdoG*::*kan*). The competitive index (CI) was calculated as [mutant/wild-type]$_{output}$/[mutant/wild-type]$_{input}$. All experiments were performed at least twice with a minimum total of 10 mice.

Competition of *S*. Typhimurium in medium. For competition in either LB or EGC, initial cultures were inoculated from LB agar into 10 ml of LB or EGC and grown overnight. *S*. Typhimurium SL1344 and SL1344 Δ*mdoG* at ~2 x 10^6 CFU/ml were mixed at a ratio of 1:1 and then subcultured 1:1000 into fresh LB or EGC and incubated at 37°C overnight. Samples from each culture were extracted at the start of the cultivation, diluted,

and plated on LB plates with and without antibiotic to confirm the 1:1 ratio between SL1344 and the Δ*mdoG* mutant. The two populations were monitored every 2 h by serial dilution in PBS and plating on LB agar plates containing nalidixic acid (50 μg/ml) or kanamycin (50 μg/ml). Each competition experiment was performed in duplicate or triplicate and repeated at least twice. To monitor the role of the starting ratio of the strains, *S*. Typhimurium SL1344 and the Δ*mdoG* mutant cultures were mixed 25:1 in 10 ml LB or EGC medium. The starting ratio was confirmed by plating as described above. Cultures were grown and analyzed as described above.

Competition of *S*. Typhimurium onto polyethersulfone membranes on fresh-cut fruits. 'Roma' tomatoes, cucumbers, honeydews, cantaloupes, watermelons, 'Granny Smith' apples, 'Red Delicious' apples, and 'Fuji' apples were obtained from local grocery stores. These fruit surfaces were sterilized using 70% ethanol and sliced with a sterile knife. Approximately 2- to 3-mm-thick slices were placed on sterilized Petri-dishes and polyethersulfone membranes (0.22 μm thick; Durapore membrane, Millipore Inc., Bedford, MA) were autoclaved before they were laid on fresh-cut slices (Gawande and Bhagwat, 2002). A 10 μl mixture (containing approximately 10^7 cells) of *Salmonella* Typhimurium WT cells with Δ*mdoG* mutant at a 1:1 (v/v) ratio were placed on individual polyethersulfone membranes and allowed to incubate at room temperature for 20 min in order to allow the cells to adhere to the polyethersulfone disk on the sliced fruit surface. The cells on the polyethersulfone disk were extracted in saline to determine the recovery of cells for control. After this, the fruit slices on a Petri-dish in a closed container were incubated at 15°C. At 3 days and 5 days after incubation, the cells on the polyethersulfone disk were extracted in saline, and serial dilutions in PBS were used for

viable-count determinations on LB agar plates containing nalidixic acid (50 μg/ml) or kanamycin (50 μg/ml).

Carbon source utilization

Phenotypic analysis was carried out using Phenotype MicroArrays (PMs) in collaboration with Dr. Ben Tall from the U.S. Food and Drug Administration. Respiration rates of *S.* Typhimurium parental and mutant strains were determined using various carbon sources in BIOLOG plates (Biolog Inc., Hayward, CA). Cells from frozen stock were streaked onto EGC minimal medium agar and were grown overnight at 37°C. Cells were then re-streaked on EGC agar and again grown overnight at 37°C. Colonies were picked from the surface of the EGC plate using a cotton swab. These cells were suspended in GN/GP-IF (inoculating fluid) at a density corresponding to 85% transmittance (optical density, 0.05) in the Biolog turbidimeter using a 20-mm diameter tube, and the suspension was added to PM Microplates (Biolog Inc., Hayward CA) containing various substrates. The microtiter plates were incubated for 48 h at 37°C. Three independent replicate assays were performed for each strain. The color change resulting from the reduction of tetrazolium violet by respiration was monitored according to the manufacturer's instructions.

4.4. Results

Competitive infection of mice with *Salmonella* Typhimurium SL1344 (wild-type) and the Δ*mdoG* mutant

Infection of mice with *S.* Typhimurium results in a systemic disease whereby bacteria replicate primarily in the spleen and liver (Kingsley et al., 2000). In competitive

index assays (CI), the relative abilities of two bacterial strains to colonize the host were measured. These experiments provide a more sensitive measure of the contribution of a given *Salmonella* gene to virulence than the traditional time-to-death studies.

To investigate if MDO play a role in virulence, the ability of the Δ*mdoG* mutant bacteria to infect mice was compared to the WT. After 6 days, infected intestines, spleens, and livers were collected and homogenized. The bacteria were differentiated on antibiotic-containing plates. We had previously observed that in time-to-death studies, infection with a Δ*mdoG* mutant was distinguishable from that by the WT bacteria (see Chapter 3). In the more sensitive CI assay, this mutant was out-competed by the WT SL1344 strain, which established normal levels of colonization, showing virulence attenuation (Fig. 14). The mean CI has statistical significance in intestine and spleen ($P < 0.01$ in intestine; $P < 0.05$ in spleen, one-sample *t* test) in that they are much lower than 1 (Fig. 14). In liver ($P > 0.2$), the data is skewed because one data point is abnormally high, making the mean is closer to 1 (Fig. 14). In summary, mutant survival in the liver can be considered to be as poor as survival in any other organ. Thus, it was concluded that the function of *mdoG* is adequately required such that a Δ*mdoG* mutant suffers a reduced virulence in mice.

