

***Salmonella* Enteritidis Thin Aggregative Fimbriae and the Extracellular Matrix**

by

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### ABSTRACT

The formation of the *Salmonella* extracellular matrix is a multicellular behavior important for environmental persistence. It is comprised of uniquely but ill-defined assembled thin aggregative fimbriae (Tafi), cellulose and uncharacterized polysaccharides. Consequently, investigations were launched into further clarifying Tafi assembly and the polysaccharide constituents of the extracellular matrix.

In the *Salmonella agfBAC* Tafi operon, the transcription and role of *agfC* has been elusive. In this study using the clinical isolate, *Salmonella* Enteritidis 27655-3b, *agfBAC* transcripts were detected using a reverse transcriptase and transcription was not enhanced by replacement of a stem-loop structure immediately preceding *agfC*. AgfC-his was purified, localized to the periplasm, and found to specifically bind non-crystalline cellulose suggesting an association with the extracellular matrix. An in-frame  $\Delta agfC$  mutant displayed an abundance of 20 nm fibers, which could be complemented with *agfC in trans*, in addition to Tafi and an increase in cell hydrophobicity. Depolymerization of purified 20 nm fibers required exceptionally stringent conditions to release what proved to be AgfA subunits revealing the 20 nm fibers as AgfA assemblages of unique morphology. The role of AgfC in Tafi assembly was investigated further via a novel, quantitative antibody-capture assay of in-frame *agf* mutants. A soluble antibody-accessible form of AgfA was captured in *wt*,  $\Delta agfB$  and  $\Delta agfF$  strains in support of the extracellular nucleation-precipitation pathway of Tafi assembly, but not in  $\Delta agfC$  or  $\Delta agfE$  mutants. These results suggest that AgfC and AgfE are required for AgfA's extracellular assembly and thus may act as atypical AgfA-specific chaperones which facilitate Tafi assembly. The implications of these results are presented in an assembly model for Tafi.

Additional investigations revealed that *Salmonella* produces an O-Antigen capsule co-regulated with the extracellular matrix. Structural analysis of purified extracellular polysaccharides (EPS) yielded a repeating oligosaccharide unit similar to

that of lipopolysaccharide O-Antigen with modifications. Putative carbohydrate transport and regulatory operons important for capsule expression, designated *emcA-H* and *emcIJ*, were identified by screening a random transposon library with immune serum generated to the capsule. The absence of capsule was confirmed by generating various in-frame  $\Delta emc$  mutants where *emcG* and *emcE* were shown to be important in capsule assembly and translocation. Luciferase-based expression studies showed that, AgfD differentially regulated the *emc* operons in coordination with extracellular matrix genes. Survival assays demonstrated the capsule is important for desiccation tolerance. The *emc* genes were found to be conserved in *Salmonellae* and thus, the O-Antigen extracellular matrix capsule may be a conserved survival strategy important for environmental persistence.

Finally, a compositionally unique acidic EPS was found associated with the extracellular matrix. In-frame  $\Delta bcsA$ ,  $\Delta emcG$  and  $\Delta agfA$  mutants but neither  $\Delta agfA\Delta bcsA$  nor  $\Delta agfD$  mutants bound calcofluor, a  $\beta$ -glucan binding fluorescent agent, suggesting that multicellular behavior itself and not necessarily AgfD alone was influencing EPS expression. A transposon library was screened by ELISA using serum generated against purified EPS. This identified mutations inactivating genes involved in quorum sensing AI-2 degradation, flagella repression and Tafi and TolA expression. All mutations resulted in the loss of multicellular behavior and immunologically decreased levels of Tafi. This is the first report that implicates quorum sensing AI-2 degradation and flagella repression as part of the regulatory circuit for Tafi expression.

Together, the results reveal Tafi uses assembly factors to facilitate extracellular polymerization which likely assists the formation of a network of branched, amorphous fimbriae. Tafi together with EPS form the extracellular matrix: Tafi stabilizes the EPS on the microbial communities; EPS imparts it with physical properties such as hydration, charge and diffusion barriers that protect it from adverse environmental conditions such as desiccation and antimicrobials. This probably contributes to *Salmonella* survival in the environment and facilitates its cyclic lifestyle.

## TABLE OF CONTENTS

ABSTRACT .....	iii
TABLE OF CONTENTS.....	v
LIST OF TABLES .....	x
LIST OF FIGURES .....	xi
LIST OF ABBREVIATIONS.....	xiv
ACKNOWLEDGEMENTS .....	xx
DEDICATION .....	xxii
PUBLICATIONS.....	xxiii
<b>1.0 LITERATURE REVIEW .....</b>	<b>1</b>
<b>1.1 <i>Salmonellae</i> .....</b>	<b>1</b>
1.1.1 Taxonomy .....	3
1.1.2 <i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Enteritidis.....	4
1.1.3 <i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Enteritidis 27655-3b...	5
<b>1.2 <i>Salmonella</i> Pathogenesis .....</b>	<b>6</b>
1.2.1 Non-host environments .....	7
1.2.2 Host environments .....	10
<b>1.3 Fimbriae .....</b>	<b>10</b>
1.3.1 Biogenesis of fimbriae .....	11
<b>1.4 <i>Salmonella</i> fimbriae .....</b>	<b>15</b>
1.4.1 Thin aggregative fimbriae.....	15

<b>1.5</b>	<b>Bacterial polysaccharides .....</b>	<b>24</b>
1.5.1	LPS.....	25
1.5.2	EPS and capsules .....	31
<b>1.6</b>	<b><i>Salmonella</i> polysaccharides .....</b>	<b>36</b>
1.6.1	LPS.....	36
1.6.2	EPS: Vi-Ag, cellulose and colanic acid .....	39
<b>1.7</b>	<b>Biofilms.....</b>	<b>45</b>
1.7.1	Lifecycle and physiology.....	48
1.7.2	Extracellular matrix.....	49
1.7.3	Bacterial persistence and transmission .....	51
<b>1.8</b>	<b><i>Salmonella</i> biofilms .....</b>	<b>52</b>
1.8.1	Extracellular matrix.....	53
1.8.2	AgfD regulation of biofilm formation .....	56
<b>2.0</b>	<b>HYPOTHESES, OBJECTIVES, SIGNIFICANCE AND SPECIFIC AIMS</b>	<b>60</b>
2.1	Hypotheses .....	60
2.2	Objectives and significance .....	60
2.3	Specific aims .....	60
<b>3.0</b>	<b>AgfC and AgfE of the <i>Salmonella enterica</i> Enteritidis thin aggregative fimbriae operon facilitate fimbrial assembly.....</b>	<b>61</b>
<b>3.1</b>	<b>Introduction .....</b>	<b>61</b>
<b>3.2</b>	<b>Material and methods .....</b>	<b>64</b>
3.2.1	Bacterial strains, growth conditions and culture media .....	64
3.2.2	RT-PCR.....	64
3.2.3	Expression and purification of hexa-histidine tagged AgfC .....	65
3.2.4	Production of polyclonal serum against hexa-histidine tagged AgfC....	65
3.2.5	Cellular localization .....	65

3.2.6	N-terminal sequencing of AgfC-his.....	66
3.2.7	Cellulose binding assay.....	66
3.2.8	Generation of <i>Salmonella</i> Enteritidis 3b mutant strains .....	66
3.2.9	Recombinant DNA Techniques .....	67
3.2.10	Transmission Electron Microscopy .....	67
3.2.11	Purification of aberrant Tafi from <i>Salmonella</i> Enteritidis 3b <i>ΔbcsAΔagfC</i> .....	68
3.2.12	Hydrophobicity assay.....	68
3.2.13	Antibody capture of Tafi subunits in <i>Salmonella</i> Enteritidis 3b <i>ΔbcsA</i> strains	68
3.2.14	SDS-PAGE, protein staining and immunoblotting .....	69
<b>3.3</b>	<b>Results .....</b>	<b>70</b>
3.3.1	<i>agfC</i> is co-transcribed with <i>agfBA</i> .....	70
3.3.2	Cell localization of AgfC .....	71
3.3.3	Purified histidine tagged AgfC binds non-crystalline cellulose.....	72
3.3.4	The <i>Salmonella</i> Enteritidis 3b <i>ΔagfC</i> mutant expressed an aberrant form of Tafi.....	74
3.3.5	The cell surface of <i>Salmonella</i> Enteritidis 3b <i>ΔagfC</i> is more hydrophobic relative to the <i>wt</i> .....	77
3.3.6	AgfC and AgfE are Tafi assembly factors .....	78
<b>3.4</b>	<b>Discussion.....</b>	<b>81</b>
<b>4.0</b>	<b><i>Salmonella</i> produces an O-Antigen capsule translocated by the <i>emc</i> operons, regulated by AgfD and important for environmental persistence. ....</b>	<b>92</b>
<b>4.1</b>	<b>Introduction .....</b>	<b>92</b>
<b>4.2</b>	<b>Material and methods .....</b>	<b>95</b>
4.2.1	Bacterial strains, culture media and growth conditions .....	95

4.2.2	EPS purification .....	96
4.2.3	Generation of polyclonal serum against EPS and capsule.....	97
4.2.4	Scanning Electron Microscopy .....	97
4.2.5	Transmission Electron Microscopy .....	97
4.2.6	Transposon Mutagenesis and screening for capsule-negative mutants .	98
4.2.7	Generation of <i>Salmonella</i> Enteritidis 3b mutants .....	99
4.2.8	ELISA .....	99
4.2.9	Immunofluorescent Microscopy .....	99
4.2.10	SDS-PAGE, protein staining and immunoblotting.....	100
4.2.11	Generation of <i>lux</i> reporters .....	100
4.2.12	Desiccation Experiment .....	101
4.2.13	Etest® antibiotic testing.....	101
<b>4.3</b>	<b>Results .....</b>	<b>101</b>
4.3.1	<i>Salmonella</i> EPS.....	101
4.3.2	<i>Salmonella</i> Enteritidis 3b produces an O-Ag capsule.....	104
4.3.3	The <i>emc</i> operons are important for capsule assembly and translocation	105
4.3.4	Capsule expression is synchronized with Tafi and flagella production	110
4.3.5	The <i>emc</i> genes are differentially regulated via AgfD in coordination with the extracellular matrix genes .....	111
4.3.6	Conservation of <i>emc</i> operons and biosynthesis in <i>Salmonellae</i> .....	116
4.3.7	The O-Ag capsule promotes environmental persistence.....	117
<b>4.4</b>	<b>Discussion.....</b>	<b>118</b>
<b>4.5</b>	<b>Supplementary Material.....</b>	<b>126</b>
4.5.1	Predicted operator sites in the <i>emc</i> intergenic region .....	126
4.5.2	The O-Ag capsule protects against diffusion dependent antibiotics....	128
<b>5.0</b>	<b>A unique acidic polysaccharide of the <i>Salmonella</i> extracellular matrix is associated with multicellular behavior.....</b>	<b>130</b>

<b>5.1</b>	<b>Introduction .....</b>	<b>130</b>
<b>5.2</b>	<b>Material and methods .....</b>	<b>132</b>
5.2.1	Bacterial strains, growth conditions and culture media .....	132
5.2.2	EPS purification .....	133
5.2.3	Compositional analysis .....	133
5.2.4	Generation of polyclonal serum against EPS.....	133
5.2.5	Transposon Mutagenesis.....	134
5.2.6	EPS mutant screening and gene identification.....	134
5.2.7	Tafi expression.....	135
5.2.8	SDS-PAGE, protein staining and immunoblotting.....	135
<b>5.3</b>	<b>Results .....</b>	<b>136</b>
5.3.1	A <i>Salmonella</i> acidic EPS associated with the extracellular matrix .....	136
5.3.2	Composition of the novel EPS .....	137
5.3.3	Mulicellular behavior is important for EPS surface expression .....	138
5.3.4	Quorum sensing AI-2 degradation, flagella repression and Tafi and TolA expression are important for EPS surface expression. ....	139
5.3.5	Multicellular behavior of EPS mutants.....	142
5.3.6	Quorum sensing AI-2 degradation, flagella repression and TolA expression influences Tafi expression .....	144
<b>5.4</b>	<b>Discussion.....</b>	<b>145</b>
<b>6.0</b>	<b>GENERAL DISCUSSION AND OVERALL CONCLUSIONS .....</b>	<b>149</b>
<b>7.0</b>	<b>FUTURE INVESTIGATIONS.....</b>	<b>150</b>
<b>8.0</b>	<b>REFERENCES.....</b>	<b>154</b>

## LIST OF TABLES

Table 1.I.	Top 20 most frequently reported <i>Salmonella</i> serotypes from Human sources reported to CDC in 2003 adapted from Brenner (2004).....	2
Table 3.I.	Immunological comparison of AgfA and AgfB from <i>Salmonella</i> Enteritidis 3b and isogenic <i>agf</i> mutant strains.....	81
Table 3.II.	Primers used in this study.....	89
Table 4.I.	<i>Salmonella</i> Enteritidis 3b Tn10dCm mutants deficient in O-Ag capsule production.....	113
Table 4.II.	Conserved Domain Hits of the proteins encoded by the <i>emc</i> ( <i>yih</i> ) operons (Marchler-Bauer and Bryant, 2004).....	114
Table 4.III.	Primers used in this study. ....	124
Table 4.IV.	Antibiotic characteristics. ....	129
Table 4.V.	Etest® for MIC determination of <i>Salmonella</i> Enteritidis 3b strains.....	129
Table 5.I.	Glycosyl composition analysis of <i>Salmonella</i> Enteritidis 3b EPS. ....	138
Table 5.II.	<i>Salmonella</i> Enteritidis 3b Tn10dCm mutants deficient in EPS production.....	142
Table 5.III.	Multicellular behavior of <i>Salmonella</i> Enteritidis 3b $\Delta bcsA$ Tn10dCm mutants deficient in EPS production. ....	143

## LIST OF FIGURES

Figure 1.1. The route to human infection by <i>Salmonella</i> Enteritidis. ....	6
Figure 1.2. Life cycle of <i>Salmonella</i> . ....	9
Figure 1.3. Fimbrial biogenesis pathways. ....	14
Figure 1.4. Transmission electron micrograph of Tafi fimbriae associated with cellulose. ....	19
Figure 1.5. Comparative analysis of the antibody recognized epitopes and primary structure between AgfB and AgfA. ....	20
Figure 1.6. A stereo ribbon diagram of the predicted tertiary structure of AgfA and AgfB. ....	21
Figure 1.7. Molecular surface comparison between predicted AgfB and AgfA parallel $\alpha$ -helix models. ....	22
Figure 1.8. Intercellular complementation of Tafi between <i>Salmonella</i> Enteritidis 3b <i>agfB</i> donor and <i>agfA</i> recipient strains. ....	23
Figure 1.9. O-Antigen assembly models. ....	30
Figure 1.10. Colanic acid structure, genetic organization and biosynthetic pathway. ....	44
Figure 1.11. Approaches used in biofilm studies. ....	47
Figure 1.12. Detection of immunoreactive Tafi-associated material in different enterobacterial species. ....	55

Figure 1.13. Detection of HMW immunoreactive material in <i>Salmonella</i> Enteritidis 3b and $\Delta wcaJ$ strains.....	56
Figure 1.14. AgfD regulatory network.....	59
Figure 3.1. Reverse transcriptase (RT)-PCR of <i>agfC</i> . ....	71
Figure 3.2. Immunoblot analysis of AgfC in cellular fractions. ....	72
Figure 3.3. SDS-PAGE analysis of a cellulose binding assay of AgfC-his.....	73
Figure 3. 4. Transmission electron micrographs of <i>Salmonella</i> Enteritidis 3b cell surface fibers. ....	76
Figure 3.5. SDS-PAGE and immunoblot analysis comparing cellular proteins in <i>Salmonella</i> Enteritidis 3b and the $\Delta agfC$ mutant .....	77
Figure 3. 6. Antibody capture of AgfA and AgfB subunits in <i>Salmonella</i> Enteritidis 3b strains.....	80
Figure 3.7. Model for the growth of normal and aberrant Tafi fimbriae in <i>Salmonella</i> . ....	88
Figure 4.1. The <i>Salmonella</i> EPS associated with the extracellular matrix. ....	103
Figure 4. 2. The <i>Salmonella</i> Enteritidis 3b capsular polysaccharide structure. ....	105
Figure 4. 3. Structure of the <i>emc</i> operons involved in assembly and translocation of the <i>Salmonella</i> O-Ag capsule.....	108
Figure 4. 4. Immunological analysis of capsule expression on <i>Salmonella</i> Enteritidis 3b <i>emc</i> mutants. ....	109

Figure 4. 5.	Immunological analysis of capsule expression on <i>Salmonella</i> Enteritidis 3b <i>agf</i> mutants.....	111
Figure 4. 6.	The <i>emc</i> genes are differentially regulated via AgfD in coordination with the extracellular matrix genes.....	112
Figure 4. 7.	Conservation of <i>emc</i> operons in <i>Salmonellae</i> .....	116
Figure 4. 8.	The capsule is required for survival against desiccation stress. ....	118
Figure 4. 9.	Predicted operator sites in the <i>emc</i> intergenic region. ....	128
Figure 5. 1.	The <i>Salmonella</i> EPS associated with the extracellular matrix. ....	137
Figure 5.2.	Calcofluor binding of <i>Salmonella</i> Enteritidis 3b and extracellular matrix mutants. ....	139
Figure 5. 3.	Immunological analysis of EPS expression on <i>Salmonella</i> Enteritidis 3b $\Delta$ <i>bcsA</i> Tn10dCm mutants. ....	141
Figure 5. 4.	Immunological analysis of Tafi expression on <i>Salmonella</i> Enteritidis 3b EPS mutants.....	144

**LIST OF ABBREVIATIONS**

$A_x$	absorbance at x nm
aa	amino acid
Abe	Abequose
AcCoA	acetyl coenzyme A
ADH	adipic acid dihydrazine
AEPS	acidic extracellular polysaccharide
AIDS	autoimmune deficiency syndrome
ATM	adherence test medium (60 mM NaCl, 30 mM NaHCO <sub>3</sub> , 20 mM KCl, 111 mM glucose, pH 8.4)
bdar	brown, dry and rough when colony grown on Luria agar without salt supplemented with 40 µg/mL of CR and 20 µg/mL of Coomassie brilliant blue
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indoylphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
c-di-GMP	cyclic diguanylic acid
CBM17	family 17 cellulose binding module
CFA	colonization factor antigen media (1% casaamino acids, 0.15% yeast extract, 1M MgSO <sub>4</sub> x7H <sub>2</sub> O, 0.1M MnCl <sub>2</sub> x4H <sub>2</sub> O, 1M Na <sub>2</sub> PO <sub>4</sub> , 1M KH <sub>2</sub> PO <sub>4</sub> , ph 7.5, +/- 2% agar)
CFU	colony forming units
CLSM	confocal laser scanning microscopy
CR	Congo red
CTAB	cetyltrimethylammoniumbromide

3D	3-dimensional
d	diameter
dH <sub>2</sub> O	deionized water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ECA	enterobacterial common antigen
EDTA	(ethylene diamine) tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ENP	extracellular nucleation-precipitation
EPEC	enteropathogenic <i>E. coli</i>
EPS	extracellular polysaccharide
EtBr	ethidium bromide
EtOH	ethanol
Eunits	repeat units
FA	formic acid
FITC	fluorescein isothiocyanate
Fru	D-fructose
Fuc	L-fucose
g	grams
Gal	D-galactose
GalA	D-galacturonic acid
Glc	D-glucose
GlcA	D-glucuronic acid
GlcNAc	2-deoxy-N-acetylglucosamine
h	hour
HMW	high molecular weight
H-NS	histone-like nucleoid-associated protein