_S_. Typhimurium SL1344 competition with the Δ*mdoG* mutant in LB medium

To monitor growth and death of the subpopulations of co-cultures, competition experiments were performed with a spontaneous antibiotic resistant mutant of the WT strain. The antibiotic resistance markers did not confer an increased or decreased fitness under the conditions assayed as both antibiotic resistant strains remained fully viable in 1:1 overnight mixes. In an attempt to understand the mice organ colonization properties

of *S.* Typhimurium strains, we tested the WT strain SL1344 against the Δ*mdoG* mutant in LB *in vitro*, a rich medium containing many of the carbon sources, vitamins, and energy sources necessary for growth. Under LB, the Δ*mdoG* mutant out-competed the WT strain. Although the WT strain constituted 50% of the population at the start of the cultivation, it ended up constituting of only 4% (Fig. 15). The Δ*mdoG* mutant constituted of about 97% of the population after only 4 h. However, this result contrasts with the CI assay in mice. To further investigate the advantage of the Δ*mdoG* mutant, it was competed against WT SL1344 with a starting ratio of 1: 10. Although the WT constituted 90% of the population at the start of the experiment, it accounted for only 31% of the population after 12 h (data not shown). Taken together, the data show that the Δ*mdoG* mutant is able to outgrow the *S.* Typhimurium SL1344 WT strain.

To demonstrate how the Δ*mdoG* mutant outcompetes the WT strain, we compared the generation time between WT and mutant in LB. Both strains grew well in LB, with doubling times 38.8 ± 0.1 min for WT and 38.1 ± 2.3 min for the Δ*mdoG* mutant (data not shown). No significant difference in generation time of the Δ*mdoG* mutant relative to the parental strain SL1344 was apparent, indicating that the ability of the Δ*mdoG* mutant to out-compete the WT was not due to different growth characteristics. These data might also indicate that increased growth was due to an enhanced ability to acquire or more efficiently utilize available nutrients during and after exponential growth.

_S._ Typhimurium SL1344 competition with the Δ*mdoG* mutant in EGC medium

Since amino acids are the most abundant nutrients, we competed the WT strain SL1344 against the Δ*mdoG* mutant on EG minimal medium supplemented with casamino acids, a mixture of monomeric amino acids, to determine if the mixture of amino acids

had any effect on competition. Under EGC, the Δ*mdoG* mutant outcompeted the WT strain like on LB, indicating that the *mdoG* mutation conferred on bacteria a significantly faster growth on the mixture of the amino acids. Although the WT strain constituted 50% of the population at the start of the cultivation, it ended up constituting only 3% (Fig. 16). This showed that the Δ*mdoG* mutant constituted about 97% of the population after 6 h, which is 2 h later than in LB. However, this result also contrasts with the competitive index (CI) assay in mice where WT dominated the mutant. We also tested the Δ*mdoG* mutant against the WT SL1344 with a starting ratio of 1: 10. Although the WT constituted 90% of the population at the start of the experiment, the Δ*mdoG* mutant dominated in the population after 12 h (data not shown). Taken together, these data show that the Δ*mdoG* mutant is able to outgrow *S*. Typhimurium SL1344 WT strain either in LB rich medium or in EGC minimal medium. The generation time between the WT and mutant in EGC medium was examined, and there was no difference of doubling time between the WT and mutant. This result indicated that the superiority of the Δ*mdoG* mutant against the WT was not due to different growth characteristics in EGC medium. Interestingly, in *S*. Typhimurium FIRN, the strain with the *mdoG* mutation did not outgrow the WT FIRN strain, indicating that the *mdoG* mutation confers a growth advantage on laboratory media *in vitro* only in *S*. Typhimurium SL1344 background.

_S_. Typhimurium SL1344 competition with the Δ*mdoG* mutant in fresh-cut produce

In order to determine if the nutrient composition of fresh-cut fruits affects competition between the WT and mutant, a mixture of *Salmonella* Typhimurium SL1344 and the Δ*mdoG* mutant at a 1:1 (v/v) ratio were inoculated onto polyethersulfone membranes on various fresh-cut fruits (Figs. 17, 18, 19, and 20).

On tomato and cucumber slices, after 3 days, the population of the Δ*mdoG* mutant was slightly lower than WT strain SL1344.; the WT strain constituted 50% of the population at the start of the experiment and its population increased to 60% after 3 days (Figs. 17 and 18). However, when examining the percentage of each individual strain in the other fresh-cut fruits inoculated with the mixture of the WT and Δ*mdoG* mutant (1:1, v/v), we observed an equal combination of SL1344 and Δ*mdoG* mutant through 5 days (Figs. 19 and 20). Growth of the strains on fresh-cut fruits was similar for both the WT and mutant strains, while subtle differences in growth of the bacteria were observed between WT SL1344 and the Δ*mdoG* mutant in tomato and cucumber slices.

Carbon substrate utilization

The carbon utilization profile of parental and Δ*mdoG* mutant strains was determined by testing carbon compounds present on Biolog plates. While many carbon sources were utilized by both strains, differences between the strains in their response to several substrates were observed. Table 6 provides a list of carbon compounds for which utilization by the Δ*mdoG* mutant strain was greater than that by the parental strain. The compounds for which the largest difference in utilization was observed were amino acids or their derivatives (L-cysteine, L-alanine, L-threonine, D-galactosamine, L-tryptophan, L-glutamic acid, ser-gln, arg-lys, lys-asp, lys-phe, ala-pro, gly-asn), and α-cyclodextrin. Other carbon sources, such as dextrin, D-arabinose, D-xylose, L-asparagine, 2-deoxy-D-ribose, D-saccharic acid, L-proline, sorbic acid, D-mannose, D-glucosamine, D-galactose, and D-trehalose were also utilized consistently less efficiently in the parental strain, but their utilization rate was lower than that in the mutant strain (data not shown). All compounds listed above are those for which we observed a difference in utilization

between the mutant and parental strains in at least two out of three replicated experiments. The carbon sources listed in Table 6 need to be tested for their ability to support growth of the parental and Δ*mdoG* mutant strains in minimal medium.

4.5. Discussion

The goals of this research were to examine the impact of various host environmental conditions on the levels and distribution of microbial competition between *S*. Typhimurium SL1344 WT and the Δ*mdoG* mutant and to determine their phenotypic properties and colonization levels in experimentally inoculated hosts.

Since *S*. Typhimurium is highly adaptable and capable of growth and survival in a wide variety of habitats, in this study, we inoculated the mixture of two bacterial strains at a 1:1 ratio (v/v) into mice, a laboratory medium, and fresh-cut fruit slices. The Δ*mdoG* mutant was outcompeted by the WT in mice organs *in vivo* but the Δ*mdoG* mutant outcompeted the WT in a medium *in vitro*. Comparative population studies revealed clearly that the MDO producing parental strain of *S*. Typhimurium SL1344 overall outcompeted the Δ*mdoG* mutant strain in the mouse intestine, indicating that MDO production conferred an advantage on *S*. Typhimurium in the enteric colonization of this host. This was further supported by our observation that *S*. Typhimurium SL1344 invaded mouse spleen and liver to a significantly higher extent than its Δ*mdoG* mutant.

Observations indicate that presence of wild type MDO confers an advantage in mouse virulence, but seems to give no benefit in LB and EGC media. In spite of the fact that mutant and parental strain were both co-cultured in the laboratory media at a 1:1 ratio, comparative population studies revealed that the Δ*mdoG* mutant strain outcompeted

the WT strain in LB and EGC media. Data indicates that increased growth of the Δ*mdoG* mutant was due to an enhanced ability to acquire or more efficiently utilize available nutrients. We also found that the colonization in fresh-cut fruits was similar for both the WT and mutant strains. Apparently, the competitiveness of mutant bacteria is reduced during colonization in a fruit environment. This might be of great importance in natural environments where nutrients are scarce and competition for them is intense (Finkel, 2006). However, the following observation does not support a significant role for MDO production in the growth and survival of *S.* Typhimurium in relation to virulence in mice: (i) the superiority of the Δ*mdoG* mutant when the mutant strain was co-cultured with the WT in a laboratory environment, despite a lower inoculation ratio of mutant strain to parental strain (1:1), and (ii) the similar behavior of the Δ*mdoG* mutant and the parental strain when they were coinoculated at a 1:1 ratio onto fresh-cut fruit slices. Thus, our observations indicate that the synthesis of MDO by *S.* Typhimurium in both a laboratory medium and on fresh-cut fruit surfaces does not measurably affect its fitness (defined as growth rate) in those environments.

In this study, the role of a mutation in *mdoG* and the influence of increased competitive ability on bacterial adaptation under a laboratory medium is of interest. The fact that bacteria with a mutation take over the population under laboratory conditions has been studied among several bacterial species (Notley-McRobb et al., 2002; Farrell et al., 2003; Zinser et al., 1999; Roos et al., 2006). For *E. coli* populations in a stationary phase, cell debris can serve as a nutrient source supporting survival and even growth as shown by cells exhibiting the growth advantage in stationary phase (GASP) phenotype, which resume growth in the stationary phase, scavenging nutrients released from dead

cells (Zambrano et al., 1993; Zinser et al., 1999). Whether a mutation confers mainly a cost or a benefit depends on several factors, including environmental stability, how well adapted a bacterium is to a particular niche, and the population structure. As shown by studies in laboratory media and animals, a mutation in *rpoS* that results in an attenuation of RpoS activity confers a benefit because of adaptation to basic pH and enhanced ability to utilize amino acids. In *E. coli*, strain 83972 grows very well in human urine *in vitro*, with shorter doubling times, in competition experiments against UPEC strains by outcompeting all of them, but this strain is not able to cause symptoms in the human host due to a loss in ability to express functional type 1 and P fimbriae (Roos et al., 2006).

An interesting question is whether and how an exchange of competitive ability exists between adaptations to different niches. For example, it has been shown that *E. coli* adapted to a mouse intestine shows lower fitness in a laboratory environment (Giraud et al., 2001). Conversely, it is known that propagation of pathogens under laboratory conditions often results in a loss of virulence (Siebers et al., 1999; Somerville et al., 2003 2004, Roos et al., 2006). The reasons for these exchanges are unclear but one proposal is that they result from mutations in genes that are not under selection in the primary environment. An alternative explanation is that there are genetic or physiological exchanges such that adaptive mutations that increase fitness in particular niches will inevitably cause a loss of fitness in a dissimilar secondary environment.