IFAT	immunofluorescence antibody technique
IHF	integration host factor
IM	inner membrane
IPTG	isopropyl- $\beta$ -D-thiogalactoside
K-Ag	<i>E. coli</i> capsule
kDa	kilodalton
KDO	2-keto-3-deoxyoctonate
KLH	keyhole limpet hemocyanin
kV	kilovolt(s)
l	litre(s)
L-Ara <sub>4</sub> N	4-amino-4-deoxy-L-arabinose
Lb	Luria-Bertani media (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4, +/- 1.5% agar)
L, D-Hep	L-glycero-D-mannoheptose
LMW	low molecular weight
LPS	lipopolysaccharide
M9MM	Minimal medium (47.7 mM Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O, 0.238 M KH <sub>2</sub> PO <sub>4</sub> , 0.25% NaCl, 0.5% NH <sub>4</sub> Cl, 2 M MgSO <sub>4</sub> , 0.1 mM CaCl <sub>2</sub> , 1.5% agar, pH 7.2)
M	molar
mAb	monoclonal antibody
M-Ag	colanic acid
MALDI-TOF	matrix-assisted laser desorption / ionization time-of-flight
Man	D-Mannose
MCC	microcrystalline cellulose
mg	milligram
MIC	minimum inhibitory concentration
mL	millilitre

mm	millimetre
mM	millimolar
ms	mass spectrometry
MW	molecular weight
m/z	mass/charge
NBT	4-Nitro Blue Tetrazolium chloride
NC	non-crystalline cellulose
ng	nanogram
nm	nanometer
OAc	O-acetyl
O-Ag	O-Antigen
OD	optical density
OM	outer membrane
ORF	open reading frame
O-unit	O-antigen repeat unit
P	phosphate
PEP	phosphoenolpyruvate
PP	pyrophosphate
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
Pyr	pyruvate
rdar	red, dry and rough colony grown on Luria agar without salt supplemented with 40 µg/mL of CR and 20 µg/mL of Coomassie brilliant blue
Rha	D-Rhamnose

RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
SARB	<i>Salmonella</i> reference collection B
SARC	<i>Salmonella</i> reference collection C
saw	smooth and white colony grown on Luria agar without salt supplemented with 40 µg/mL of CR and 20 µg/mL of Coomassie brilliant blue
sdH <sub>2</sub> O	sterile deionized water
SDS	sodium dodecal sulphate
SEF	<i>Salmonella</i> Enteritidis fimbriae
SEM	scanning electron microscopy
sp.	species
SPI	<i>Salmonella</i> pathogenicity island
Tafi	thin aggregative fimbriae
TCR	T medium with 10 µg/mL Congo red
T media	tryptone (1% bacto tryptone, pH 7.2, +/- 1.5% agar)
TBS	tris buffered saline
TE	10 mM Tris, 1mM EDTA, pH8
TEM	transmission electron microscopy
T <sub>m</sub>	melting temperature
TyrR	Tyrosine repressor protein
Tyv	Tyvelose
U	units
UA	uranyl acetate
µg	microgram(s)

$\mu$ l	microlitre(s)
Und-P	undecaprenol phosphate
UDP	uridine diphosphate
UPEC	uropathogenic <i>E. coli</i> .
U.S.	United States of America
USDA	United States Department of Agriculture
WHO	World Health Organization
<i>wt</i>	<i>wild type</i>
x g	gravitational force
X-gal	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside
Xyl	xylose

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## **DEDICATION**

This thesis is in honor of my beautiful Aunt Myrna, who bravely battles cancer,  
and her children who face each day with her.

## PUBLICATIONS

- Gibson, D.L.**, White, A.P., Rajotte C.M. and W.W. Kay (2005) AgfC and AgfE of the *Salmonella* Enteritidis thin aggregative fimbriae operons facilitate fimbrial assembly. *J.Bac.* (submitted).
- Gibson, D. L.**, White, A. P., Surette M., and W. W. Kay and Snyder, S. D., Heiss, C., Azadi, P. and W.W. Kay (2006) *Salmonella* produces an O-Antigen capsule translocated by the *emc* operons, regulated by AgfD and important for environmental persistence. *Mol. Microbiol.* (submitted).
- White, A. P., **Gibson, D. L.**, Kim, W., Kay, W. W. and M.G. Surette. (2006) Multicellular behavior and environmental persistence of *Salmonella*. *J.Bac.* (accepted).
- Snyder, S. D., **Gibson, D. L.**, Heiss, C., Kay, W. W., and P. Azadi. (2006) Structure of The O-Antigen *Salmonella* capsule. *Carbohydrate Research.* (submitted).
- White, A.P., **Gibson, D.L.**, Banser, P.A., and W.W. Kay. (2003) Extracellular polysaccharides associated with thin aggregative fimbriae (Tafi) of *Salmonella enterica* serovar Enteritidis. *J.Bacteriol.* **185**, 5398-407.
- White, A.P., Collinson, S.K., Banser, P.A., **Gibson, D.L.**, Paetzel, M., Strynadka, N.C., and W.W.Kay. (2001) Structure and characterization of AgfB from *Salmonella enteritidis* thin aggregative fimbriae. *J. Mol. Biol.* **311**, 735-749.
- Gibson, D.L.**, White, A.P., Snyder, D.S., Azadi, P., and W. W. Kay (2006) A unique acidic polysaccharide of the *Salmonella* extracellular matrix is associated with multicellular behavior. *J.Bac.* (in preparation).
- Barak, J.D., Jahn, E.J., **Gibson, D.L.**, and A.O. Charkowski (2006) The role of cellulose and O-Antigen capsule in the colonization of plants by *Salmonella enterica*. *Molecular Plant Microbe Interactions* (in preparation).

White, A.P., **Gibson, D.L.**, Banser, P.A., Potter, A., Malo, D., and W.W. Kay  
*Salmonella* thin aggregative fimbriae (Tafi) as a scaffold for heterologous  
epitopes. *Vacc.* (in preparation).

Snyder, S. D., **Gibson, D. L.**, Heiss, C., Kay, W. W., and P. Azadi. Structure of a novel  
*Salmonella* extracellular polysaccharide. *Carbohydrate Research.* (in progress).

## 1.0 LITERATURE REVIEW

### 1.1 *Salmonellae*

*Salmonellae*, named after Dr. Daniel Salmon, are gram-negative, motile, rod-shaped, non-spore forming, facultative anaerobes that are a genus of the family *Enterobacteriaceae* (Brenner *et al.*, 2000). *Salmonella* are the etiological agents of the human diseases typhoid fever and gastroenteritis, both of which are major public health threats (Pang *et al.*, 1995). The WHO has estimated that globally, there are 16.6 million cases of typhoid fever resulting in 3.6% deaths and 1.3 billion cases of human gastroenteritis resulting in 0.23% deaths each year (Pang *et al.*, 1995). In the U.S. alone there was an estimated 2-4 million cases of human salmonellosis costing \$2 billion per year (Pang *et al.*, 1995). In 2003, a total of 33,589 *Salmonella* human isolates were reported from participating U.S. Public Health laboratories representing cases reported for 11.6 per 100,000 people (Brenner, 2004). This represented a 9% decrease compared with 1993 and a 3% increase over 2002 (Brenner, 2004). Typhoid fever is declining worldwide and mostly remains a disease of the developing world, but gastroenteritis has been increasing possibly in part due to increased automation in food processing (Pang *et al.*, 1995).

*Salmonella* is isolated most frequently from children under 5 years of age, accounting for 25% of isolates (Brenner, 2004) and in immunocompromised individuals such as those with AIDS (Kankwatira *et al.*, 2004). The twenty most common serovars from 2003 (Table 1.I.) represented 78% of all isolates where *Salmonella* serovars Typhimurium and Enteritidis accounted for 34%. This has been a similar trend since 1993 (Brenner, 2004). Unfortunately, a growing proportion of these isolates are multidrug resistant (<http://www.cdc.gov/narms/>) which has significantly increased the concern about these pathogens. *Salmonella* are also isolated from non-human sources where serovar Typhimurium is commonly isolated from bovine and chicken sources and serovar Enteritidis from chicken sources (Brenner, 2004). Human consumption of

Table 1.I. Top 20 most frequently reported *Salmonella* serotypes from Human sources reported to CDC in 2003 adapted from Brenner (2004).

Rank	Serovar	Reported	Percent
1	Typhimurium	6631	19.7
2	Enteritidis	4863	14.5
3	Newport	3847	11.5
4	Heidelberg	1810	5.4
5	Javiana	1659	4.9
6	Montevideo	849	2.5
7	Saintpaul	823	2.5
8	Muenchen	781	2.3
9	Oranienburg Infantis	554	1.6
10	Braenderup	539	1.6
11	Agona	530	1.6
12	Thompson	510	1.5
13	I 4,[5],12:i:-	494	1.5
14	Mississippi	489	1.5
15	Typhi	438	1.3
16	Paratyphi B*	359	1.1
17	Hadar	331	1.0
18	Bareilly	280	0.8
19	Stanley	234	0.7
20	<b>Sub Total</b>	224	0.7
	All Other Serotyped	<b>26245</b>	<b>78.1</b>
	Unknown	5239	15.6
	Partially serotyped	735	2.2
	Rough or nonmotile	1351	4.0

<b>Sub Total</b>	19	0.1
<b>Total</b>	<b>7344</b>	<b>21.9</b>
	<b>33589</b>	<b>100</b>

\*, var. L(+) tartrate+ve

contaminated foods including beef, poultry, eggs, fresh vegetables and dried foods such as almonds, chocolate, squid chips, potatoe chips, milk powder are reported routes of transmission (Brenner, 2004; Hiramatsu *et al.*, 2005). Additionally reptilian species, adult and larval forms of ground beetles, centipedes and adult muscoid flies (including the common housefly, *Musca domestica*) can serve as other forms of transmission (Brenner, 2004).

### 1.1.1 Taxonomy

*Salmonellae*'s nomenclature has been complex and controversial. Before the mid 1980s, the existence of multiple *Salmonella* species was taxonomically accepted but with the development of genomics a high degree of DNA identity (95 - 99%) was discovered within *Salmonellae* (Crosa *et al.*, 1973). Consequently, all isolates were classified into two species: *Salmonella bongori* and *Salmonella enterica*, the last being further subdivided into 7 subspecies (I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae* and VI, *indica*; VII; Brenner *et al.*, 2000). Originally *Salmonella bongori* was designated *S. enterica* subspecies V and thus commonly referred to as "subspecies V" (Brenner *et al.*, 2000). To avoid confusion between serovars and species, Le Minor and Popoff (1987) proposed that serovar names be printed in Roman type starting with a capital letter (e.g. *Salmonella enterica* subspecies *enterica* serovar Enteritidis). Since genus abbreviation (*Salmonella* = *S.*) is only authorized when followed by the species name, serovars are written as *Salmonella* Enteritidis (Brenner *et al.*, 2000).

The antigenic classification or serotyping of *Salmonella* refers to antibody interactions with bacterial surface antigens (Kauffmann, 1950). Specific serovars have been defined by immunologically distinct antigenic combinations of the LPS O-, flagellar H-, and polysaccharide Vi-Antigens. This has resulted in the identification of 2,541 *Salmonella* serovars as of 2003, most of which were named for the cities or hosts where they were isolated from (Brenner, 2004). A less extensive classification system is used in clinical microbiological laboratories, where only O-Ag serotyping is used to define common serovars into serogroups. There are currently 46 serogroups and 57 O-Ags (Brenner, 2004). This grouping system can be used clinically to confirm genus and serogroup however it cannot identify the associated disease because of common epitopes. For example, serovar Enteritidis, the common cause of gastroenteritis, and serovar Typhi, which causes Typhoid (enteric) fever, are both group D (factor 9). The serum specifically recognizes the common Tyv residue found as part of their O-unit. This is differentiated from group B, which includes serovar Typhimurium because the Tyv residue is replaced with an Abe residue providing immunological distinction.

Phage typing is another common method used to differentiate serogroups of *Salmonella*. This was developed in the late 70s and was manifested by observations that different phages produced various patterns of lysis to different serogroups, different serovars within serogroups and different strains within in serovars (Gershman, 1977). Commonly, the phage pattern is referred to as “Phage type (PT)”.

### 1.1.2 *Salmonella enterica* subspecies *enterica* serovar Enteritidis

The majority (59%) of the *Salmonella* serovars are classified as *S. enterica* subspecies *enterica* which causes ~ 99% of the *Salmonella* infections in humans and warm-blooded animals including typhoid fever and gastroenteritis. Gastroenteritis is the most common disease caused by *S. enterica* where the most common serovars associated with this disease are Typhimurium and Enteritidis (Finlay, 1994).

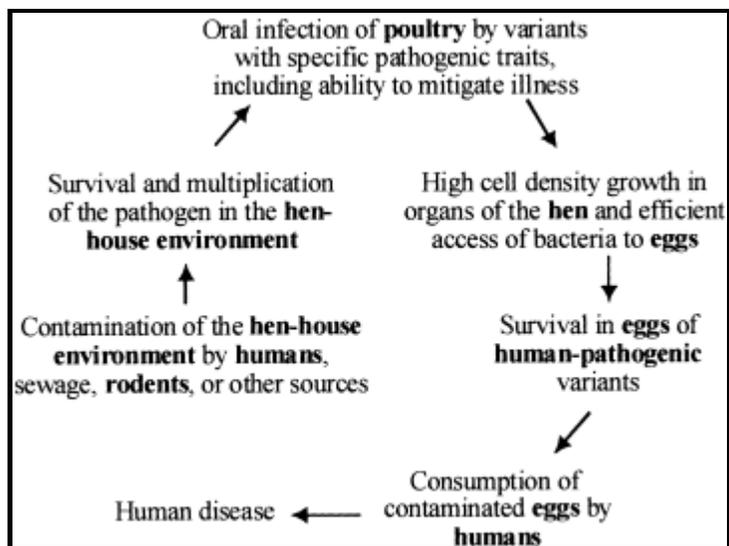
Human illness caused by *Salmonella* Enteritidis has steadily increased worldwide beginning in the early-1970s and in 1990 replaced serovar Typhimurium as the primary cause of salmonellosis (Baumler *et al.*, 2000). In 1997, *Salmonella* Enteritidis accounted for 85% of all cases of salmonellosis in Europe and US, predominated by PT4 and fortunately since then the incidence has declined (Baumler *et al.*, 2000). This may in part be due to its ability to efficiently contaminate the hen egg and cause human illness (Hogue *et al.*, 1997). To support this idea, epidemiological studies linked the decreased illness to egg targeted interventions like refrigeration and on-farm prevention and control measures (Guard-Petter, 2001). Baumler (2001) hypothesized that eradication of *Salmonella* Gallinarum from poultry farms resulted in the loss of flock immunity against the O9-Ag (Tyv) shared by both *Salmonella* Gallinarum and *Salmonella* Enteritidis, enabling the later to colonize and spread amongst poultry (Rabsch *et al.*, 2001).

The *Salmonella* Enteritidis pandemic involves adaptation to multiple environments, including hen houses, birds, eggs and humans (Fig. 1.1; Guard-Petter, 2001). Although, *Salmonella* Enteritidis resembles other pathogenic *Salmonellae* with regard to virulence mechanisms involved in host cell invasion, survival and growth in the host, it has been hypothesized that specific characteristics could be responsible for its frequent association with the hen egg including a biofilm phenotype referred to as lacy associated with glucosylated HMW LPS (Guard-Petter *et al.*, 1996), swarming and motility phenotypes and high cell density (Guard-Petter, 2001).

### **1.1.3** *Salmonella enterica* subspecies *enterica* serovar Enteritidis 27655-3b

The strain studied here is *Salmonella enterica* subspecies *enterica* serovar Enteritidis 27655-3b PT4 (*Salmonella* Enteritidis 3b). *Salmonella* Enteritidis 27655 was originally isolated from human faeces in India (Feutrier *et al.*, 1986). After incubation on CFA agar for 18-24 hr at 37°C two colonial morphologies were observed:

smooth (3a) and rough (3b; Baloda *et al.*, 1988). Initial comparative analysis showed that *Salmonella* Enteritidis 3b was hyper-fimbriated (Muller *et al.*, 1989).



**Figure 1.1. The route to human infection by *Salmonella* Enteritidis.**

Host and non-host environments are colonized or infected *Salmonella* Enteritidis. Epidemiologists suggest the route is linear where humans are the end host but evidence suggests a cyclical route considering humans can transmit infection to chickens. This figured was copied with permission from Guard-Petter (2001).

## 1.2 *Salmonella* Pathogenesis

Nearly all the work on *Salmonella* pathogenesis has been done on serovar Typhimurium because there is a well-developed mouse model mimicking typhoid fever in humans and molecular genetic tools are widely available (Santos *et al.*, 2001). It would be useful to study other *Salmonella* sp. but this is difficult because different disease manifestations occur in different hosts. For example, in humans *Salmonella* Enteritidis causes severe but self-limiting gastroenteritis with occasional bacteremia whereas systemic disease develops in rats and mice; *Salmonella* Typhimurium causes

typhoid fever in mice, but enteritis in calves and humans; *Salmonella* Dublin causes bacteremia in humans but cows become asymptomatic chronic carriers with a high prevalence for abortion (Santos *et al.*, 2001; Rabsch *et al.*, 2001). Thus, different hosts exhibit strikingly different responses to different *Salmonella* infections. Host-adapted *S. enterica* serotypes are characterized by their ability to cause systemic disease associated with high mortality rates in their respective host reservoirs. These include the poultry-adapted *Salmonella* Gallinarum (fowl typhoid and pullorum disease caused by biotypes Gallinarum and Pullorum, respectively), the porcine-adapted *Salmonella* Choleraesuis (pig paratyphoid), the bovine-adapted *Salmonella* Dublin (bacteremia), and human-adapted *Salmonella* Typhi (typhoid fever) (Rabsch *et al.*, 2002). Although *Salmonella* is one of the most highly studied organisms to date, we still know very little about the biochemical nature of its surface and its interactions with the host resulting in disease, although at least one degree of host-adaptation has been shown to involve specific fimbrial types expressed in each serovar (Emmerth *et al.*, 1999). However, a simple correlation between host range and the presence of a single fimbrial operon is unlikely (Townsend *et al.*, 2001). Fundamental questions remain: these include what determines host tropism and what factors determine which disease manifestations?