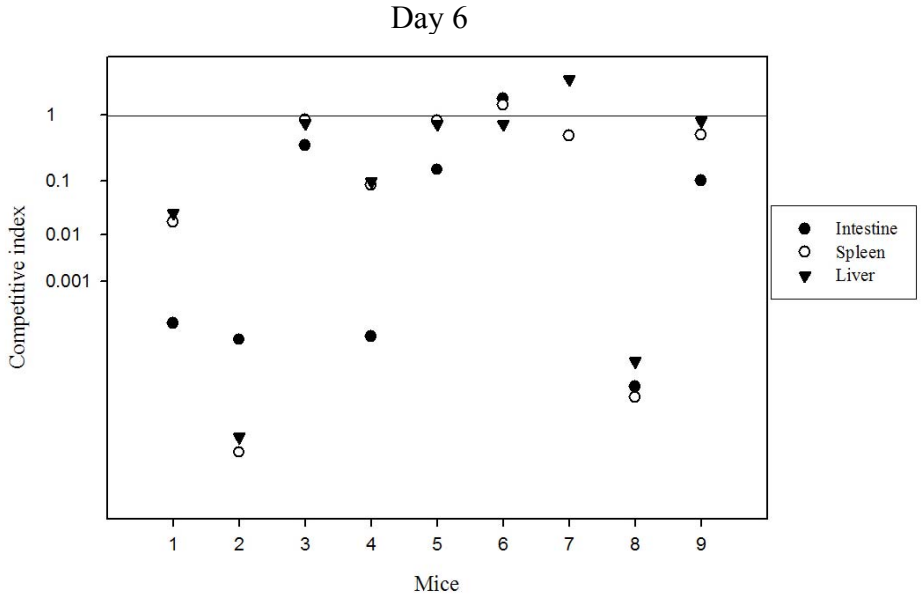
However, in mouse organs, the virulence requires WT *mdoG* activity. We have to determine what plays a particularly important role in determining the mutation in *mdoG* that confers a fitness advantage. The major difference observed in the contribution of MDO synthesis to the fitness of *S*. Typhimurium in mice compared to that in a laboratory

medium or on fresh-cut fruits may be related to differences in the chemical environment in mouse organs, a medium, and in the fruits. Indeed, examination of carbon utilization profiles revealed that the Δ*mdoG* mutant strain utilized certain amino acids and their derivatives, as well as organic acids, more efficiently than the parental strain but that most carbohydrates were utilized at a similar rate in both strains. However, this observation needs to be supported by growth rate studies in minimal medium with single carbon sources.

Our studies provide evidence that MDO production has a niche-specific benefit in *S*. Typhimurium. Although a wide variety of amino acids and organic acids are present on cut plant surfaces, albeit in small amounts (Morgan et al., 1964), assuming that *S*. Typhimurium behaves similarly on fruits, it was observed that MDO synthesis would be of little benefit to growth of *S*. Typhimurium on fruit slices. In contrast, amino and organic acids, which are likely to be present in high amounts in the mouse intestine due to the breakdown of proteins from food, may be an important nutrient source for *S*. Typhimurium, in addition to simple carbohydrates. Thus, the parental strain of *S*. Typhimurium SL1344 may have benefited from higher utilization rates of these compounds and competitively excluded its isogenic Δ*mdoG* mutant in the mouse intestine. However, this observation is not related to the relative competitive ability of a mutant versus a WT in a laboratory medium. It could be predicted that, based on our carbon utilization data, MDO synthesis would not give an advantage to the growth of *S*. Typhimurium in LB culture and minimal medium containing casamino acids. Perhaps the degree of variability in the competitive ability of bacteria from various niches is

dependent on the number of nutrients available and types and degree of various stresses present.

A



B

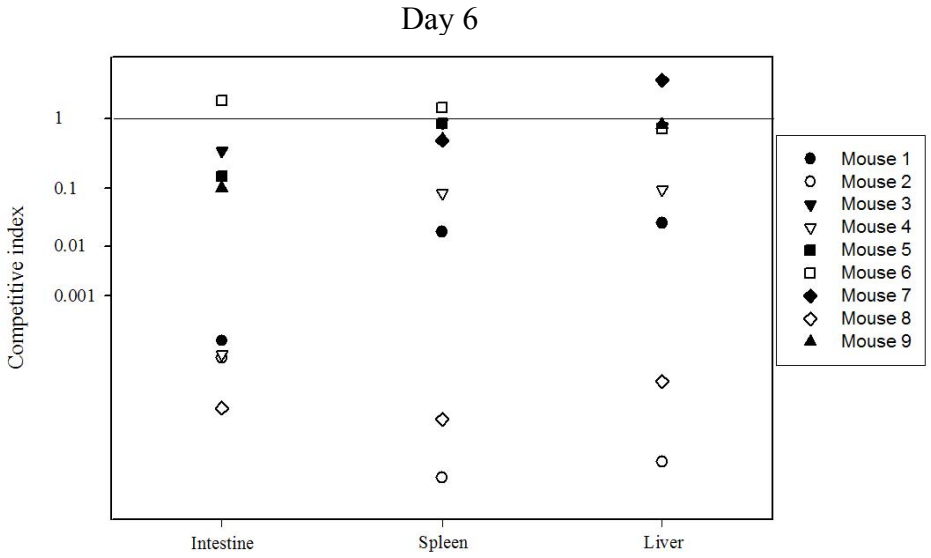


Fig. 14. Competitive infection of *Salmonella* Typhimurium SL1344 (wild-type) and the Δ*mdoG* mutant in the intestines, spleens, and livers of mice. Growth defects of MDO in

mixed infections. Groups of ten mice were infected orally with a 1:1 mixture of SL1344 (wild-type) and SG111 (Δ*mdoG*::kan) (3 x 10^7 CFU per mouse). At day 6 postinfection, the intestine, spleen, and liver were collected and the number of CFU for each strain was determined. Nine mice are shown in graph because neither wild-type nor SG111 were recovered from one of the mice. The CI for SG111 was calculated as the ratio of SG111 to SL1344 recovered from the various organs. During the competitive infection, recovery of SG111 was significantly lower in the intestine, spleen, and liver. (A) shows the CI (Competitive Index) versus mice while (B) shows the CI versus mouse organs. A mean CI is significantly different from 1 in intestine and spleen ($P < 0.01$ in intestine; $P < 0.05$ in spleen, one-sample *t* test) but not in liver ($P > 0.2$).