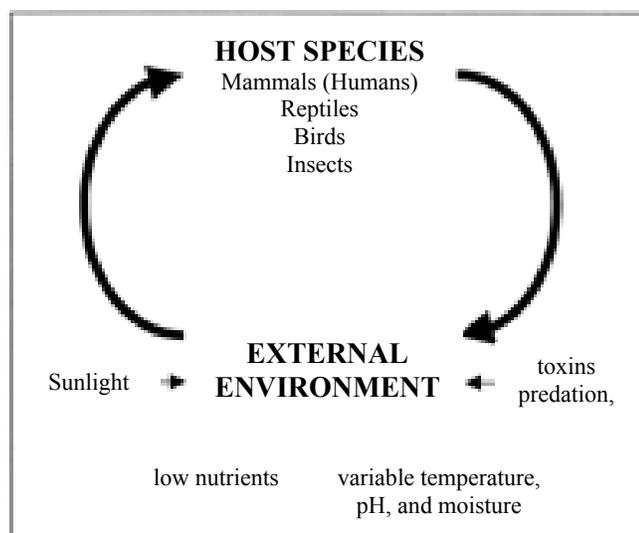
### 1.2.1 Non-host environments

*Salmonella* pathogenesis is generally envisioned from the host perspective but a non-host or environmental perspective is also important. This is because *Salmonella* has a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Fig. 1.2; Winfield and Groisman, 2003). Since environmental persistence or survival is a critical stage to the cycle, *Salmonella* must use factors to enhance its survival under adverse conditions such as those found outside an animal where conditions such as temperature and pH fluctuate and where stressors abound such as limited nutrient availability, desiccation and the presence of antimicrobials.

Remarkably, *Salmonella* has the ability to survive long-term or persist in the environment. For example, *Salmonella* has been detected within cow farms, pig farms, and slaughterhouses, both before and after sacrifice (Winfield and Groisman, 2003). Some examples of these studies include, but are not limited to: *Salmonella* sp. which were isolated from > 90% of dairy farms, conventional or organic (n=110; Fossler *et al.*, 2004); the same *Salmonella* clone from a cow herd was recovered from processed ground meat (Millemann *et al.*, 2000); the same *Salmonella* clone was collected over a 2-year period from animals, their environment, and their feed (Baloda *et al.*, 2001); *Salmonella* genotypes persisted within swine wallows for > 5 months and genetically indistinguishable *Salmonella* was isolated from outdoor sow faeces indicating that the bacteria were cycling between swine and their environment (Callaway *et al.*, 2005). *Salmonella* Enteritidis PT4 was also found in soil samples between 8 - 13 months and in faeces from wild mice, foxes and cats (Davies and Breslin, 2003). Moreover, and perhaps the most disturbing was the same *Salmonella* clone persisted in a poultry house for over a year, despite routine disinfections (Davies and Wray, 1995). Collectively, these results indicate that the same *Salmonella* clonal population can persist long-term in the environment, which provides opportunities for the microbe to spread from one host to another (Winfield and Groisman, 2003).

*Salmonella* can withstand a variety of stresses associated with environmental fluctuations and persist in water environments (Winfield and Groisman, 2003). Therefore, water sources serve as a reservoir for *Salmonella* transmission between hosts because of constant shedding into the environment from the waste of infected humans, farm animals, pets, and wildlife despite sanitization efforts (Baudart *et al.*, 2000). Additionally, *Salmonella* spreading between hosts can occur by passage of bacteria from infected farm animals to vegetables as a result of field fertilization with raw, contaminated manure thereby perpetuating the presence of *Salmonella* species outside animal hosts (Winfield and Groisman, 2003). This is concerning because *Salmonella* can infiltrate, colonize and persist on tomato plants (Guo *et al.*, 2002) and other plant

tissues such as alfalfa sprouts (Barak *et al.*, 2005). Furthermore, *Salmonella* has been detected frequently, for up to a year, in soil samples collected from both agricultural and recreational areas (Lemunier *et al.*, 2005; Winfield and Groisman, 2003; Zaleski *et al.*, 2005). Adhesion of *Salmonella* cells to soil particles probably correlates with cell surface hydrophobicity due to OM modifications in response to changes in environmental conditions. This could represent a critical adaptation to its cyclic lifestyle involving host and nonhost environments (Winfield and Groisman, 2003). Finally, the presence of *Salmonella* on household surfaces can also serve as a source of propagation between hosts (Winfield and Groisman, 2003). For example, *Salmonella* can persist in biofilms for several weeks in bathrooms and on toilets following bacterium-induced illness (Barker and Bloomfield, 2000).



**Figure 1.2. Life cycle of *Salmonella*.**

*Salmonella* actively cycles through host and nonhost environments. This figure was copied with permission from Winfield and Groisman (2003). Copyright © 2006, the American Society for Microbiology. All rights reserved.

### 1.2.2 Host environments

Extensive research has gone into studying *Salmonella* pathogenesis in host environments (Finlay, 1994; Kaufmann *et al.*, 2001; Rosenberger *et al.*, 2001). This is an important area of research however, only a very brief and general introduction to this field will be covered in this thesis since most of the results here are associated with the environment rather than the host.

*S. enterica* is a facultative intracellular pathogen that resides inside macrophages and requires both antibodies and a cellular immune response for clearance (Finlay, 1994). In humans, there are three major diseases caused by *Salmonella*: typhoid fever, gastroenteritis and bacteremia. Orally ingested bacteria that survive stomach acidity penetrate the intestinal mucosa via M-cells and migrate to the spleen and liver via the lymph nodes, where they reside and replicate intracellularly within macrophages (Finlay, 1994). *Salmonella* enters host cells by specifically altering the host membrane using a type III secretion system and several effectors encoded on a pathogenicity island, SPI-1. The effectors are inserted into the host cell where they disrupt normal cellular functions (Kaufmann *et al.*, 2001). *Salmonella* avoids fusing with lysosomes and uses a second type III secretion system (SPI-2) to survive inside macrophage cells (Kaufmann *et al.*, 2001). Intracellularly, *Salmonella* macrophage infection causes significant changes in host gene expression including those for chemokines, cell surface receptors, signaling molecules, and transcriptional activators as well as changes in bacterial gene expression such as those encoding LPS, porins, fimbrial proteins, flagella, lipoproteins, glycoproteins and peptidoglycan (Eckmann *et al.*, 2000; Rosenberger *et al.*, 2001).

### 1.3 Fimbriae

Bacteria are capable of expressing a variety of surface structures in response to diverse environmental conditions. Many of these structures consist of one or more proteinaceous subunits assembled into filamentous appendages extending out from the

cell surface. These polymeric non-motile structures are referred to as fimbriae, and are historically called pili. These structures generally mediate contact between the bacterium and a eukaryotic cell surface, tissue matrix or serum protein, or to bacteria of the same or different species. These interactions are committed steps leading to subsequent colonization of epithelial surfaces, entry into host cells, conjugation, or development of biofilms, colonies or multicellular fruiting bodies.

Some fimbrial proteins are specifically induced during stationary phase growth. These include Pap pili-related proteins, Tafi subunit proteins, and fimbriae-like structures associated with aggregation in *Vibrio sp.* (Low, 1996). In general, fimbrial expression is activated by poor growth conditions (low glucose and amino acid levels) and is repressed by temperatures lower than 28°C (Low, 1996). Tafi fimbriae, however, are maximally expressed in *wt* isolates at 28°C (Arnqvist *et al.*, 1992) but upon iron starvation can be expressed at 37°C (Gerstel and Romling, 2001; Romling *et al.*, 1998).

The adhesive properties of fimbriae are often determined by specific adhesins. In some cases, such as in *E. coli* type I fimbriae, the fimbrial protein containing adhesin activity is a minor component, distinct from the major fimbrial subunit. In contrast, *E. coli* K88 fimbriae confer adhesin activity to the major fimbrial subunit (Low, 1996). Typically, the receptor is a carbohydrate moiety of a glycolipid or glycoprotein (Low, 1996).

### 1.3.1 Biogenesis of fimbriae

Typically, the genes involved in the biogenesis of fimbriae are organized in contiguous transcriptional units as one or multiple operons. At least one of these encodes a regulatory protein that either activates or represses fimbrial gene expression. In addition, most fimbrial operons are controlled by global regulatory networks coordinating fimbrial expression with other types of gene expression in response to environmental stimuli (Low, 1996). Fimbrial subunits and accessory proteins are

transported through the IM by export machinery encoded by the *sec* genes and a signal peptidase. In the periplasm, specific proteins involved in fimbriae biogenesis can be divided into at least four distinct assembly mechanisms: the chaperone-usher pathway, the general secretion pathway, the alternate chaperone pathway and the extracellular nucleation-precipitation (ENP) pathway (Soto and Hultgren, 1999).

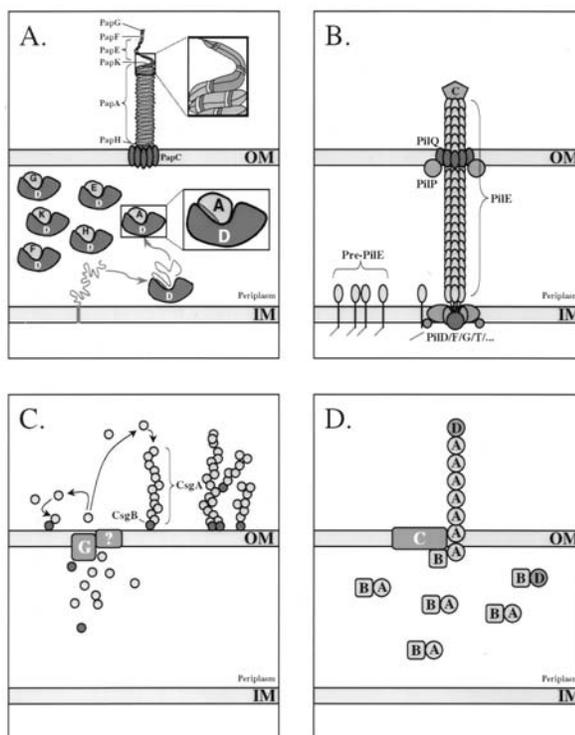
The assembly pathways of different fimbriae, including the correct incorporation of individual subunits in a predefined order and the prevention of premature aggregation between subunits, have been determined to some degree in the first three pathways (Figure 1.3 A, B and D; Soto and Hultgren, 1999). In all cases these structures undergo assembly from the base, (i.e. the distal end containing the adhesin is assembled first). The use of the fourth pathway, the ENP model, represents an important deviation from this where fiber formation has been proposed to occur from outside of the microbe by the precipitation of secreted soluble subunits into thin fibers on the surface of the microbe (Figure 1.3 C).

The biogenesis of the chaperone-usher fimbriae has been studied extensively and is consequently the best understood of the fimbrial systems (Sauer *et al.*, 2004). The fimbrial subunits have a missing secondary structure, which results in a hydrophobic groove and is the site for both chaperone protein binding and fimbrial subunit-subunit interaction. The chaperone facilitates efficient subunit folding, subunit stabilization, prevention of subunit aggregation and/or degradation and provides the missing secondary structural element through a mechanism termed "donor strand complementation" (Sauer *et al.*, 1999). This strand is exchanged for the N-terminal extension of the subunit through a mechanism termed "donor-strand exchange" (Sauer *et al.*, 1999). The energy that drives polymerization is thought to be provided by the conformational change from chaperone-bound to subunit-bound. In the periplasm, subunits appear to be maintained in a semi-unfolded state due to stabilization by the interaction with the chaperone that caps the subunit's interactive surfaces and thus inhibits premature fiber formation (Sauer *et al.*, 2004). The chaperone is therefore

required for assembly and in its absence subunits misfold, aggregate and are proteolytically degraded.

Chaperone–subunit complexes in the periplasm are targeted to the OM associated ‘usher’ protein. The usher is where the process of donor strand exchange occurs and somehow the usher destabilizes the chaperone–subunit complex thus releasing the chaperone. The usher forms a ring with a pore 2–3 nm in diameter, wide enough to allow passage of a tip fibrillum and the unwound pilus rod. Thus, it has been proposed that the pilus winds into its final structure once it has crossed the usher pore and is outside the cell. The chaperone–adhesin complex binds tightly to the usher, positioning the adhesin at the tip of the pilus. The fiber then grows by the addition of subunits to the base.

The other well studied fimbrial system is the general secretion system of type IV pili which are responsible for the multicellular mobility behaviour “twitching motility” (Craig *et al.*, 2003). The fimbrial subunits consist of a highly conserved extended N-terminal hydrophobic  $\alpha$ -helix region followed by a globular C-terminal domain containing  $\beta$ -strands. The hydrophobic N-terminal  $\alpha$ -helices are predicted to form the core of the pilus fiber and the C-terminal domains are putatively exposed to the surface, which is thought to stabilize the structure to withstand the force generated during twitching motility. Minor pilins (i.e. PilE, PilV, PilW, PilX, FimT and FimU of *P. aeruginosa*) are involved in assembly because in their absence surface piliation or associated functions are lost. However, clear definitions of these proteins do not exist because of the difficulty to detect these proteins as they are present in minute quantities. They may play roles in priming of pilus extension or prevention of pilus retraction; in control of pilus length; or in pilus-specific functions including adherence, transformation competence or motility. No chaperone functions are predicted for the minor subunits of the general secretion pathway probably because this system uses a translocation scaffold that crosses the periplasm.



**Figure 1.3. Fimbrial biogenesis pathways.**

(A) Assembly of P fimbriae from *E. coli* via the chaperone-usher pathway. The rod is comprised of PapA, the tip fibrilla is comprised of PapE and joined to the rod by the PapK adaptor, the adhesin PapG is joined to the distal end of the fibrilla by the PapF adaptor, and PapH links the rod to the OM. In the periplasm, subunits interact with a “chaperone”, PapD, which escorts the subunits to the OM where they interact with an “usher” protein, PapC which allows the subunits to pass through to form the fiber. (B) Assembly of type IV fimbriae from *N. gonorrhoeae* via the general secretion pathway. Prepilin is processed by the PilD signal peptidase, mature PilE subunits are assembled by the PilF, PilG and PilT complex and translocated through the OM by a gated multimeric PilQ with the assistance of PilP and the PilC adhesin. (C) Assembly of curli from *E. coli* (*Salmonella* Tafi homologue) by the ENP pathway. Curli’s main subunit, CsgA, is secreted across the OM. Surface-localized CsgB serves to nucleate the assembly of CsgA into curli. CsgB is also found the length of the fiber. CsgG (G) is an OM-localized lipoprotein that is necessary for the secretion of CsgA. CsgE and CsgF play an undefined role and are not shown. (D) Assembly of CS1 pili from *E. coli* by the alternate chaperone pathway. The chaperone, CooB, forms periplasmic complexes with the fiber subunits, CooA and CooD. CooC may function as an OM channel for passage of the fiber. This figure was copied with permission from Soto (1999). Copyright © 2006, the American Society for Microbiology. All rights reserved.

## 1.4 *Salmonella* fimbriae

There are at least 13 putative fimbrial operons in *Salmonella* Typhimurium named *agf* (*csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti* and *stj* (Townsend *et al.*, 2001). *In vitro* expression of only Pef, Fim and Agf has been demonstrated (Humphries *et al.*, 2003); whereas *in vivo* expression of BcfA, FimA, LpfA, PefA, StbA, StcA, StdA, StfA and StiA has been demonstrated from *Salmonella* infected bovine intestinal ileal loop (Humphries *et al.*, 2003). *Salmonella* Enteritidis has at least one more expressed fimbrial operon, SEF14 which are < 2 nm diameter and expressed in broth culture at > 30°C in stationary phase of growth (Clouthier *et al.*, 1994).

### 1.4.1 Thin aggregative fimbriae

Thin aggregative fimbriae, Tafi, previously called SEF17 (*Salmonella* Enteritidis fimbriae, 17 kDa) were identified in *Salmonella* Enteritidis by Collinson *et al* (1991). Tafi are unusually insoluble in that 90% formic acid (FA) is required to promote depolymerization (Collinson *et al.*, 1991). Tafi expression is associated with multicellular aggregation, pellicle formation and Congo red (CR) binding (Collinson *et al.*, 1993), which have more recently been referred to as a rdar morphotype (red, dry and rough on CR plates; Romling *et al.*, 1998). Tafi are morphologically thin (d = 4-6 nm), curled and branch points are revealed in the absence of cellulose (Fig.1.4. A, C; White *et al.*, 2003). In *wt* cells Tafi are co-expressed and intimately associated with cellulose and thus the ultrastructure of Tafi is not easily discernable but rather appears as a tangled amorphous matrix (Fig.1.4. B, D).

The genes involved in Tafi production are organized into two adjacent, divergently transcribed operons: *agfDEFG* and *agfBAC*. Both operons are required for the production of functional Tafi (Collinson *et al.*, 1993). Tafi are primarily comprised of a major subunit, AgfA, and a minor subunit, AgfB and both are required for polymerization (White *et al.*, 2001). While AgfB is the first product of the *agfBAC* operon, it is a minor component of the final fimbrial complex at a stoichiometry of > 20

AgfA per AgfB molecule (White *et al.*, 2001). AgfB is cell associated and found at branch points throughout the length of the fiber (Loferer *et al.*, 1997; White *et al.*, 2001). AgfG is a lipoprotein required for AgfA and possibly AgfB's secretion outside the cell (Chapman *et al.*, 2002; Loferer *et al.*, 1997). AgfD is a biofilm control point transcriptional regulator that positively regulates the production of Tafi (Gerstel *et al.*, 2003; Romling *et al.*, 1998). AgfE and AgfF have undefined roles in assembly where an *agfF* mutant results in less fiber formation and an *agfE* mutant produces morphologically aberrant fibers (Chapman *et al.*, 2002). Before this study, AgfC was not predicted to have a role in Tafi assembly.