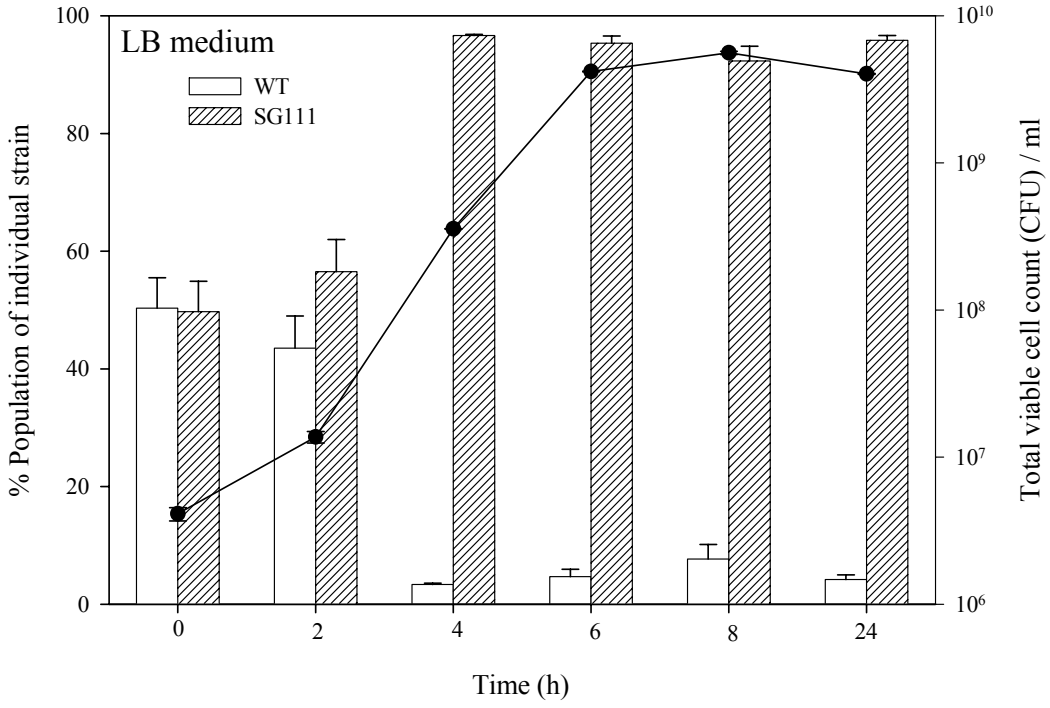


Fig. 15. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in LB medium. Values are means of triplicates, and error bars indicate SE.

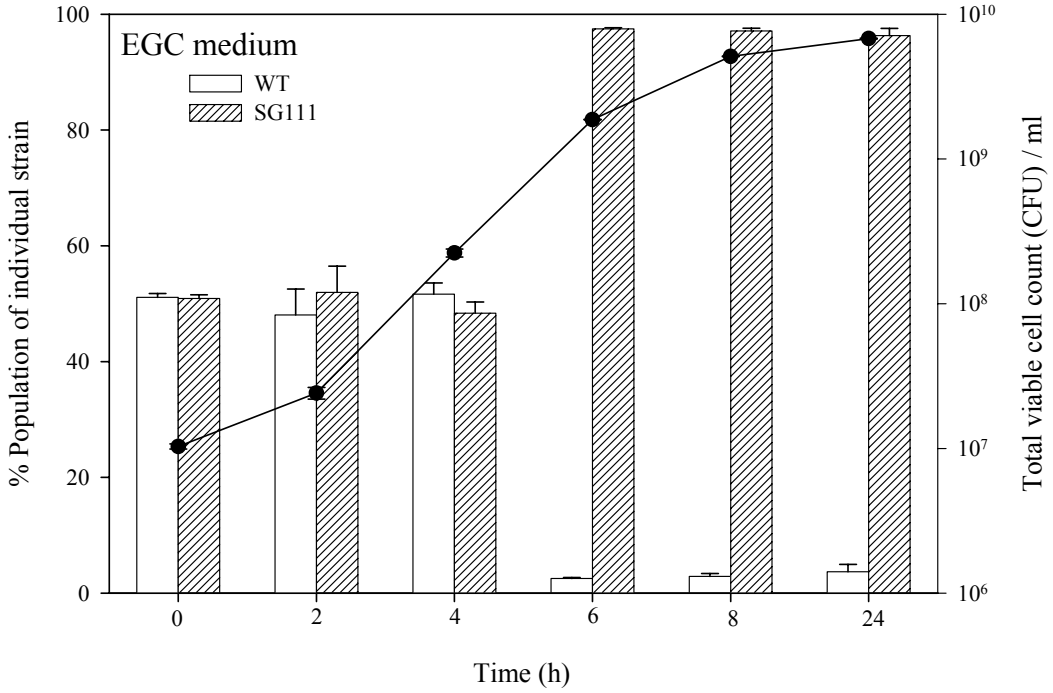


Fig. 16. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in EGC medium. Values are means of triplicates, and error bars indicate SE.

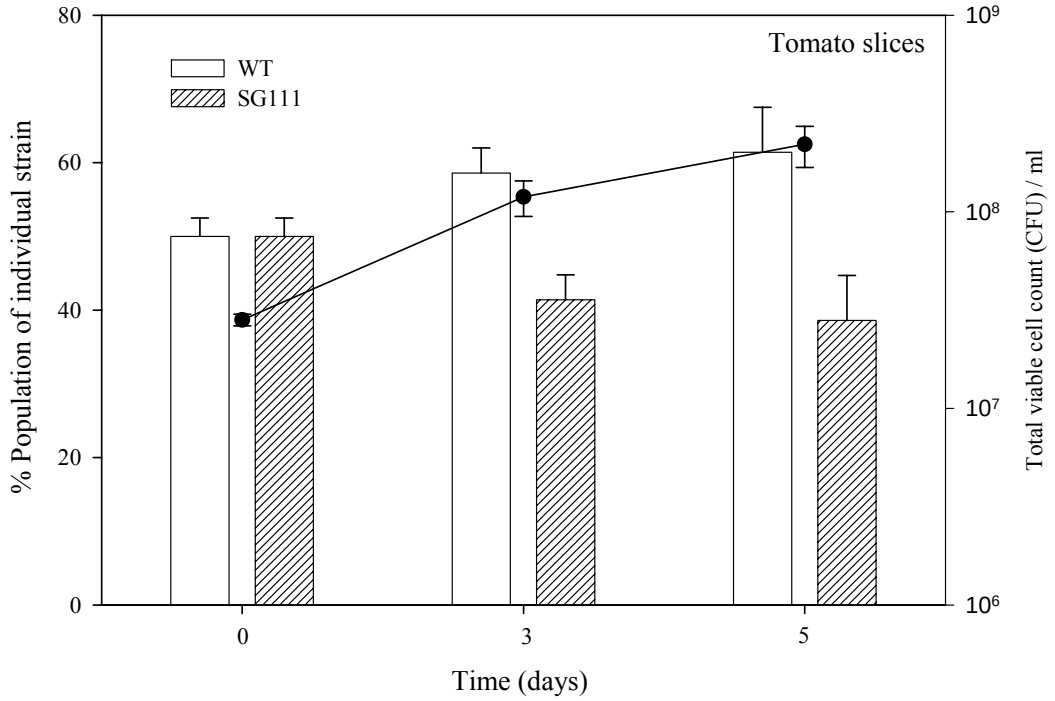


Fig. 17. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in tomato slices. Values are means of triplicates, and error bars indicate SE.

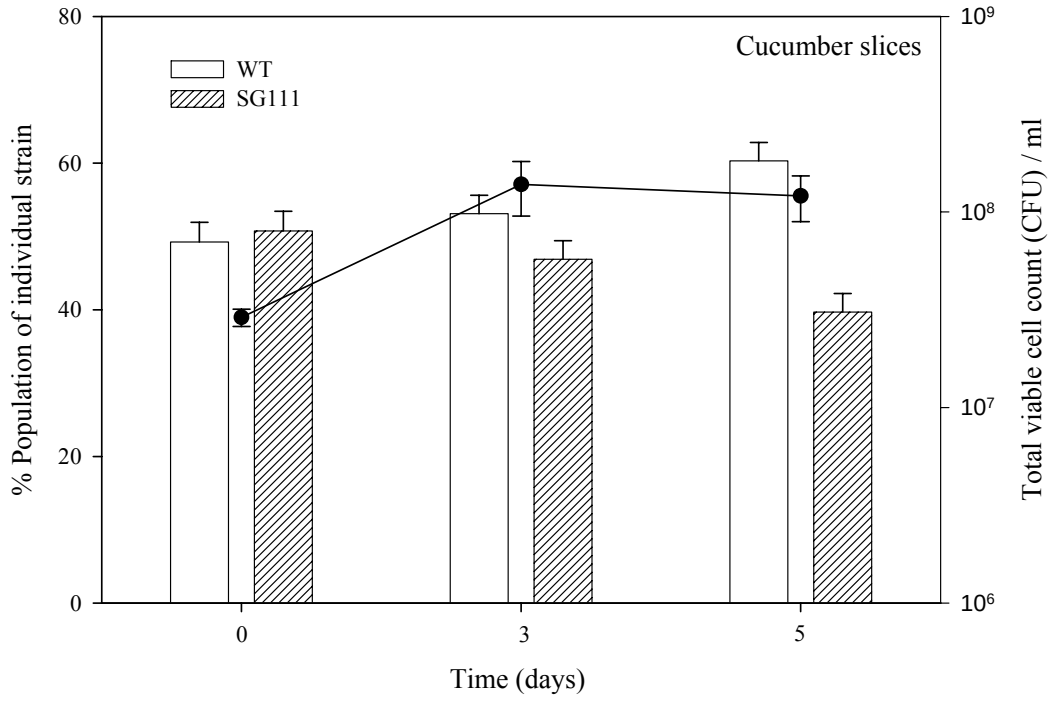


Fig. 18. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in cucumber slices. Values are means of triplicates, and error bars indicate SE.