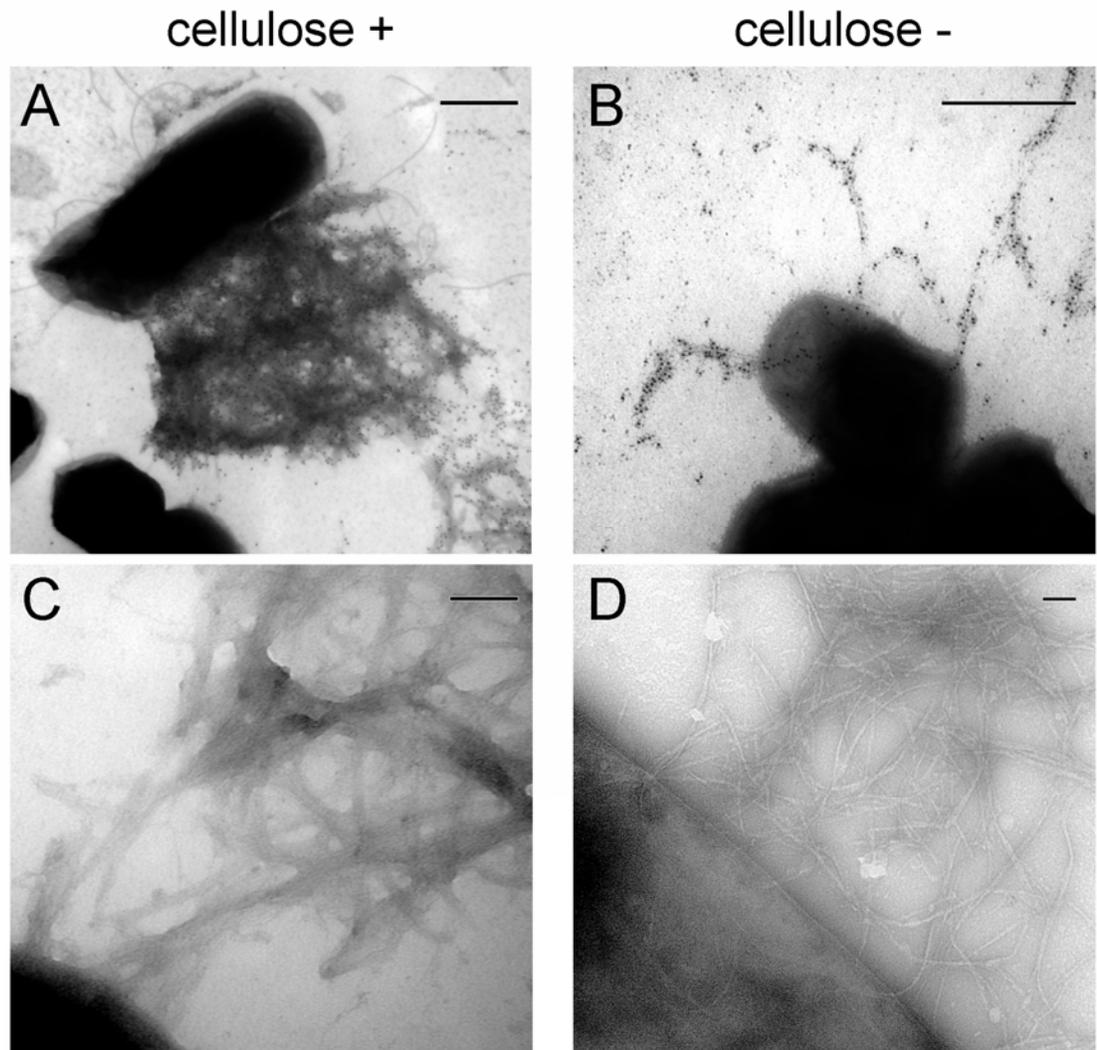
Tafi subunits have a unique amino acid composition and are not related to other families of fimbrial subunits (Collinson *et al.*, 1992). Structural studies of these proteins have not been achieved due to their insolubility; however structural predictions have been made. The primary sequence of both subunits can be divided into an N-terminal region distinct from the major C-terminal core region and have 51% overall sequence similarity (Fig. 1.5. A, B; Collinson *et al.*, 1999; White *et al.*, 2001). The subunit's N-terminal sequences differ chemically where overall AgfB is polar and AgfA is non-polar and glycine-rich. The C-terminal core of both subunits contains a novel four or five, 18 residue, tandem 5-fold repeat sequence with a consensus sequence,  $x_6QxGx_2NxAx_3Q$ . Secondary structural predictions indicate the subunits adopt a series of tandem  $\beta$ -strand conformations centered around the conserved alternating polar and non-polar residues at positions 2–6 and 13–17 of each of the repeat sequence (Fig. 1.5 C). Molecular modeling indicates that AgfA and AgfB similarly form highly compact parallel  $\beta$ -helix tertiary structures with regular hydrogen bonding patterns between residues within each  $\beta$ -strand and between residues in adjacent  $\beta$ -strands of the helix likely contributing to their intrinsic stability (Fig. 1.6; Collinson *et al.*, 1999; White *et al.*, 2001). The conserved A, N, G and Q residues are internalized and probably stabilize the closely aligned helically arrayed  $\beta$ -strands. Intriguingly, stable chimeric AgfA subunits can be generating containing 16-21 amino acid residues of foreign

epitopes replacing most of the predicted  $\beta$ -strands revealing the structural stability of these unique proteins (White *et al.*, 1999; unpublished). Although, AgfA and AgfB are similar in size and structural features they have different biochemical properties: AgfB is amphiphilic where one face is hydrophilic with cationic patches and the other face is hydrophobic whereas AgfA has less hydrophobicity overall with one hydrophobic face and the other face is hydrophilic with anionic patches (Fig. 1.7; White *et al.*, 2001). These charge differences may allow an electrostatic interaction between the two subunits, which may in part also play a role in fiber insolubility (White *et al.*, 2001).

The *agf* genes are highly conserved throughout the family *Enterobacteriaceae* (Doran *et al.*, 1993; Romling *et al.*, 2003; White *et al.*, 2005). In *E. coli*, the Tafi homologue was discovered and termed curli (Collinson *et al.*, 1992; Arnqvist *et al.*, 1992) and most researchers often refer to Tafi as curli encoded by *csg* genes. Tafi can bind host serum tissues (Olsen *et al.*, 2002), plant tissues (Barak *et al.*, 2005), modulate an inflammatory response (Bian *et al.*, 2000; Bian *et al.*, 2001; Tukul *et al.*, 2005), however, there is conflicting data as to whether it is important for virulence (Allen-Vercoe *et al.*, 1998; Gophna *et al.*, 2002) and host persistence (La Ragione *et al.*, 1999; Weening *et al.*, 2005). Since Tafi is normally expressed at 28°C, has been shown to protect cells against environmental stressors (White *et al.*, 2005), and is important in biofilm formation (Austin *et al.*, 1998), it is thought that Tafi may be important in the environment. Whether or not it is also important in facilitating invasion and host persistence is unresolved.

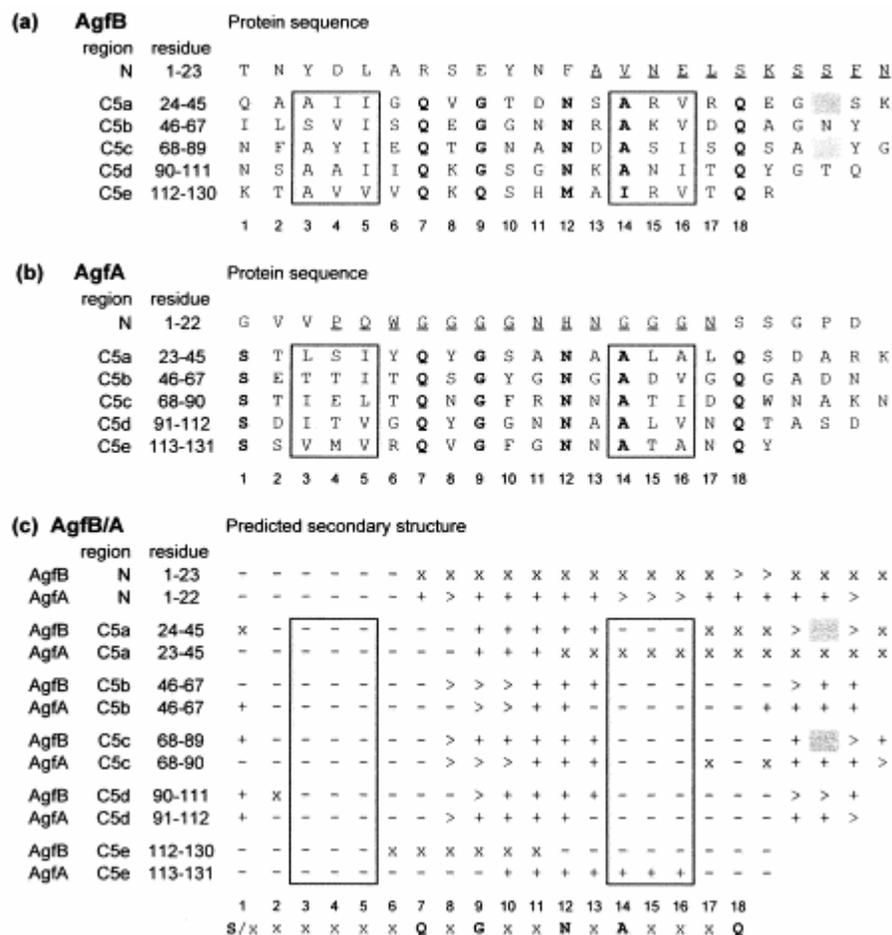
The assembly of Tafi has not been elucidated but a conceptual model has been proposed based on genetic evidence of *E. coli* curli mutants and is termed the ENP pathway (Fig. 1.3 C; Hammar *et al.*, 1996). Importantly, this model has been proposed to facilitate our understanding of other aggregative fibers such as prion related fibers (Chapman *et al.*, 2002). The subunits in *E. coli* are called Csg. CsgB acts as an OM-associated nucleator protein, which seeds polymerization of CsgA subunits. Soluble monomeric CsgA subunits are induced to form insoluble polymeric fibers by either

CsgB interaction or subsequent interactions with polymerized CsgA. Although counter-intuitive, the elongation of the fiber has been assumed to proceed from the distal end (subunits are added to the free tip of the growing fiber). For this reason, the CsgB-induced change in CsgA properties is supposed to be propagated among CsgA subunits in the growing fiber after one initial CsgB-CsgA interaction. Once this interaction has occurred, new CsgA subunits are added to the growing fiber without direct interaction with CsgB. These assumptions are based on the demonstration of intercellular complementation of curli production in *E. coli*. A *csgA* mutant cannot make curli but expresses a functional CsgB protein. A *csgB* mutant cannot make curli but produces a functional CsgA protein, which in the absence of CsgB is secreted into the medium as soluble monomers. These subunits are polymerization-competent in that they are capable of forming curli on the cell surface presenting a functional CsgB protein on an adjacent cell. In this experiment, a *csgA* mutant (recipient) grown beside a *csgB* mutant (donor) on CR containing media revealed a zone of CR binding on the recipient strain. This is known to correspond with CsgA polymerization. Recently, White *et al* (2003) challenged this model. The intercellular complementation between in-frame  $\Delta agfA$  and  $\Delta agfB$  mutants could not be reproduced in *Salmonella* Enteritidis 3b. However, intercellular complementation could be detected between these strains only when both donor and recipient were LPS O-Ag mutants (Fig. 1.8; White *et al.*, 2003). Therefore, O-Ag was found to be an impenetrable barrier to AgfA assembly between cells but not within individual cells. Consistent with these findings, the original *E. coli* MC4100 strain used in the original intercellular complementation experiment is a K-12 derivative, which does not produce LPS O-polysaccharide (Liu and Reeves, 1994) or cellulose (Zogaj *et al.*, 2001). Therefore, beyond being a system to reveal the mobility of soluble CsgA and CsgB subunits, the significance of intercellular complementation in the Tafi assembly pathway in *wt* cells has been questioned (White *et al.*, 2003).



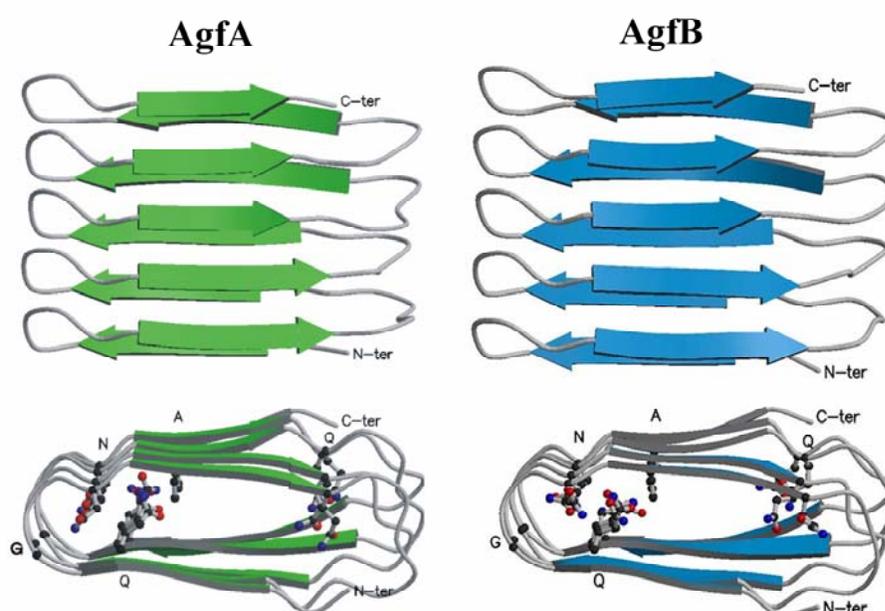
**Figure 1.4. Transmission electron micrograph of Tafi fimbriae associated with cellulose.**

*Salmonella* Enteritidis 3b (cellulose positive) (A and C) and the  $\Delta bcsA$  mutant (cellulose negative) (B and D) were immunogold labeled with AgfA-specific mAb followed by goat anti-mouse immunoglobulin-10-nm-diam gold (A and B) or negatively stained with UA (C and D). Bars, 500 nm (A and B) or 100 nm (C and D). This figure was copied with permission from White (White *et al.*, 2003).



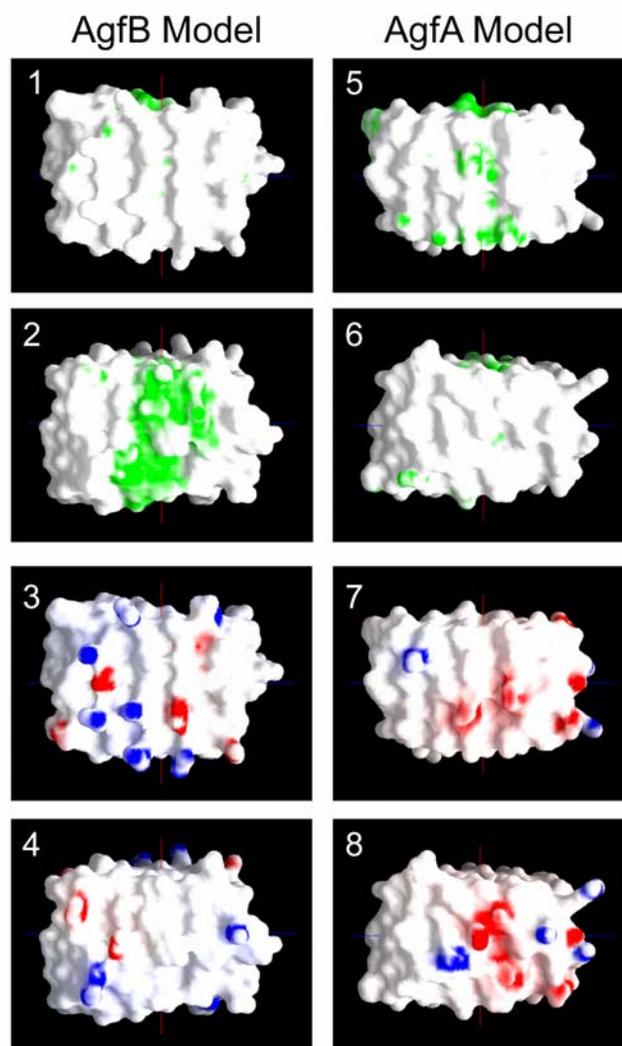
**Figure 1.5. Comparative analysis of the antibody recognized epitopes and primary structure between AgfB and AgfA.**

(a) The AgfB and (b) AgfA aa sequence is aligned to highlight the distinct N-terminal region (N) from five repeat sequences (a-e) of the C-terminal core region (C). The N-terminal aa residues comprising the linear epitope(s) identified within AgfB and AgfA that are recognized with  $\alpha$ -AgfB and  $\alpha$ -Taf<sub>i</sub> immune serum is underscored in the aa sequence. Spaces (shaded regions) were introduced to optimize alignments and conserved residues (bold) of the 18-residue consensus sequence are within each sequence and below the Figure. Boxed regions indicate non-polar/polar/non-polar triplet motifs at positions 3–5 and 14–16 of each C-terminal core repeat. (c) The predicted secondary structure of each region of AgfB and AgfA. This figure was copied with permission from Collinson (1999) and White (2001).



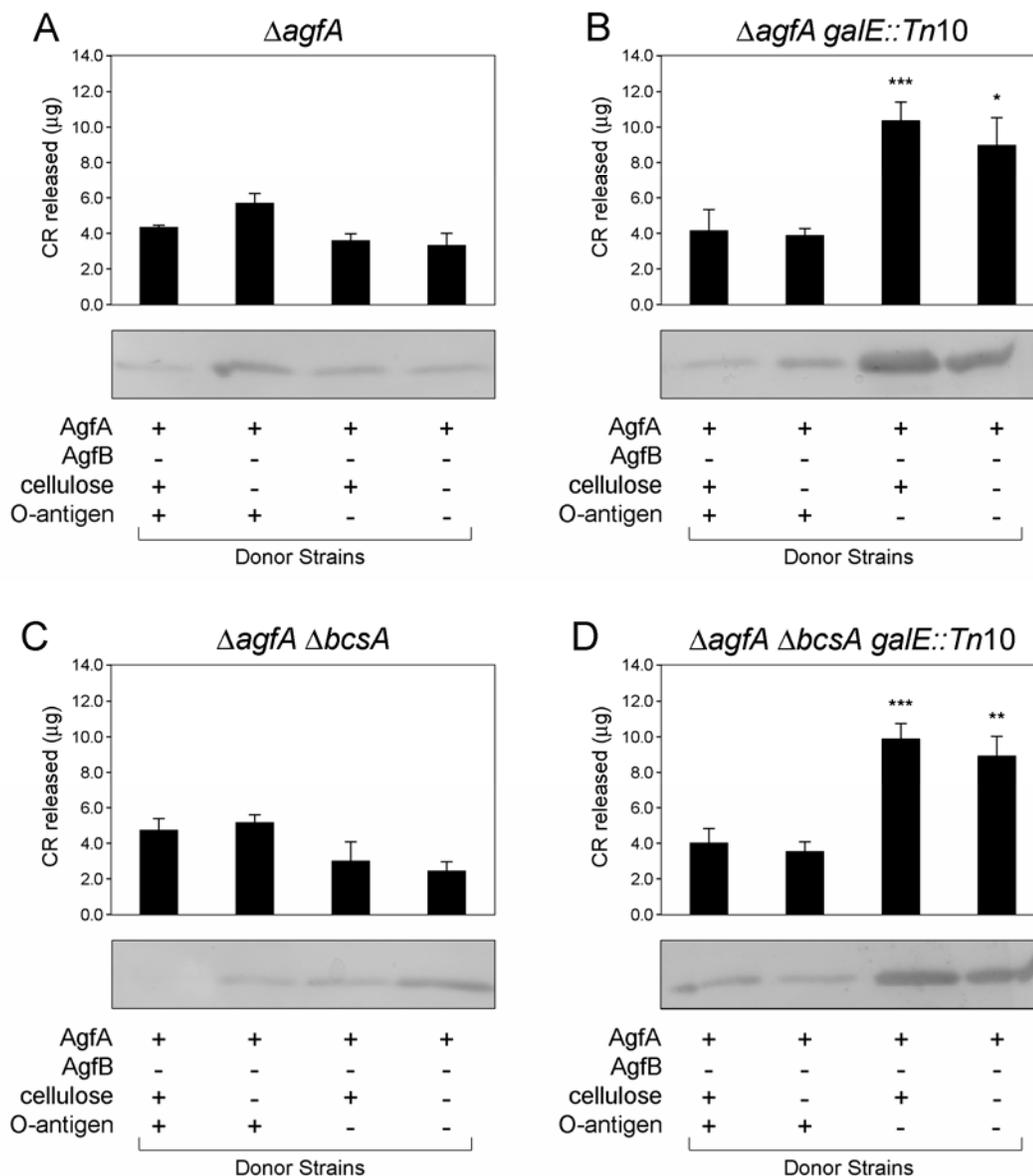
**Figure 1.6.** A stereo ribbon diagram of the predicted tertiary structure of AgfA and AgfB.

The model is rotated  $90^\circ$  around the *X*-axis, showing in ball-and-stick the conserved residues displayed that are oriented toward the center of the molecule. Single-letter aa abbreviations are used as labels for the conserved residues. N-ter represents the beginning of the core region whereas C-ter represents the C-terminal residue. AgfA is depicted in green and AgfB is depicted in blue. This figure was adapted with permission from Collinson (1999) and White (2001).