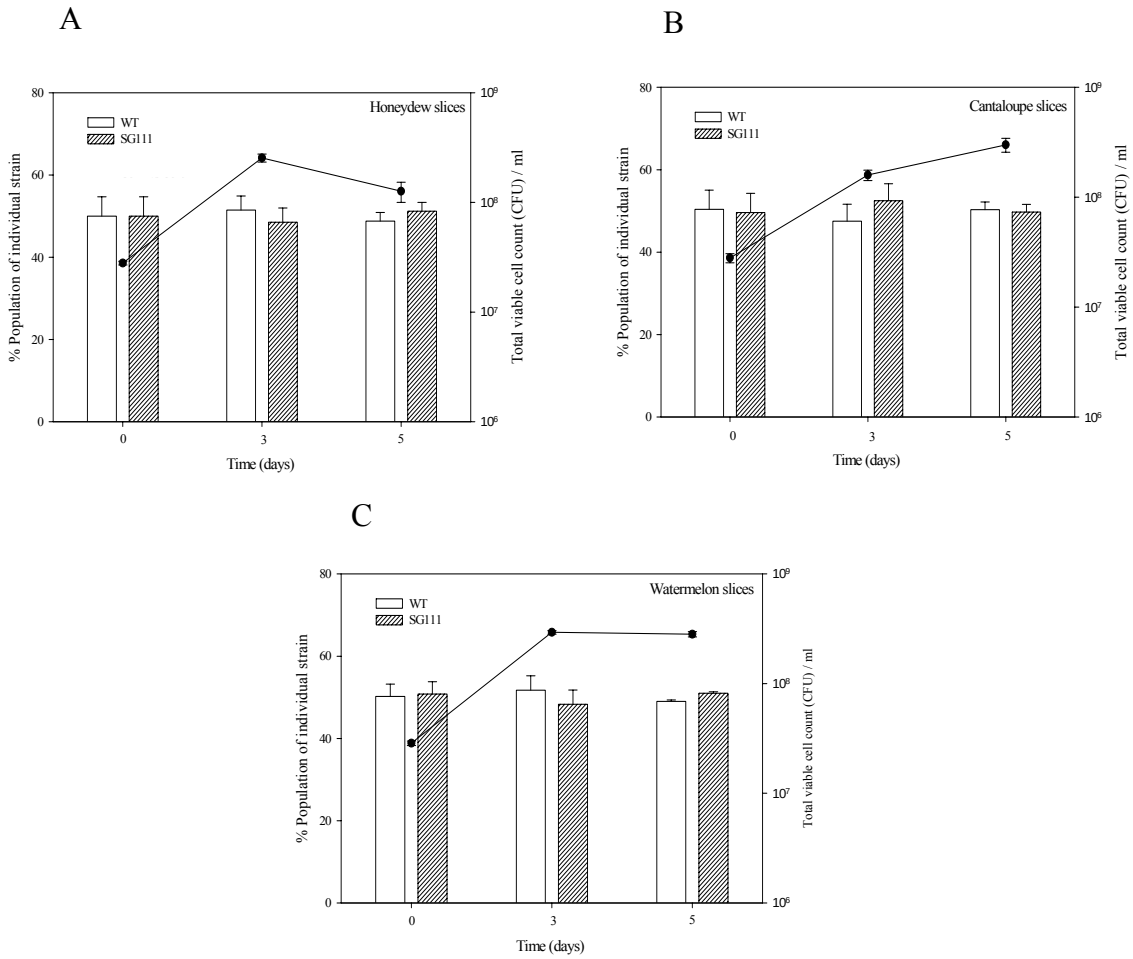


Fig. 19. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in honeydew (A), cantaloupe (B), and watermelon (C) slices. Values are means of triplicates, and error bars indicate SE.