**Figure 1.7. Molecular surface comparison between predicted AgfB and AgfA parallel  $\alpha$ -helix models.**

AgfB and AgfA  $\beta$  helix models viewed from the front side (1, 3, 5, 7) as in Figure 1.6 (a) or the back side (2, 4, 6, 8). Molecular surface of the models color coded by hydrophobicity as designated in green (panels 1, 2, 5, 6) or by electrostatic potential where blue represents basic residues and red represents acidic residues (panels 3, 4, 7, 8).



**Figure 1.8. Intercellular complementation of Tafi between *Salmonella* Enteritidis 3b *agfB* donor and *agfA* recipient strains.**

CR binding of different *agfA* recipient strains grown together with *agfB* donor strains is shown. Recipient strain genotypes are indicated above each graph, while *agfB* donor strain phenotypes are listed at the bottom. CR binding values for each combination of mutants were normalized by subtracting values for the corresponding *agfB* or *agfA* strains grown individually. Each bar shows the averages and standard errors from four separate experiments, and asterisks indicate significant differences within each group (\*, \*\*,

$P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  [Tukey-Kramer multiple comparisons test]). The panels below the bar graphs show immunoblot analysis performed with immune serum raised to purified Tafi. The amount of AgfA fimbrial material associated with recipient and donor cells scraped off the TCR agar is noted for each recipient-donor combination. This figure was copied permission from White (2003).

## 1.5 Bacterial polysaccharides

The surfaces of most natural isolates of bacterial are covered by polysaccharides. These can be in the form of secreted or linked extracellular polysaccharides (EPS), the former termed capsular, as well as complex glycolipids such as lipopolysaccharide (LPS) and enterobacterial common antigen (ECA). EPS is distinguished from capsules by the presence of a covalently attached lipid anchor (Roberts, 1996). EPS are present on the cell surfaces of both Gram-negative bacteria such as *Nisseria meningitidis*, *Haemophilus influenzae*, *E. coli* and Gram-positive bacteria such as *Streptococci* and *Staphylococci* (Sutherland, 1994). Their structures are made up of either monosaccharides producing a homopolymer like  $\alpha$ -(2,8)-linked sialic acid found on the surfaces of *N. meningitidis* and *E. coli* K1, or from repeating units consisting of di- to hexasaccharides producing heteropolymers and can be anchored to the OM. In contrast, LPS is a major component of the OM in Gram-negative bacteria by virtue of its complex lipid anchor and comprises the entire outer lipid leaflet of the outer membrane. The polysaccharide component can be divided into three distinct regions: inner core, outer core and O-Ag where the later can be either a homopolymer or a heteropolymer. The sugar polymers making up either capsules (K-Ag) or LPS O-Ag are serotype-specific and variations in sugar composition, linkage specificity, as well as substitution with non-carbohydrate residues result in 186 different O-serogroups and more than 80 polysaccharide K-Ags in *E. coli* (Reeves *et al.*, 1996) 54 O-Ag in *Salmonella enterica* (Minor, 1997), 193 distinct O-Ag in *V. cholerae*, and 20 different O-Ag structures in *Pseudomonas aeruginosa* (Raymond *et al.*, 2002). This clearly demonstrates the enormous diversity of O-Ag in Gram-negative bacteria. The O- and K-Ag provide

recognized virulence determinants (Orskov *et al.*, 1977) where O-Ag is important for resistance to complement-mediated serum killing (Whitfield and Paiment, 2003), and K-Ag is responsible for resistance against phagocytosis (Caroff and Karibian, 2003). ECA is a common polysaccharide of gram-negative bacteria that is composed of a trisaccharide repeat unit comprised of 4-acetamido-4,6-dideoxy-D-galactose, N-acetyl-D-mannosaminuronic acid and N-acetyl-D-glucosamine and is found in several forms: attached to a phosphoglyceride moiety (ECA<sub>PG</sub>), linked to LPS lipid A-core (ECA<sub>LPS</sub>), and as a water-soluble cyclic form ECA<sub>CYC</sub> (Erbel *et al.*, 2003). ECA may play a role in acid resistance (Barua *et al.*, 2002).

### 1.5.1 LPS

LPS is essential for physiological membrane functions, growth, survival and pathogenesis, in particular septic shock, of Gram-negative bacteria (Weintraub, 2003). These molecules are comprised of: a highly conserved endotoxic lipid moiety called lipid A, a glycosidic moiety comprised of approximately 7-10 monosaccharides, referred to as the core further subdivided into inner and outer units and a third variable region consisting of repetitive subunits of one to eight monosaccharides named O-Ag. This last region is found in *wt* bacterial strains and is responsible for much of the immunospecificity of the bacterial cell. LPS is heterogenous: the smallest LPS molecules are composed of lipid A and inner core units; the slightly larger molecules have lipid A and complete core structure; and the largest contain lipid A, a complete core and a variable number of O-Ag repeat units (Caroff and Karibian, 2003). Therefore, LPS populations appear as a ladder starting at the bottom through to the top in a silver stained gel with each successive rung having an additional O-unit normally ranging from 16-34 (Whitfield *et al.*, 1997) but observations of very long chain LPS in *Salmonella* has been estimated to have over 100 O-units (Murray *et al.*, 2003).

LPS biosynthesis takes place as two separate pathways, which has mostly been characterized in *E. coli* (Raetz and Whitfield, 2002). The first biosynthetic pathway

involves lipid A-core synthesis whereby lipid A is synthesized on the cytoplasmic leaflet of the IM followed by the transfer of core sugar residues catalyzed by specific glycosyltransferases. The completed lipid A-core is subsequently translocated by ATPase-dependent MsbA to face the periplasmic side of the IM. The second biosynthetic pathway involves the synthesis and export of the O-unit by one of three postulated mechanisms: Wzx/Wzy-dependent, ATP binding cassette transporter (ABC)-dependent or synthase-dependent (Fig.1.9; Raetz and Whitfield, 2002). O-units are synthesized on a lipid carrier molecule, C<sub>55</sub>-polyisoprenoid derivative, undecaprenol phosphate (Und-P). Heteropolymeric O-units are thought to use the Wzx/Wzy-dependent pathway where the O-unit is somehow flipped to the periplasmic face of the IM by an IM permease-like protein, Wzx, and subsequently polymerized from the reducing terminus by the IM associated Wzy where Wzz controls the O-Ag length (Fig. 1.5.1A; Raetz and Whitfield, 2002). The homopolymeric O-Ags are completed to their full lengths in the cytoplasm by addition of monosaccharides to the non-reducing terminus of the growing polymer. In most cases, the entire polymer is somehow translocated to the periplasmic space by an ABC transporter system, composed of homologues of KpsD and KpsE. In only one case, *Salmonella* Borreze plasmid-encoded O:54 Ag composed of poly-N-acetylmannosamine, the synthase-dependent pathway is used where a synthase catalyzes polysaccharide polymerization while simultaneously extruding the emerging polymer across the IM (Keenleyside and Whitfield, 1995). After synthesis of hetero- or homopolymeric O-Ag, WaaL ligates the polymer from the Und-P to the preformed lipid A-core molecule. Interestingly, *E. coli* K-12 WaaL can link its lipid A-core to O-Ag with structurally diverse polymers arising from any of the known biosynthetic pathways indicating there is a lack of discrimination for donor structures (Raetz and Whitfield, 2002). The completed LPS molecule is translocated onto the OM by a still unknown mechanism but may involve generalized OM assembly functions such as the TolA protein in *E. coli* (Gaspar *et al.*, 2000).

The structure of lipid A is composed of a phosphorylated N-acetylglucosamine dimer attached to 6-7 saturated fatty acids and is highly conserved amongst many Gram-negative bacteria, especially *Enterobacteriaceae* (Wyckoff *et al.*, 1998). The enzymology and molecular genetics of the conserved steps of lipid A biosynthesis are best characterized in *E. coli* (Raetz, 1996; Raymond *et al.*, 2002). The *lpx* genes seem to be constitutively expressed and appear scattered throughout the chromosome. *lpxX* is beside *msbA*, the product of which is used to transport phospholipids and lipid A. The resulting products of *lpx* are located in the cytoplasm or on the inner surface of the IM. Initially, the sugar nucleotide UDP-GlcNAc is acylated by LpxA, a UDP-GlcNAc acyltransferase, which is specific for  $\beta$ -hydroxymyristate carried on an acyl carrier protein. This leads to deacetylation of UDP-3-*O*-(acyl)-GlcNAc by LpxC. A second  $\beta$ -hydroxymyristate moiety is incorporated by LpxD to generate UDP-2,3-diacylglucosamine, which is subsequently cleaved at its pyrophosphate bond by LpxH, a pyrophosphatase to form 2,3-diacylglucosamine-1-phosphate (lipid X). LpxB catalyzes the condensation of lipid X with another molecule of UDP-2,3-diacylglucosamine creating a disaccharide, which is phosphorylated by LpzK generating lipid IV<sub>A</sub>. Two KDO residues are next transferred to lipid IV<sub>A</sub> by the bifunctional enzyme, WaaL, and finally acyltransferases add lauroyl and myristoyl residues from acyl carrier proteins to the distal glucosamine unit, generating acyloxyacyl moieties. *E. coli* and *Salmonella Typhimurium* contain additional enzymes for modifying lipid A with phosphoethanolamine, L-Ara<sub>4</sub>N and/or palmitate groups. Normally these enzymes are latent in *E. coli* K-12 but are expressed in various stresses. For example, phosphoethanolamine and L-Ara<sub>4</sub>N moieties are added to lipid A following exposure to mildly acidic conditions, or by mutation. These modifications can be important for bacterial survival and virulence. For example, the presence of the L-Ara<sub>4</sub>N group confers resistance against the antimicrobial polymyxin (Gunn *et al.*, 1998) and palmitate modifications is controlled by the PhoP/PhoQ system, which is activated by low

concentrations of  $Mg^{2+}$ , as would be encountered inside phagolysosomes (Guina *et al.*, 2000).

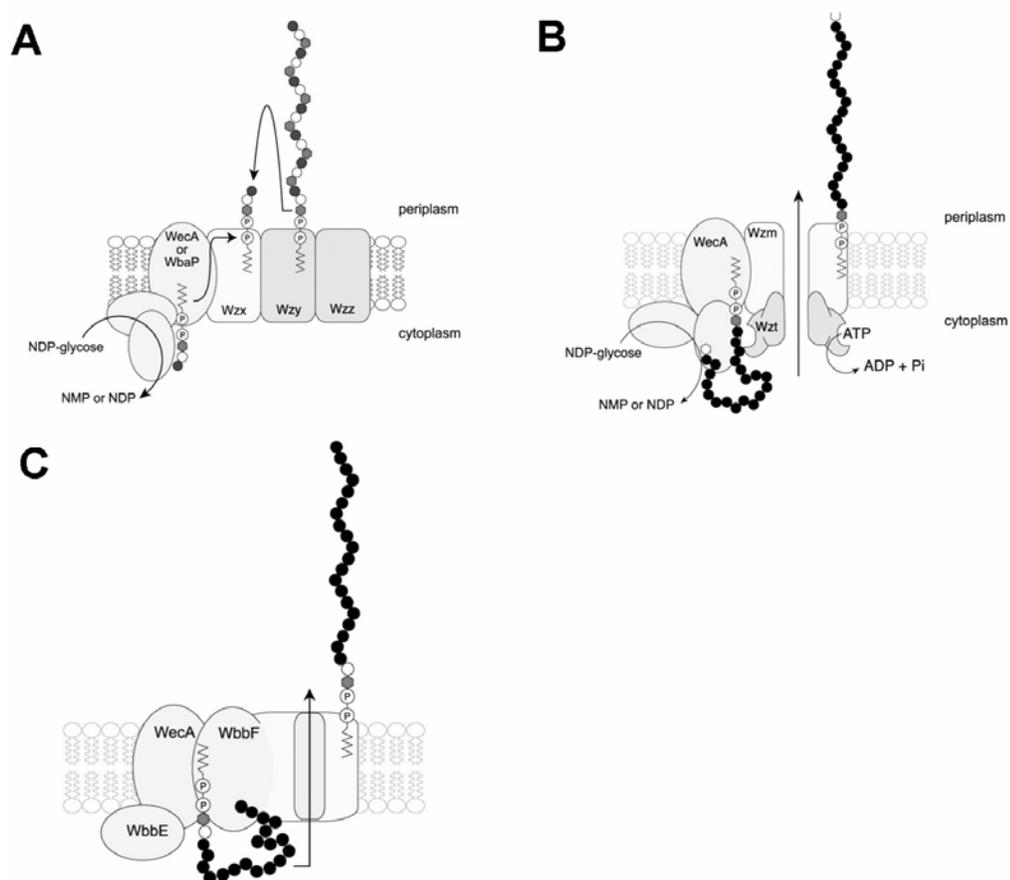
As with lipid A, the core oligosaccharide has been studied intensively in *E. coli* and *Salmonella* (Raetz and Whitfield, 2002). The chromosomal *waa* region (formerly *rfa*) contains the major core-oligosaccharide assembly operons and in *E. coli* and *Salmonella*, the *waa* locus consists of three operons. The core oligosaccharides are divided into two regions: inner core (lipid A proximal) and outer core, which provides an attachment site for O-Ag. Within a genus or family, the structure of the inner core tends to be well-conserved typically containing residues of KDO and L, D-Hep. The inner core is often modified with nonstoichiometric additions of other sugars, phosphate, pyrophosphorylethanolamine, or phosphorylcholine again adding to the heterogeneity of LPS molecules. The outer core compared to the inner core seems to show more structural diversity within in given species. For example, in *E. coli* there are five known core types (R1, R2, R3, R4, and K-12). All are found amongst commensal isolates (Amor *et al.*, 2000), whereas the R1 and R3 type are associated with the pathogenic isolates. There are two known outer cores from *Salmonella* where the one found in serovar Typhimurium is common to isolates from routine human infections (*S. enterica* subspecies *enterica*), and the structure found in Arizonae IIIA predominates in other subspecies (Raetz and Whitfield, 2002). All of the *E. coli* and *Salmonella* outer cores have a glucose residue as the first sugar in the outer core initiated by a conserved  $\alpha$ -1,3-glycosyltransferase, WaaG. The subsequent transferases form  $\alpha$ -1,3- and  $\alpha$ -1,2-linkages, and depending on the structure the enzymes transfer glucose or galactose to an acceptor of either glucose or galactose. The corresponding transferases are all members of family 8 with highly conserved motifs, making assignments of their specificity difficult in the absence of biochemical evidence. These enzymes are specific for their linkages whereby formation of an  $\alpha$ -linkage in the product is from an  $\alpha$ -linked donor. Within *E. coli*, the R1 and R4 core types are the only ones involving a  $\beta$ -

glycosyltransferase and thus an  $\alpha$ -linked donor is used to generate a  $\beta$ -linked product by an inverting mutarotase enzyme (Raetz and Whitfield, 2002).

The O-unit structures can differ in the monomer glycoses, numbers of monosaccharides, the position and stereochemistry of the O-glycosidic linkages, the presence or absence of noncarbohydrate substituents (O-acetylation) or nonstoichiometric modifications (glycosylation) in addition to being linear or branched. The *rfb* genes involved in O-Ag biosynthesis are generally found clustered on the chromosome and the O-Ag structural variation is mirrored by genetic variation within these clusters (Samuel and Reeves, 2003). *rfb* genes appear to be constitutively expressed, are close to each other, and typically have a lower GC content than the genome average, which suggests that the clusters have been laterally transferred from a different bacterial species (Samuel and Reeves, 2003). The *rfb* genes encode proteins involved nucleotide activated sugars biosynthesis, the glycosyltransferases on Und-P and the proteins involved in polymerization and translocation (Reeves *et al.*, 1996).

The precursors of several of the sugars commonly found in O-units are part of other pathways including UDP-Glc, UDP-Gal, and UDP-GlcNAc. Presumably because these products have housekeeping functions, the genes involved in their synthesis are not duplicated in the O-Ag gene cluster. Instead, only the sugars unique to the O-units have their corresponding synthesis genes found in the gene cluster. Therefore each O-Ag gene cluster is expected to have nucleotide sugar biosynthesis pathway genes corresponding to each of the non-housekeeping sugars present in their respective O-Ag (Samuel and Reeves, 2003).

The glycosyltransferase genes involved in the transfer of the UDP-glycoses are commonly found dispersed throughout the O-Ag cluster (Samuel and Reeves, 2003). The extensive range of sugars found in O-Ag allows for numerous combinations of donor sugar, acceptor sugar and acceptor carbon atom for the glycosidic linkages. This provides for a very large number of linkage specificities and thus of glycosyltransferase specificities. Different glycosyltransferase genes have been identified but since their



**Figure 1.9. O-Antigen assembly models.**

(A) Wzy-dependent pathway. Individual Und-PP-linked O-units are transferred across the membrane by a process involving Wzx. The intermediates act as substrates for the polymerase, Wzy, in the periplasm where extension occurs at the reducing terminus with the nascent chain being transferred from the nonreducing terminus of the Und-PP-linked subunit. The extent of polymerization is determined by Wzz. The polymer is ligated to lipid A-core and translocated to the OM (B) ABC-transporter-dependent pathway. The ABC-transporter formed by Wzm and Wzt is required for transfer of the undecaprenyl-linked polymer to the periplasmic face of the membrane, where it is ligated to lipid A-core and translocated to the OM. Within the polymer, the primer is the filled hexagon, the residues of the repeating-unit by filled circles, and the chain terminator by the open hexagon. (C) Synthase-dependent pathway. WbbF serves as a glycosyltransferase and an exporter that moves undecaprenyl-linked intermediates to the periplasm. The polymer is ligated to lipid A-core and translocated to the OM (Raetz and Whitfield, 2002). Reprinted, with permission, from the Annual Review of Biochemistry, Volume 71 (c) 2002 by Annual Reviews ([www.annualreviews.org](http://www.annualreviews.org)).

diversity is reflected in the heterogeneity of their DNA sequences it has been difficult to identify the specificities of each transferase without biochemical data (Samuel and Reeves, 2003). Unfortunately, only a small proportion of the putative transferases have been studied biochemically, and thus there are only a few cases where all the transferases in a particular O-Ag cluster have been identified. In *E. coli*, the first step in O-Ag biosynthesis involves the formation of an Und-PP-linked GlcNAc by transfer of a sugar-1-P residue to Und-P by an initiating conserved transmembrane glycosyltransferase, WecA. This glycosyltransferase is differentiated from the simple glycosyltransferases by its ability to recognize the hydrophobic lipid carrier rather than the sugar acceptor (Jiang *et al.*, 1991). The energy of the sugar-P linkage in the donor molecule is conserved in the resulting Und-PP-linked intermediate and is subsequently used to drive polymerization of the lipid A-core to O-Ag (Raetz and Whitfield, 2002). The resulting Und-PP-GlcNAc acts as a primer for saccharide chain extension. In the Wzx/Wzy-dependent pathway where the enzyme is required for each lipid-linked O-unit, GlcNAc is found within the repeat unit structure itself while in the ATP-translocase dependent pathway, the GlcNAc residue will be transferred to lipid A-core during ligation and only occurs once per chain (Samuel and Reeves, 2003).

### 1.5.2 EPS and capsules

Many bacteria synthesize EPS that are either secreted or linked through a covalently attached lipid to the cell as capsules. Notably, capsules can also be released into the environment as a consequence of the instability of the phosphodiester linkage between the polysaccharide and the phospholipid anchor (Roberts, 1996). EPS are key components of biofilm matrices by promoting adherence of bacteria to surfaces and to each other as well as have important roles in immune evasion and tolerance toward antibacterial agents (Roberts, 1996). EPS also play a role in desiccation tolerance because the molecules are highly hydrated holding up to 95% water (Costerton *et al.*, 1981) that protects the cells (Roberson and Firestone, 1992). For example, capsules

confer desiccation tolerance to *E. coli*, *Acinetobacter calcoaceticus* and *Erwinia stewartii* where at least in *E. coli*, desiccation triggers EPS gene expression (Ophir and Gutnick, 1994). This could be due to changes in external osmolarity, which triggers increased EPS synthesis (Roberts, 1996). At least in *P. aeruginosa*, alginate synthesis increases in response to high external osmolarity (Berry *et al.*, 1989). Capsules also facilitate swarming, a multicellular surface motility behaviour, in *Proteus mirabilis* by providing the cells with lubricant and thus reduce friction over the cell surface (Gygi *et al.*, 1995).

By far most known EPS molecules are neutral or polyanionic (Sutherland, 2001). Enzymatic alteration of EPS is believed to significantly change its physio-chemical and immunological properties and thus function. For example, *Staphylococcus epidermidis* PIA, a homopolymer of  $\beta$ -1,6-linked *N*-acetylglucosamine residues encoded by *icaADBC* genes is immunologically distinct from its deacetylated polymer and acetylation is responsible for evasion of host immune defense and virulence (Vuong *et al.*, 2004). *E. coli* strains produce more than 80 chemically and serologically distinct capsules, called K-Ag in addition to two common EPS, cellulose and colanic acid. The capsule genes clusters are used as a paradigm for capsules in other bacteria. The capsular K-Ags have been separated into four groups on the basis of chemical composition, molecular weight, intergenic relationships, and regulation of expression. The majority of extraintestinal isolates of *E. coli* associated with invasive disease express group 2 capsules, with certain capsules being associated with certain diseases. For example, the expression of *E. coli* K13 Ag is associated with uropathogenic *E. coli* (UPEC; Vann and Jann, 1979). Some *E. coli* K-Ags have identical polysaccharide repeat units but differ in modifications such as K13, K20 and K23 where each has the structure 3- $\beta$ -D-Rib-(1,7)- $\beta$ -KDO-2 but K13 is O-acetylated at KDO and K20 is O-acetylated at Rib (Vann *et al.*, 1983). Different species of bacteria can produce chemically identical capsules such as the *N. meningitidis* group B capsule and the *E. coli* K1 capsule or the *H. influenzae* capsule and the *E. coli* K18, K22 and K100 capsules

(Roberts, 1996; Jann and Jann, 1990). EPS associated gene operons are clustered on the chromosome, presumably allowing for co-ordinated expression of biosynthesis and export (Roberts, 1996). Capsules appear to use similar mechanisms of export as do LPS O-Ags (Amor and Whitfield, 1997; Whitfield, 1995). Group 1 and 4 K-Ags follow the Wzx/Wzy-dependent polymerization pathway similar to that of heteropolymeric O-Ag (Amor and Whitfield, 1997). Group 2 and 3 K-Ags are assembled by an ABC-transporter-dependent pathway, similar to that of homopolymeric O-Ag. EPS common to most *wt E. coli* and *Salmonella* strains are ECA, colanic acid and cellulose. Both ECA and colanic acid are synthesized by the Wzx/Wzy-dependent pathway (Roberts, 1996) and cellulose is probably synthase dependent (Romling, 2002).

Group 1 K-Ags are acidic polysaccharides which may contain amino sugars, expressed at all temperatures, regulated by RcsA (Roberts, 1996) and have two different forms: LMW K-Ag oligosaccharides ( $K_{LPS}$ ) comprised of one or a few K-units linked to lipid A-core and capsular (MacLachlan *et al.*, 1993). The capsular K-Ag is linked to the cell through an unknown lipid anchor but it is not lipid A-core and the presence of both forms indicates they are exported from the cell by different pathways (Roberts, 1996). *E. coli* K1 strains may also modify their capsules by O-acetylating sialyl units at the carbon 7 or 9 hydroxyl, thereby altering polysialic acid immunogenicity and susceptibility to glycosidases (Deszo *et al.*, 2005). At least two genetic locations have been identified for the group 1 capsule gene loci: near the *trp* locus and between the *his* and *rfb* loci (Roberts, 1996). Group 1 genes can occupy the same general location (*rfb*) as the colanic acid gene locus, and this would explain why the expression of group 1 capsules and colanic acid appear to be mutually exclusive (Roberts, 1996). The genes for the production of group 1 capsules have been identified in *E. amylovora*, *E. stewartii*, *P. solanacearum* and *Klebsiella pneumoniae* but there are some differences in genetic organization (Roberts, 1996).

Group 4 capsules used to be considered a part of group 1 (Roberts, 1996) but because they comprise structurally identical O-units they are now their own group and

are known as the “O-Ag capsules” (Whitfield and Roberts, 1999). Half of the O111-Ag is found as an LPS fraction and the remainder is in an LPS-unlinked capsular form (Goldman *et al.*, 1982). It was recently shown that O-Ag capsule mutants are more susceptible to complement mediated killing (Peleg *et al.*, 2005).

Group 2 capsules are generally acidic, expressed above 20°C and linked to the cell via an  $\alpha$ -glycerophosphatidic acid moiety and, in some *E. coli*, KDO provides a 'linker' between the lipid and the reducing terminus of the polysaccharide. Group 2 capsule gene clusters are mapped near the *ser* locus and have a common organization consisting of three regions: variable region 2 encoding the enzymes for serotype specific polysaccharide biosynthesis which is flanked by conserved regions 1 and 3, encoding six (KpsF; putative regulator, KpsE; IM protein involved in periplasmic transport, KpsD; periplasmic protein involved in transport, KpsU; activated sugar nucleotide synthase, KpsC and KpsS; cytoplasmic proteins) and two (KpsM and KpsT; ABC IM transporters) proteins respectively. These regions constitute putative capsule regulatory proteins and the ABC transport machinery for capsule export out of the cell. Each region is a single transcriptional unit where region 1 is divergent from regions 2 and 3 (Roberts, 1996). Any mutation in one of the genes in regions 1 or 3 significantly reduces membrane transferase activity indicating that biosynthesis and export are linked. It is thought that a multi-protein complex exists on the IM and may extend through to the OM (Roberts, 1996). At least two IHF binding site consensus sequences have been identified 110 bp 5' and 130 bp 3' of the transcription start site. Genetic organization of *E. coli* group 2 capsules resembles those of *N. meningitidis* group B and *H. influenzae* type B. Again, there are some differences; *N. meningitidis* has several more genes originally thought to be organized in 5 transcription regions but at least two sections have now been shown to be transcribed as one unit and in some *H. influenzae* strains the gene locus is duplicated (Kroll *et al.*, 1988).

Group 3 capsules are encoded by *wcb* genes in *E. coli* K-10. Although there is no direct chemical evidence for an  $\alpha$ -glycerophosphate moiety at the terminus of group 3

capsules, genetic data are consistent with a common anchor for group 2 and group 3 capsules. However groups 3 are genetically distinguishable from group 2 and thus are classified as their own group (Roberts, 1996).

Moving HMW polymers ( $M_r > 100\ 000$ ) across the OM is not well understood and the mechanism of capsule surface assembly and translocation is a major question. Current models for the assembly and translocation of capsular polysaccharides predict a "scaffold" comprised of translocation machinery that transfers the capsule across the OM by forming a transient link between the membranes. This hypothesis is based on EM imaging techniques showing translocation of *E. coli* group 1 and 2 capsules to the cell surface was observed to occur at specific sites where the membranes appeared to come into close contact (Roberts, 1996; Whitfield and Valvano, 1993). The group 1 capsules require a conserved OM lipoprotein, Wza, which associates with another conserved protein kinase, Wzc, which is required for efficient surface expression. The Wzb protein is an acid phosphatase. The translocation system for group 4 capsules originally appeared to be different from group 1 (Whitfield and Paiment, 2003). However recently discovered genes involved in the assembly and export of the O-Ag capsule from enteropathogenic *E. coli* (EPEC) include a putative polysaccharide secretion system encoded by a seven gene operon (*ymcDCBA*, *yccZ*, *etp*, *etk*) required for capsule expression and many of these predicted proteins are Wza, Wzb and Wzc homologues (Peleg *et al.*, 2005). Etk is predicted to be a structural homologue to Wzc. As well, Ycc is predicted to be an OM protein and may be functionally similar to Wza whereas Etp is predicted to be a cytoplasmic phosphatase and thus may be functionally similar to Wzb. Each gene is required for surface translocation in EPEC and a mutation in any of them results in the loss of cell surface capsule expression. The operon is present in some *E. coli* strains but not expressed in *E. coli* K12. The translocation of group 2 capsules to the cell surface is mediated by the KpsE and KpsD proteins, which form an ABC module (Bliss and Silver, 1996; Roberts, 1996). The KpsE

and D proteins appear to be linked to an IM complex that forms a translocase between the membranes (Roberts, 1996).

## 1.6 *Salmonella* polysaccharides

Considering that there are at least 19 uncharacterized, putative polysaccharide operons involved in either biosynthesis or transport, found in the sequenced chromosome of *Salmonella* Typhimurium ([http://cmr.tigr.org/tigr-scripts/CMR/shared/GeneList.cgi?Sub\\\_org=Salmonella+typhimurium](http://cmr.tigr.org/tigr-scripts/CMR/shared/GeneList.cgi?Sub\_org=Salmonella+typhimurium)), it seems that polysaccharides have been understudied in *Salmonellae*. Like *E. coli*, members of *Salmonellae* have ECA, a diverse selection of serovar specific LPS, cellulose and colanic acid. Additionally, some host-adapted serovars of *Salmonella* synthesize Vi-Ag. Considering there have been > 80 capsules reported in *E. coli* and many capsules reported for other members of the *Enterobacteriaceae*, it seems odd that no capsules have been reported in *Salmonellae*.

### 1.6.1 LPS

*Salmonella* LPS biosynthesis follows similar pathways of synthesis and export as already described above for *E. coli*. Differences between the two genera include the initiating enzyme in O-unit synthesis. *E. coli* initiates with WecA whereas *Salmonella* initiates with RfbP, which transfers a Gal-1-P from UDP-Gal to Und-P and consequently most *Salmonella* repeat units begin with Gal residues (Whitfield, 1995). The strains that do not start with Gal may use a different initiating transferase. Sequence homology does not exist between the two initiating transferases but both are predicted integral membrane proteins. The 2,541 different serovars of *Salmonellae* are derived from considerable diversity seen within *Salmonella* O-Ag structures. As discussed previously, the basis of the variation in O-Ag structure is represented by differences in sugar composition, arrangement of the sugars in the O-unit, the specific linkages between the O-units and the addition of modifying side groups.

Much of *Salmonella* O-Ag variation is a consequence of the extensive genetic diversity within *rfb* gene clusters, which encode many of the enzymes involved in O-Ag biosynthesis and assembly. For example, *Salmonella* Enteritidis contains Tyv in its O-unit and thus has genes specific for Tyv synthesis and incorporation whereas *Salmonella* Typhimurium contains Abe in its O-unit and thus has the genes specific for Abe synthesis and incorporation. Also, there is variation in the linkage between O-units: serovar Typhimurium O-units are linked by  $\alpha$ -1,2 linkage whereas other strains have an  $\alpha$ -1,6 linkage. The Wzy polymerase for these linkages can be located within or outside the O-Ag gene cluster and sometimes even encoded by a phage (Wang *et al.*, 2002b).

The *rfb* gene clusters from 10 *Salmonella* serogroups have been studied: A, B, C<sub>1</sub>, C<sub>2</sub>, D<sub>1</sub>, D<sub>2</sub>, D<sub>3</sub>, E<sub>1</sub>, O:54 and O:35 (Fitzgerald *et al.*, 2003). The *rfb* regions from serogroups A, B, C<sub>2</sub>, D, and E are similar and all have a trisaccharide O-unit containing Man, Rha and Gal. In contrast, the *rfb* gene cluster from serogroup C<sub>1</sub> O-unit is unrelated, which has composed of four Man residues, one *N*-acetylglucosamine residue, and a Glc side branch. A number of *Salmonella* O-Ags genes have been reported to be bacteriophage encoded, such as factors O1 and O34A and a plasmid-encoded *rfb* gene cluster has also been reported necessary for biosynthesis of the O54 antigen in *Salmonella* Borreze.

*Salmonella* Enteritidis is group D1 and has four residues in its O-Ag: Man, Gal, Rha and Tyv. The initial structural studies indicate that the Man residue is found in  $\beta$  form (Hellerqvist, 1968; Staub, 1959) whereas the second report indicated that both anomers were detected and thus no assignment was made (Hellerqvist *et al.*, 1969). Finally, the third and four reports indicated that it was in the  $\alpha$  form determined by acid instability of the anomeric carbon and enzymatic degradation (Fukuda *et al.*, 1971; Hellerqvist *et al.*, 1971). Each of these studies used different isolates and different techniques and some did not mention growth conditions making it difficult to compare results. Some studies have shown that group D is subdivided into 3 groups. Group D1 which includes *Salmonella* Enteritidis and Typhi have the  $\alpha$ -Man configurations, D2

has the  $\beta$  configuration whereas group D3 has either or both configurations (Curd *et al.*, 1998). The initial study of *Salmonella* Enteritidis detected both  $\alpha$ - and  $\beta$ -Man residues adding some doubt as to which mannose configuration serovar Enteritidis is in (Hellerqvist *et al.*, 1969). Interestingly, in *Salmonella* group E, phage infection has been attributed to a change from  $\alpha$  Man to  $\beta$  Man (Robbins and Uchida, 1962) due to phage conversion (Nghiem *et al.*, 1992). Current literature references *Salmonella* Enteritidis in either conformation (Rahman *et al.*, 1997; Whitfield, 1995). Thus, there is some confusion as to which configuration the Man residue takes in *Salmonella* Enteritidis and it appears this molecule is pliant in group D and E O-Ag structures.

The genes for O-Ag modification such as acetylation and glucosylation are found outside of O-Ag gene clusters. These include transferase genes whose products modify O-Ag after polymerization. For example, the *S. enterica* B O-Ag can be modified by addition of Glc to the Gal residue. This is either via a 1,4 linkage, determined by chromosomal genes, or a 1,6 linkage, determined by a gene carried by P22 lysogenic phage (Makela, 1973). Glycosyl transfer occurs after the O-unit has been translocated to the periplasmic face of the IM. The transfer is from Und-P-Glc, which is synthesized and translocated by the products of the *gtrBAC* genes (Wang *et al.*, 2002b). The third gene varies genetically and encodes a specific transferase that determines linkage specificity (Wang *et al.*, 2002b). The group D serovar Typhi also is glucosylated at the Gal residue and serovar Enteritidis displays heterogeneity in glucosylation where HMW O-Ag is highly glucosylated and LMW O-Ag is not (Parker *et al.*, 2001). O-Acetyl transferases, encoded by *oafA*, modify the group B O-Ag by acetylation at the Abe residue (Slauch *et al.*, 1996). O-acetylation modification of the Abe residue in *Salmonella* Typhimurium significantly alters the 3-D conformational structure of the O-Ag molecule and thus creates and destroys antigenic determinants (Slauch *et al.*, 1995). *oafA* has homologous genes in other members of the *Enterobacteriaceae* and other bacteria such as *Xanthomonas campestris* (Slauch *et al.*, 1996).

### 1.6.2 EPS: Vi-Ag, cellulose and colanic acid

Historically, *Salmonella* Vi-Ag is probably the most well known EPS. Throughout the literature, Vi-Ag is referred to as capsular but actually by definition, it is not because it is not associated with the cell through a covalently attached lipid. This EPS is composed of a linear homopolymer of poly- $\alpha$ -1,4-2-GalANAc that is variably O-acetylated at the C-3 position (Szu *et al.*, 1991). Serum antibodies elicited to Vi-Ag confer protective immunity against typhoid fever and immunogenicity is closely related to its degree of O-acetylation (Szu *et al.*, 1991). Expression of Vi-Ag is controlled by two gene loci, *viaA* and *viaB* located at distinct sites on the chromosome (Makela and Stocker, 1969). The *viaA* locus, proposed to be involved in regulation of *viaB* genes, is present not only in Vi-Ag expressing *Salmonella* and *Citrobacter* strains but also in *E. coli* (Johnson and Baron, 1969). The *viaB* locus is limited to Vi-Ag expressing strains of *Salmonella* serovars Typhi, Dublin, and Paratyphi C and *C. freundii* and is located to a pathogenicity island identified as SPI7 (Pickard *et al.*, 2003). The *viaB* locus consists of 10 genes: *tviBCDE* for Vi polysaccharide biosynthesis and *vexABCDE* for export, as well as *tviA*, a regulatory protein positively controlling the transcription of *tviBCDE* and itself (Virlogeux *et al.*, 1995). *tviA* is activated by the RcsC/AB signal transduction system (Arricau *et al.*, 1998) and OmpR/EnvZ signal transduction system (Pickard *et al.*, 1994) in response to low osmolarity and pH. At least eight strains of Typhi (n=120), including SARB 64, one of the strains in the SARB set of strains constructed by Boyd (1993), are Vi-Ag negative due to SPI7 deletions (Boyd *et al.*, 2003; Nair *et al.*, 2004). Vi-Ag negative *Salmonella* Typhi strains retain pathogenicity indicating that Vi-Ag is not essential for infection to be established in humans (Bueno *et al.*, 2004; Mehta and Arya, 2002). Vi-Ag negative strains can escape immune protection resulting from the use of Vi-Ag as a vaccine which is concerning because Vi-Ag is currently the basis for a licensed injectable vaccine against typhoid fever (Nair *et al.*, 2004) although an oral attenuated vaccine is also available.