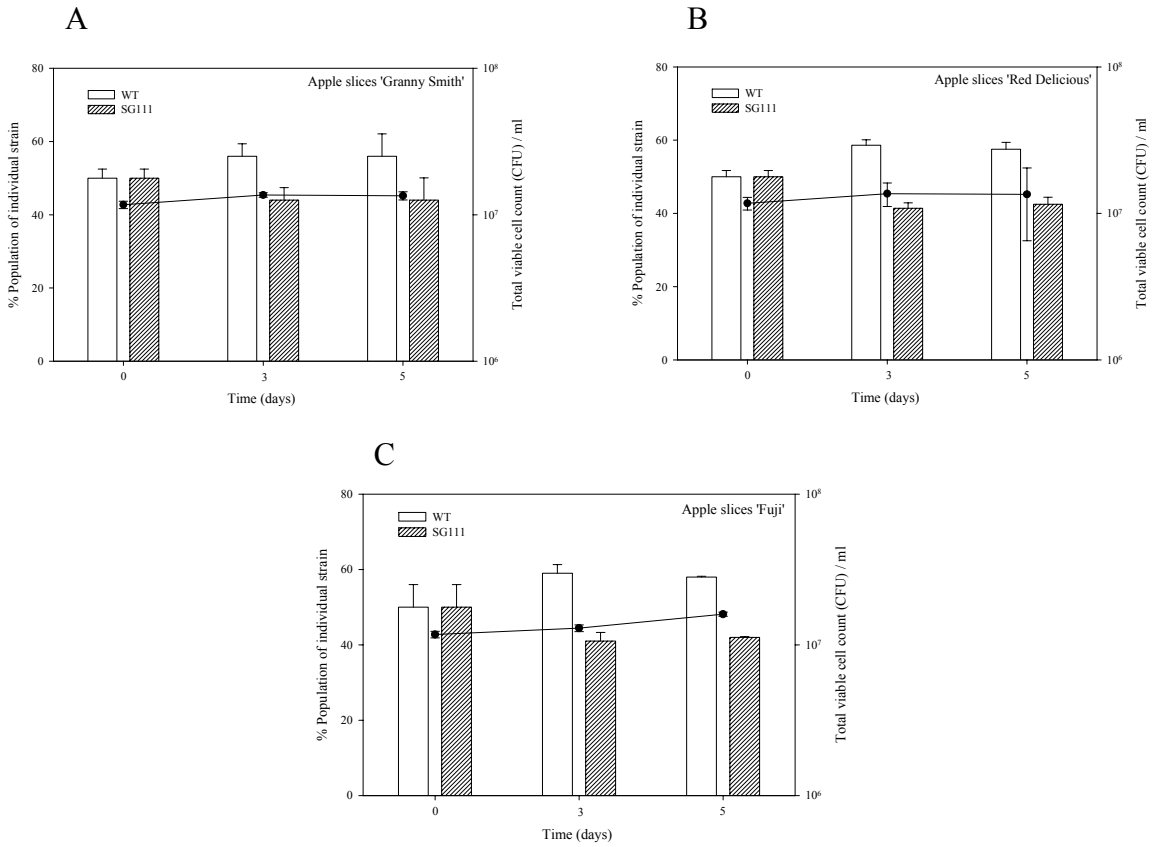


Fig. 20. Competition experiment between SL1344 (wild-type) and SG111 (Δ*mdoG* mutant) mixed 1:1 at the starting point in apples 'Granny Smith' (A), 'Red Delicious' (B), and 'Fuji' (C) slices. Values are means of triplicates, and error bars indicate SE.

Table 6. Carbon compounds utilized differentially by the *S*. Typhimurium SL1344 parental and Δ*mdoG* mutant strains in the Biolog assay.

Carbon compound	Mutant	Parental	Mutant/ Parental[a]
	Arbitrary units (Respiration)		
L-Cysteine	3484	2013	1.7
Ser-Gln	2366	787	3.0
Arg-Lys	3264	1465	2.2
Lys-Asp	3923	2064	1.9
L-Alanine	7010	259	27.1
L-Threonine	2229	83	26.9
D-Galactosamine	2730	78	35.0
L-Tryptophan	6105	3370	1.8
2`-Deoxy-Guanosine	3989	1024	3.9
α-Cyclodextrin	3594	336	10.7
L-Glutamic Acid	6579	3141	2.1
Lys-Phe	6117	2559	2.4
Ala-Pro	8296	4566	1.8
Pectin	11826	7441	1.6
Gly-Asn	9986	4408	2.3

In the Biolog assay, the color change resulting from the reduction of tetrazolium violet by bacterial respiration was measured. [a] For each compound, the data are presented as the proportion of the area obtained with the mutant to that obtained with the parental strain. Mutant strain, SG111; parental strain, SL1344

CHAPTER 5

GENERAL DISCUSSION AND FUTURE WORK

The primary goal of this research was to explore the structure of MDO that might be useful to understand if their structure is related to the function of MDO in *Salmonella* growth and virulence. However, initial NMR analysis revealed much complex and heterogeneous character of MDO and did not allow us to determine MDO structure at molecular level. In-depth carbohydrate analysis will be required and maybe using additional purification techniques and mutant strains to resolve the structure of MDO.

Next we took a genetic and physiological approach to define the role of MDO in *Salmonella* survival and virulence. The characteristics of the MDO defective mutants were investigated. As reported in Chapter 2, MDO produced by the *mdoG* deletion mutant had a different glycosyl composition compared to the WT. There appears to be a specific requirement of a certain MDO composition for optimizing mouse virulence. This is based on the fact that the *mdoG* deletion mutant synthesized MDO with an altered sugar composition and it was less virulent, except at a higher inoculum dose.

Results of 10 different *in vitro* stress-tolerance tests show that MDO of specific composition is not required for general stress-tolerance. However, for interaction with mammalian host, even *in vitro* macrophage survival assays gave significant differences based on MDO composition.

The only *in vitro* test in which we observed significant difference between wild type and mutant was using Biolog system monitoring the growth at pH 5.0. But, when we tried to reproduce Biolog data in an old fashioned growth study (counting colony forming

units (CFU)), we could not conclude that there was any growth advantage for WT MDO. Upon further investigation, we discovered in reality, Biolog system monitors cell respiration as a reporter system and not growth (turbidity). Biolog data show that the *mdoG* deletion mutant respires less than the WT. We wanted to see if reduced respiration was reflected by ATP level. ATP level, as a method to measure cell respiration, was monitored between the WT and mutant at pH 5.0 and a lower ATP level was observed in the *mdoG* deletion mutant than in the WT. Thus, it can be hypothesized from the ATP assay that virulence reduction in the *mdoG* deletion mutant must have something to do with a low ATP level (weak cells).

An altered MDO composition benefited the strain under artificial growth condition such as in LB and EGC media, indicating that MDO may also be involved in either nutrient uptake or other cellular functions.

MDO is a widely occurring substance in many gram-negative bacteria such as *Shigella*, *Vibrio*, and *Escherichia coli* 0157:H7 and the role of MDO with respect to bacterial growth and virulence is worth investigating.

List of future objectives

1. To identify the structure of MDO using NMR spectroscopy
2. To compare MDO structure in different *S.* Typhimurium strains in order to see if MDO structure is identical in different strains
3. To determine biochemical properties of the *mdoG* gene encoding the glycosyltransferase enzyme involved in MDO biosynthesis and study the structure-function relationship of the MdoG enzyme
4. To complement the *mdoG* deletion mutant by introducing the WT gene copy to recover full virulence of *Salmonella*
5. To identify the substitution of MDO in *S.* Typhimurium and the role of substituents modifying MDO in the adaptation to osmotic stress and in pathogenicity of *Salmonella* spp.

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