Cellulose is an insoluble homopolymer of  $\beta$ -(1,4) glucose with up to 10,000 glucose residues. In natural cellulose, many molecules associate in parallel to form fibrils which in turn associate to form fibers through intra-and inter-chain hydrogen bonding. Although the exact structure of cellulose in its natural state is unknown, biophysical studies of prepared cellulose samples have identified three prevalent structural classes: crystalline cellulose, amorphous cellulose, and *para*-crystalline cellulose (Atalla, 1999; McLean *et al.*, 2002). The varied structure of cellulose contributes to its recalcitrance and some cellulose binding proteins bind preferentially to the crystalline regions and others to the amorphous regions (McLean *et al.*, 2002). Although antibodies have not been raised to cellulose, non-specific dyes such as calcofluor white, a fluorescent agent which nonspecifically binds to  $\beta$ -linked polysaccharides or CR, a non-specific hydrophobic dye, can be incorporated into agar plates to determine if bacteria express cellulose (Deinema and Zevenhuizen, 1971; Romling *et al.*, 2000; Zogaj *et al.*, 2001). Using these techniques, cellulose biosynthesis has been characterized in bacteria including *Salmonella* serovars Enteritidis and Typhimurium. The biosynthetic genes in *Salmonella* are organized in two divergently transcribed operons, *bcsABZC* and *bcsEFG* (bacterial cellulose synthesis; (Solano *et al.*, 2002; Zogaj *et al.*, 2001). The *bcsA* gene is present in all species investigated of the family *Enterobacteriaceae* and expression is conserved in some members (Zogaj *et al.*, 2003). The putative functions of the *bcs* genes and cellulose biosynthetic mechanisms are based on homology to other cellulose-producing bacteria including the model organism, *Gluconacetobacter xylinus* (*Acetobacter xylinus*) where some gene functions have been experimentally determined. Cellulose biosynthesis has been thoroughly reviewed by Romling (2002) but will be summarized here. *bcs* genes are constitutively transcribed (Zogaj *et al.*, 2001) and cellulose synthesis is activated when c-di-GMP is produced by the GGDEF domain of the product of *adrA* which is activated by the Tafi regulator, AgfD (Romling *et al.*, 2000). The cellulose synthase, BcsA, is homologous to other glycosyltransferases and acts as the catalytic subunit for

cellulose biosynthesis by transferring UDP-Glc to the cellulose chain from the IM (Lin *et al.*, 1990). BcsB binds an activating second messenger molecule, c-di-GMP (Mayer *et al.*, 1991) and is localized to the IM with BcsA acting together as a cellulose-synthesizing complex (Kimura *et al.*, 2001). Cellulose synthesis is therefore modulated by the opposing action of two enzymes, diguanylate cyclase (GGDEF domain) and c-di-GMP diesterase (EAL domain), controlling the level of c-di-GMP in the cell. The mechanism of synthesis was hypothesized from EM images of freeze fractured membranes that revealed approximately 50 pores of complexes arranged in a single row along the longitudinal axis of the bacterial rod. Each complex secretes 12-25 glucan chains, which assemble into large microfibrils at the site of synthesis (Kimura *et al.*, 2001). Although the role of the other *bcs* genes have not been experimentally determined, homology searches have shown that *bcsZ* and *bcsG* encode endogluconases, *bcsC* encodes an oxidoreductase, *bcsE* encodes a protease whereas *bcsF* shows no homology to any other proteins in databases (Solano *et al.*, 2002). Cellulose plays a role in biofilm development on abiotic surfaces and confers protection against chlorine (Anriany *et al.*, 2001; Scher *et al.*, 2005; Solano *et al.*, 2002; White, 2005). Cellulose is intimately associated with Tafi (Fig. 1.4; White *et al.*, 2003) and contributes to the multicellular behavior of the microbes by connecting the cells through an elastic and stable matrix (Romling, 2002). Recently, it was found that *Salmonella* Typhimurium BJ2710 biofilms formed on Hep-2 tissue culture cells and on chicken epithelium are severely underdeveloped in the absence of cellulose indicating that cellulose also plays a role in development of biofilms on biotic surfaces (Ledeboer and Jones, 2005).

Colanic acid is a secreted EPS of unique structure (Fig. 1.10. C) that confers a mucoid phenotype to bacteria when grown at 15-20°C in high P concentration and is called the “M-Ag” in *E. coli* and *S. enterica* strains (Grant *et al.*, 1969). In *E. coli*, a cluster of 17-20 contiguous genes encoding the proteins required for the production and secretion of colanic acid, are grouped together in the *wca* (*cps*) operon separated by two genes from the O-Ag (*rfb*) gene cluster (Fig. 1.10; Rahn *et al.*, 1999; Stevenson *et al.*,

1996; Whitfield and Roberts, 1999). A similar set of genes has been identified in the sequenced genomes of *S. enterica* serovars (Stevenson *et al.*, 1996; our unpublished observations), which are likely to have similar functions but have not been studied experimentally. The *wca* genes have an unusually high GC content compared with the usual (52-57% vs. 50% in *E. coli*; 61% vs. 51% in *S. enterica*) suggesting they were acquired by lateral gene transfer from another species (Stevenson *et al.*, 1996). The synthesis of the colanic acid repeat unit requires four different nucleotide sugars; two (UDP-Glc and UDP-Gal) are used for other purposes in the cell and thus the genes (*galU*, *galTKE*) are located elsewhere on the chromosome. Synthesis of UDP-GlcA from UDP-Glc is catalyzed by UDP-Glc dehydrogenase, the product of *ugd*, and is found at a separate locus in some strains of *E. coli* and *Salmonella*. GDP-L-Fuc is derived from D-Man-6-P and requires 4 genes: *manB* (*cpsG*) encoding phosphomannomutase, *manC* (*cpsB*) encoding Man-1-P guanosyltransferase, *gmd* encoding GDP-D-Man-4,6-dehydratase and *wcaG* (*fcl*) encoding the Fuc synthetase that catalyzes both the reduction and epimerization of GDP-4-keto-6-deoxy-D-Man to GDP-L-Fuc (Andrianopoulos *et al.*, 1998). It is expected that six of the *wca* genes encode specific glycosyltransferases which would add activated sugars to the growing repeat unit; *wcaJ* putatively encodes a Glc-1-P- transferase localized to the IM, predicted to initiate synthesis by transfer of Glc-1-P to Und-P. It was found to be essential for the synthesis of the repeating unit in *Salmonella* Enteritidis 3b (our unpublished observations), *wcaA* encodes a putative transferase and *wcaCELLI* encodes other putative glycosyltransferases. *wcaB* and *wcaF* are putative acetyltransferases predicted to be involved in the addition of an acetyl group(s), one of which is attached to a Fuc residue (Stevenson *et al.*, 1996). It has been hypothesized that repeat unit assembly and export across the IM proceeds in a manner analogous to that proposed for the Wzx -dependent class of O-Ag. *wzx* encodes a Wzx-like transmembrane protein and flips the Und-PP-repeat unit from the cytoplasm into the periplasm where polymerization occurs (Liu *et al.*, 1996). *wcaD* is predicted to encode an IM protein that has a role in polymerization

of the repeating units (Stevenson *et al.*, 1996). Homologues to the group 1 and group 4 capsule export proteins, Wza (OM lipoprotein), Wxb (phosphatase) and Wzc (kinase) are used in colanic acid export (Stevenson *et al.*, 1996). When Wzc is phosphorylated, no colanic acid is synthesized, whereas when Wzc is dephosphorylated by Wzb, colanic acid is produced (Vincent *et al.*, 2000). The other products have no homologues and thus their functions are not predicted. The regulation of colanic acid biosynthesis in *Salmonella* has not been studied in any detail. However, in *E. coli*, gene expression is governed by a complex network of regulators. It is believed that the RcsC protein senses an environmental signal and, along with a second protein, RcsF, modulates the activity of RcsB through phosphorylation. RcsB can upregulate gene transcription itself and through its interaction with RcsA (Stout, 1994; Ferrieres and Clarke, 2003). Additionally, *ypdI*, encoding a putative lipoprotein, has been implicated in positively modulating colanic acid expression (Potrykus and Wegrzyn, 2004). The PhoP-PhoQ system negatively modulates colanic acid expression of genes found to be important for *Salmonella* virulence (Dominguez-Bernal *et al.*, 2004; Tierrez and Garcia-del Portillo, 2004). This would make sense if the function of colanic acid is more important for bacterial survival in the environment than in the host as predicted by some researchers (Stevenson *et al.*, 1996). To support colanic acid as an environmental factor, production is optimal at 20°C and turned off at 37°C when grown on abiotic surfaces (Danese *et al.*, 2000; Grant *et al.*, 1969; Prigent-Combaret and Lejeune, 1999; our unpublished observations). Furthermore, constitutive activation of the Rcs system attenuates *Salmonella* virulence indicating that a successful infection requires the expression of colanic acid to be off, which apparently interferes with virulence functions (Mousslim *et al.*, 2004). As well, colanic acid plays a major role in biofilm 3-D architecture on abiotic surfaces (Danese *et al.*, 2000) and protects *E. coli* O157:H7 from osmotic and oxidative stress (Chen *et al.*, 2004). In contrast to *E. coli*, *Salmonella*



biosynthetic pathway for colanic acid in *E. coli* K-12. Abbreviated enzyme names (in brackets) are shown, together with the encoding genes. Several gene assignments are speculative. CAP, colanic acid polymerase, E-units; repeat unit; Fru, fructose; GFS, GDP-L-fucose synthetase; GK, galactokinase; GLK, glucokinase; GMP, GDP-D-mannose pyrophosphorylase; GMD, GDP-D-mannosedehydratase; PEP, phosphoenolpyruvate; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; UGD, UDP-D-glucose dehydrogenase; UGE, UDP-D-galactose-4-epimerase; UGP, UDP-D-glucosepyrophosphorylase; UGT, UTP-D-galactose-1-phosphate uridylyl transferase. This figure was adapted with permission from Stevenson (1996). Copyright © 2006, the American Society for Microbiology. All rights reserved.

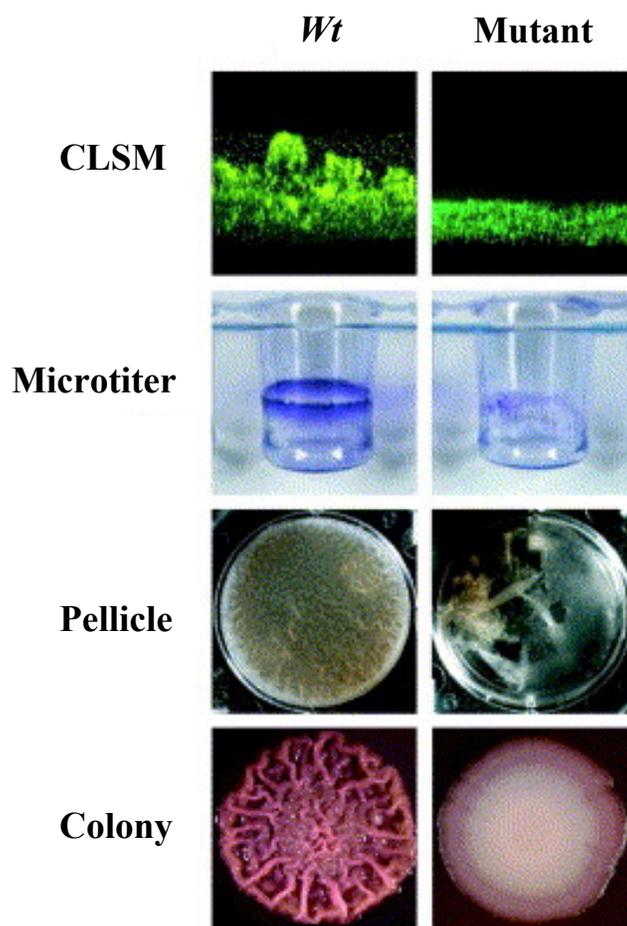
colanic acid biosynthetic genes are not required for biofilm formation on abiotic surfaces (Prouty and Gunn, 2003; Solano *et al.*, 2002; own unpublished observations) but rather play a role in the 3-D architecture of the biofilm on HEp-2 cells and chicken epithelium (Ledeboer and Jones, 2005). This indicates that *Salmonella* colanic acid production can occur at 37°C when the bacteria are exposed to eukaryotic cells in tissue culture media. Thus, colanic acid in *Salmonella* may be involved in both environmental survival and host virulence.

## 1.7 Biofilms

Biofilms are multicellular communities of surface attached microorganisms embedded within an extracellular matrix typically composed of sugar, protein and nucleic acid polymers (Stoodley *et al.*, 2002). Bacteria form biofilms on plant and animal surfaces and tissues, on medical devices, dental surfaces, sediments, soils and in water columns. Significant biological properties are attributed to biofilms, including co-operative behavior, competitive advantage, and defense against predators, antibiotics and immune systems (Davey and O'Toole, 2000; Hall-Stoodley *et al.*, 2004). Over the last few decades, it has become increasingly apparent that the predominating lifestyle of bacteria is within a biofilm where once unicellular microbes have transitioned into genetically differentiated, functionally organized and behaviorally integrated multicellular

communities (Parsek and Fuqua, 2004). However, the “pure culture” research tradition, which involves homogeneous bacterial culture grown to exponential phase, has biased researchers towards planktonic intracellular populations of microbial species. Bacteria, in their natural habitats, do not live in this manner and with this realization there has been the development of new research methods to study microbes as surface-associated biofilms. Because biofilms are recalcitrant to medical treatment and often associated with chronic bacterial infections creating medical and economic problems, the microbiologist, in order to create new ways of combating infections, must appreciate that bacteria in a biofilm have a much different physiology compared to their planktonic counterparts to create new ways to fight infections (Schembri *et al.*, 2003). It is recognized now that biofilms are a universal way of life for bacteria but even within the same species, there is extreme diversity in structure, metabolic and genetic responses to different environmental conditions thus making them difficult to study (Beloin and Ghigo, 2005).

Generally, biofilms are studied using four methods (Fig. 1.11). Bacteria that constitutively express GFP are grown in flow cell chambers which permit the biofilms to be visualized using confocal scanning microscopy and specialized computer programs (Christensen *et al.*, 1999). These studies have revealed that biofilms have an architecture that has been described as ‘mushroom-like pillars’, which are often separated by water-filled channels (Stoodley *et al.*, 2002). Biofilms can also be studied under conditions with no flow using a microtitre assay that facilitates high throughput screens to identify genes that are involved in biofilm formation (O’Toole and Kolter, 1998). Pellicles formed at the air-liquid interface of standing cultures represent another form of visualizing biofilms (Branda *et al.*, 2005; Collinson *et al.*, 1993; Romling *et al.*, 1998; Scher *et al.*, 2005). The last method is visualization of colony morphology from bacteria grown on the surface of agar-solidified media. There is a clear correlation between biofilm formation, highly structured morphologies, and the ability of a cell to



**Figure 1.11. Approaches used in biofilm studies.**

Four approaches used to study biofilms are represented by images of *wt* and mutant biofilms. In the top image, *V. cholera* biofilms were grown in flow cells and visualized by CLSM; the *wt* strain produced a biofilm that had the typical mushroom shaped pillar indicative of a mature biofilm whereas the mutant strain produced a biofilm that was relatively flat. Biofilms can be measured without flow in a microtitre dish and visualized using crystal violet staining; *E. coli* biofilms formed by the *wt* strain had more stain on the surfaces of microtiter wells than the mutant. Pellicles are biofilms formed at the air-interface of standing cultures; the pellicle produced by *Bacillus subtilis* was intact in *wt* but not in the mutant. Colony morphology indicates biofilm formation when grown on agar mediums; *P. aeruginosa* formed structured communities and was red on agar containing CR dye whereas the mutant was smooth and pink/white. Reprinted from Branda (2005), with permission from Elsevier.

produce an extracellular matrix (Anriany *et al.*, 2001; Branda *et al.*, 2005; Romling *et al.*, 1998). The cells that are able to form an extracellular matrix are dry, wrinkly, and rough and have the ability to bind CR whereas mutants are generally smooth, flat and have lost that ability (Collinson *et al.*, 1993).

### 1.7.1 Lifecycle and physiology

Using the methods defined above, researchers have been able to define the lifecycle of the biofilm that includes initiation, maturation and dispersion (Stoodley *et al.*, 2002). Initiation, mediated by the attachment of flagellated and fimbriated cells to surfaces, is often associated with genetic changes by a process called surface sensing (O'Toole *et al.*, 2000; Prigent-Combaret *et al.*, 1999). Bacteria initiate biofilm development in response to environmental cues such as nutrient availability, temperature, osmolarity, pH, iron and oxygen. Often, signals triggering biofilm formation have multiple genetic pathways indicating that there are specific subsets of genes required for various niches normally colonized by the organism (Branda *et al.*, 2005). Once attachment has been established, cells undergo physiological changes marked by the production of copious amounts of EPS, which gives the biofilm structural integrity (Branda *et al.*, 2005; Stoodley *et al.*, 2002). Properties associated with maturation are: increased antimicrobial resistance, increased resistance to UV light, increased rates of genetic exchange, increased secondary metabolite productions and greater protection against the host immune system and toxins (O'Toole *et al.*, 2000). The last, and the least understood stage is biofilm detachment where the cells or cell aggregates are sloughed off the biofilm and return to the planktonic growth mode. In *P. aeruginosa* detachment involves an EPS degrading enzyme, alginate lyase and rhamnolipid production (Boles *et al.*, 2005).

Once biofilms have matured they display a complex architecture, which can generate many microenvironments differing with respect to osmolarity, nutritional supply and cell density resulting in genetic differentiation (Hall-Stoodley and Stoodley,

2005). This may involve quorum sensing, an interbacterial communication mechanism that is dependent on population density and is associated with major (> 50%) changes in protein expression patterns (Parsek and Fuqua, 2004; Sauer *et al.*, 2002). Another common feature of a biofilm is antibiotic susceptibility patterns, where the outermost layers of cells are most susceptible and in the inner most layer cells are resistant due to metabolic inactivity in the anaerobic nutrition-depleted niches at the depths of the biofilm (Anderl *et al.*, 2003; Walters *et al.*, 2003). Interestingly, the physiology of bacteria within the biofilm is similar to planktonic cells in stationary phase of growth where a RpoS dependent general stress response is induced resulting in more tolerant phenotypes to stresses such as alterations in nutrition, cell density, temperature, pH or osmolarity (Fux *et al.*, 2005).

### 1.7.2 Extracellular matrix

The extracellular matrix is responsible for the maturation and thus the final architecture of the biofilm and is probably the most distinctive feature of biofilms cells compared to planktonic cells. The physical resilience of biofilms is the result of multiple interactions between matrix components (often EPS), bacterial surface appendages (fimbriae, flagella and aggregation factors) and coatings (LPS) and the surface colonized by the bacteria (Parsek and Fuqua, 2004; Sutherland, 2001). Over the last year increasing attention has been given to the extracellular matrix but even as little as three years ago, little was known about the biofilm matrix. It seems as though the extracellular matrix in biofilms is universally present but there is no universality in the composition or the conditions in which they are expressed. There is not only variation in biofilms formed by interspecies communities but also in different strains of a single species and major differences in biofilm architecture result from variation of the extracellular matrix composition as a result of minor environmental fluctuations (Branda *et al.*, 2005). Because of these variations, characterizing the extracellular matrix is a daunting process. Saying this, we at least know that generally, the main components of

the extracellular matrix are polysaccharides, protein polymers and nucleic acids (Parsek and Fuqua, 2004; Sutherland, 2001). The physical structure of many biofilms is not primarily the result of the expression of one matrix component, but of several interacting elements.

The EPS are considered to be the defining feature of a mature biofilm, providing structural integrity (Stoodley *et al.*, 2002). The EPS in matrices from different bacteria include alginate in *P. aeruginosa* (Davies *et al.*, 1993), cellulose in *Salmonella* Enteritidis (Solano *et al.*, 2002), and colanic acid in *E. coli* (Danese *et al.*, 2000). Several studies indicate that in the absence of the EPS, cells lack the ability to form a mature biofilm ( Danese *et al.*, 2000; O'Toole, 2000; Stoodley *et al.*, 2002; Sutherland, 2001). What is turning out to be common is that individual strains capable of forming EPS are often able to produce several different EPS (Branda *et al.*, 2005). For example, in *P. aeruginosa* alginate was generally believed to be the major EPS in the extracellular matrix of the biofilm formed by strains isolated from the lungs of Cystic fibrosis patients but recently, two other distinct EPS have also been discovered to be major components of the biofilm each with their own genetic loci (Friedman and Kolter, 2004b). One EPS, a glucose-rich polymer, is encoded by the genes *pelA-G* which are highly conserved throughout *P. aeruginosa* but the expression of these genes and the EPS varies greatly amongst strains. Notably, the common laboratory strains do not express these genes strongly (Friedman and Kolter, 2004a), suggesting that in these laboratory strains, the EPS genetic locus or regulation of this locus was subject to 'domestication' and the EPS was not necessary for laboratory survival. The genes for the other EPS, a Man-rich polymer, are present in only some *P. aeruginosa* strains (Friedman and Kolter, 2004b). Thus strain-to-strain variability can be held accountable for much of the diversity of extracellular matrices found in the biofilms.

An added level of complexity is that most microbes have the ability to form biofilms through alternate routes. For example, *P. fluorescens* biofilm defective mutants can produce biofilms in alternate environmental conditions (O'Toole and Kolter,

1998). In *V. cholera* the addition of  $\text{Ca}^{2+}$  activates a biofilm formation pathway that is independent from the EPS normally produced (Kierek and Watnick, 2003). Thus it appears that microbes have the ability to form EPS and their biofilms through multiple pathways under different environmental conditions. It is possible that the extracellular matrix structural variation provides a survival advantage by the presentation of different surface antigens in different environments.

### 1.7.3 Bacterial persistence and transmission

It is thought that biofilm formation provides a selective advantage for pathogens by increasing their ability to persist under diverse environmental conditions (Fux *et al.*, 2005). Perhaps, one of the reasons why bacteria persist within the biofilm is to facilitate the transmission of pathogens by providing a stable protective environment for the dissemination of large numbers of microbes as detached clumps (Hall-Stoodley and Stoodley, 2005). A good example of this phenomenon is in *V. cholera* where biofilm development facilitates persistence in aquatic environments leading to this pathogen's epidemic cycles. *Vibrio sp.* attach to the exoskeletons of crustaceans, aquatic insects and the cell walls of aquatic plants for attachment and initiation of biofilm formation. Once the biofilm has matured it periodically sloughs off as aggregated cells and when ingested would be expected to deliver a high concentration of bacteria for an infective dose to the host. Evidence to support this was provided when the number of cholera cases in a Bangladesh village dramatically declined following a crude method of water filtration through sari cloth (Hall-Stoodley and Stoodley, 2005). Other studies that have shown *Mycobacterium avium* isolated from hospital hot water systems corresponded to the same strains that infected AIDS patients at the same hospital (Parsek and Fuqua, 2004).

When the environmental niche of bacterial biofilms is within a host, bacterial persistence can cause a chronic infection (Parsek and Fuqua, 2004). One of most commonly studied examples of this is *P. aeruginosa* colonizing the lungs of Cystic

fibrosis patients. There is substantial evidence that *P. aeruginosa* biofilms play a role in Cystic fibrosis pathogenesis by providing a constant source of infecting bacteria that form antimicrobial resistant biofilms within the lungs (Whiteley *et al.*, 2001). Another example is chronic urinary tract infections caused by uropathogenic *E. coli* (UPEC). UPEC invades the urinary epithelium and establishes intracellular ‘pods’ of biofilms that help evade host immune responses (Anderson *et al.*, 2003). It is possible that through shedding the epithelial layers of bladder, these ‘pods’ release a constant source of bacteria that infect new tissues and again form drug-resistant biofilms. Thus biofilm formation facilitates the persistence of pathogenic organisms not only in the environment but also in their host; sometimes when the natural niche of a pathogen can be both, this can create a cyclical pattern of survival in the environment, which leads to infection, and then persistence in the host.

### 1.8 *Salmonella* biofilms

Biofilm formation is widespread among undomesticated isolates of *Salmonella enterica* serovars and often these biofilms provide a selective survival advantage in adverse conditions such as ethanol, acid, chlorine and iodophor (Anriany *et al.*, 2001; Bonafonte *et al.*, 2000; Gerstel and Romling, 2001; Jones and Bradshaw, 1996; Scher *et al.*, 2005; Solano *et al.*, 2002; White *et al.*, 2006). *Salmonella* can form biofilms on a variety of surface habitats including gallstones (Prouty and Gunn, 2003), cement (Joseph *et al.*, 2001), Teflon, stainless steel (Austin *et al.*, 1998), plastic and glass (Romling *et al.*, 1998); the last four abiotic surfaces involve Tafi expression and the last two involve cellulose expression (Solano *et al.*, 2002; Zogaj *et al.*, 2001). Similar to biofilms produced by other microorganisms, in *Salmonella* there is strain-to-strain variability in the capability to form a biofilm and environmental conditions affect the constituents of a biofilm. For example, *Salmonella* Typhimurium SL1344 is not capable of biofilm formation in nutrient-rich medium (Lb) at RT or in nutrient-deficient medium (ATM) at 37°C. In contrast, *Salmonella* Typhimurium 14028 can form a biofilm in both of these

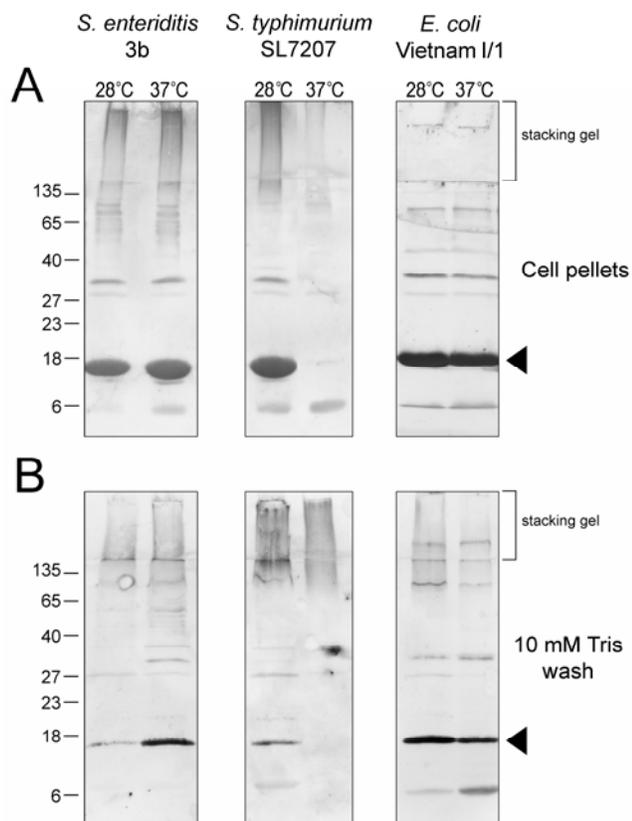
conditions. The biofilm produced in LB require genes involved in the synthesis of LPS, ECA, colanic acid, Tafi and cellulose whereas cellulose seems to be the main component of the biofilm formed when bacteria are grown in ATM (Solano *et al.*, 2002). *Salmonella* biofilms vary depending on the surface they are formed on; it has been reported that cellulose is the primary EPS formed on glass (Solano *et al.*, 2002; Zogaj *et al.*, 2001) however neither cellulose nor colanic acid were major components of biofilms formed on gallstones (Prouty and Gunn, 2003). Furthermore, the importance of flagella and LPS expression varied for *Salmonella* Typhimurium biofilms depending upon whether a biofilm formed on glass or gallstones (Prouty and Gunn, 2003). As already discussed above, *Salmonella* colanic acid is not required for biofilm formation on abiotic surfaces (Prouty and Gunn, 2003; Solano *et al.*, 2002); however, colanic acid does play a role in the 3-D architecture of the biofilm on HEp-2 cells (Ledebøer and Jones, 2005). Thus, adherence and biofilm formation may play a role in establishing persisting colonization of poultry and could also play as yet unknown roles in the *Salmonella* virulence strategy (Ledebøer and Jones, 2005). In support of this, *Salmonella* Typhimurium forms biofilms on the surface of mammalian tissue cultures (Boddicker *et al.*, 2002).

### 1.8.1 Extracellular matrix

There is a strong correlation between biofilm formation, pellicle formation, rdar (red, dry and rough) morphology, bacterial aggregation and extracellular matrix production (Allen-Vercoe *et al.*, 1997; Anriany *et al.*, 2001; Collinson *et al.*, 1993; Romling *et al.*, 1998; Romling and Rohde, 1999; Romling *et al.*, 2003; Romling, 2005; Solano *et al.*, 2002; White *et al.*, 2005). Bacteria with a rdar morphology are connected in a dense, rigid cellular network that spreads across the agar surface and can be peeled off this surface intact leaving an imprint in the agar ( Anriany *et al.*, 2001; Collinson *et al.*, 1993; Romling *et al.*, 1998; White *et al.*, 2005). These characteristics result from highly co-ordinated and organized multicellular behaviour (Romling *et al.*,

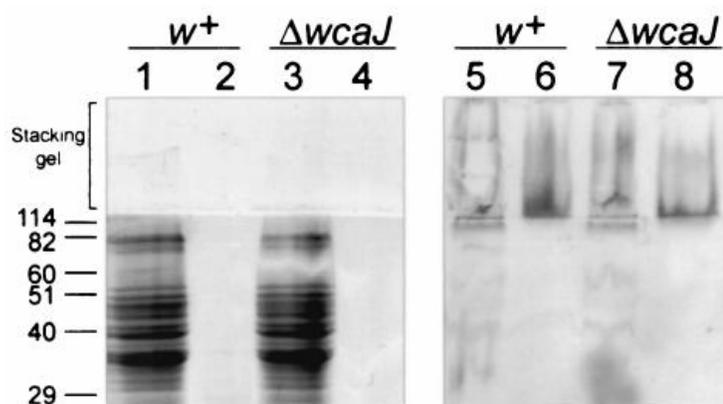
1998; Romling *et al.*, 2000; White *et al.*, 2005). Before this study, the *Salmonella* extracellular matrix was known to be composed of Tafi (Collinson *et al.*, 1993; Romling *et al.*, 1998) and cellulose (Zogaj *et al.*, 2001). Tafi organizes cells by mediating short-range cell-to-cell interactions and cellulose mediates long-range cell-to-cell interactions (Romling *et al.*, 2000). The associated rdar morphotype is highly conserved amongst *S. enterica* (Romling *et al.*, 2003; White *et al.*, 2005) and has been shown to confer resistance to USDA approved levels of chlorine which kill their planktonic counterparts (Scher *et al.*, 2005; White *et al.*, 2005). Temporal gene expression studies indicate that formation of the extracellular matrix is initiated by Tafi expression (White, 2005). During the course of this study, several researchers made observations of the presence of EPS associated with the rdar morphotype (Anriany *et al.*, 2001; Prouty and Gunn, 2003; Solano *et al.*, 2002; White *et al.*, 2003).

Previously, we found HMW immunoreactive material that appeared to be stabilized on the cell surface by cellulose and was distinct from AgfA, AgfB, and cellulose. The HMW material is present in *Salmonella* Typhimurium SL7207, and *E. coli* Vietnam I/1 (Fig. 1.12) indicating it is commonly found with Tafi in *Salmonella* and *E. coli*. Since colanic acid is common amongst *Salmonella* and *E. coli* strains (Danese *et al.*, 2000; Prigent-Combaret *et al.*, 2000) and runs as a HMW smear (Junkins and Doyle, 1992), we tested a colanic acid mutant strain (*wcaJ* mutant) but the HMW material was still present (Fig. 1.13. lanes 5 and 7), indicating that the material did not represent colanic acid. The HMW material was resistant to digestion with proteinase K (Fig. 1.13, lanes 6 and 8) and did not stain with GelCode blue (Fig. 1.13. lanes 1 to 4), indicating that it was not proteinaceous. Furthermore, the purified material tested positive for the presence of uronic acids suggesting that the HMW material represented an uncharacterized anionic EPS distinct from cellulose and colanic acid. We hypothesized that this material was a common component of the Tafi-cellulose extracellular matrix (White *et al.*, 2003).



**Figure 1.12. Detection of immunoreactive Tafi-associated material in different enterobacterial species.**

Cell pellets or acetone-precipitated proteins from a 10 mM Tris (pH 8) wash of whole cells of *Salmonella* Enteritidis 3b, *Salmonella* Typhimurium SL7207 or *E. coli* Vietnam I/1 after growth on T agar at 28 or 37°C were loaded as indicated. The brackets show the regions of immunoblots corresponding to the stacking gel from SDS-PAGE. AgfA and associated material were detected by using immune serum raised to whole Tafi; arrowheads indicate monomeric AgfA (*Salmonella*) or CsgA (*E. coli*). Molecular mass markers (in kDa) are indicated on the left. This figure was reproduced with permission from White (White *et al.*, 2003).



**Figure 1.13. Detection of HMW immunoreactive material in *Salmonella* Enteritidis 3b and  $\Delta wcaJ$  strains.**

SDS-PAGE (lanes 1 to 4) or immunoblot analysis (lanes 5 to 8) of whole cells of the *Salmonella* Enteritidis 3b ( $w^+$ ) and  $wcaJ$  strains boiled in SDS-PAGE sample buffer (lanes 1, 3, 5, and 7) and digested with 0.5 mg/mL of proteinase K for 1 h at 65°C (lanes 2, 4, 6, and 8) is shown. Proteins were detected with GelCode staining (Pierce) (lanes 1 to 4). HMW material was detected by using immune serum raised to purified Tafi (lanes 5 to 8). The bracket shows the stacking gel region of SDS-PAGE and the corresponding immunoblot. Molecular mass markers (in kDa) are indicated on the left. This figure was reproduced with permission from White (White *et al.*, 2003).

### 1.8.2 AgfD regulation of biofilm formation

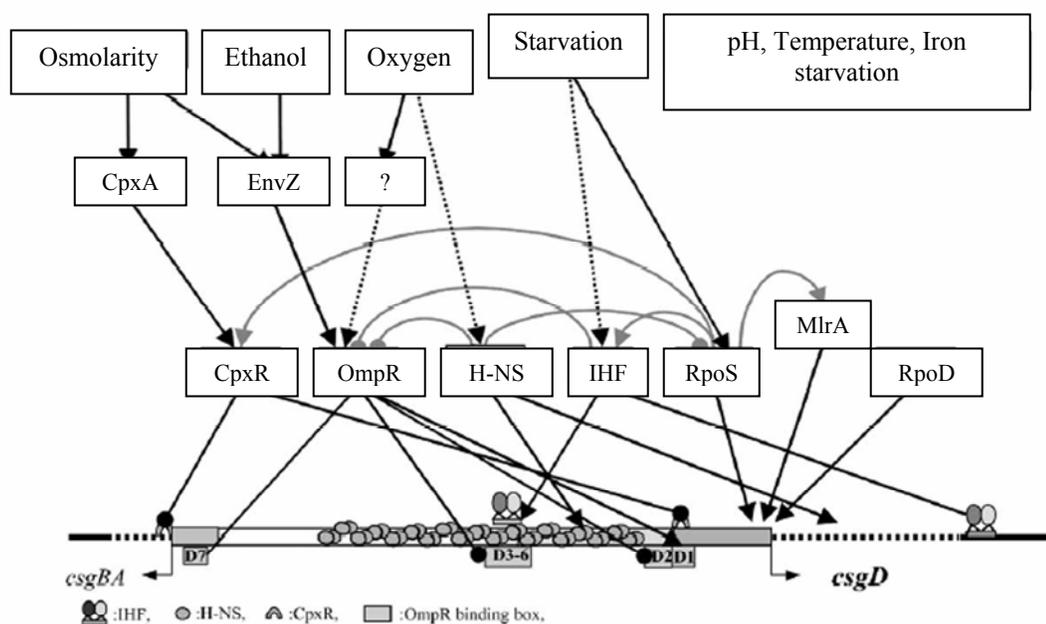
The extracellular matrix components, Tafi and cellulose are regulated by AgfD through a complex regulatory cascade and therefore AgfD is considered the regulatory checkpoint in multicellular behavior characterized by the *rdar* morphotype (Fig. 1.8.1) (Gerstel *et al.*, 2003; Gerstel and Romling, 2003). AgfD is homologous to transcriptional response regulators from the family UhpA/FixJ and is presumably activated by phosphorylation since there is a conserved aspartate in the N-terminal receiver domain (Romling *et al.*, 2000). AgfD is activated in stationary phase, limiting phosphorous and nitrogen with sufficient carbon sources, low osmolarity, pH 8.5, and 30°C or upon iron starvation at 37°C (Gerstel and Romling, 2001; Romling *et al.*, 1998). Oxygen also plays a major role in AgfD activation where maximal expression is under

microaerophilic conditions in rich media and under aerobic conditions in minimal media (Gerstel and Romling, 2001). Point mutations in the promoter region convert highly regulated expression into semi-constitutive expression where RpoS and temperature dependence is abolished and expression is maximal at pH 5.5 (Gerstel and Romling, 2001; Romling *et al.*, 1998). AgfD is further influenced by the global regulatory proteins MlrA (Brown *et al.*, 2001), RpoS (Olsen *et al.*, 1993; Romling *et al.*, 1998), OmpR (Sauer *et al.*, 2004; Vidal *et al.*, 1998), CpxR (Prigent-Combaret *et al.*, 2001), RcsB (Jubelin *et al.*, 2005), a thermosensor Crl (Arnqvist *et al.*, 1992; Bougdour *et al.*, 2004) and the architectural proteins H-NS (Arnqvist *et al.*, 1994; Olsen *et al.*, 1993) and IHF (Gerstel and Romling, 2003). Notably, biofilm formation in ATM conditions is not affected by mutations in the regulatory proteins RpoS, OmpR and AgfD suggesting that there may be an AgfD-independent pathway for cellulose production (Solano *et al.*, 2002). Also, it has been shown that out of the eight proteins predicted to synthesize c-di-GMP, only *adrA* is dependent on AgfD (Garcia *et al.*, 2004).

A putative 11 bp AgfD binding site has been predicted (CGGGKKGAKNKA; (Brombacher *et al.*, 2003) and is highly conserved amongst Tafi producing *Salmonellae* (White, 2005). In addition to *agfBAC*, the putative AgfD binding site has been observed upstream *adrA*, the product of which stimulates cellulose production via c-di-GMP as already discussed above (Romling *et al.*, 2005; Simm *et al.*, 2004). It is hypothesized that AgfD acts through different mechanisms to activate these genes since the binding sites are differentially arranged (Romling, 2005). AgfD does not regulate its own transcription (Romling *et al.*, 1998).

AgfD has been called a biofilm control point (Gerstel *et al.*, 2003). This is because in *E. coli* CsgD (AgfD), in addition to positive regulation of Tafi and cellulose, also negatively regulates genes that inhibit biofilm formation; *pepD*, encoding a broad-spectrum dipeptidase and *yagS*, encoding a molybdopterin-binding subunit of an oxidoreductase protein complex (Brombacher *et al.*, 2003). Unlike *E. coli* however, in *Salmonella* Typhimurium *pepD* does not appear to be directly regulated by CsgD

(AgfD) due to the lack of the putative DNA binding site upstream its promoter and the *yagS* is not present on the chromosome (Romling, 2005). Additionally, in *E. coli*, CsgD (AgfD) has been shown to positively regulate *glyA*, which encodes a serine hydroxymethyltransferase that is involved in glycine production and thus promotes glycine rich Tafi fimbrin, CsgA (AgfA), production (Chirwa and Herrington, 2003). Recently, a biofilm associated protein in *Salmonella* sp. was shown to be positively regulated through the action of AgfD (Latasa *et al.*, 2005). Before this study, no other AgfD regulated genes had been identified.



**Figure 1.14. AgfD regulatory network.**

The diagram depicts the 521 bp intergenic region between *agfBA(C)* and *agfD(EFG)* and the transcriptional regulators that are activated by signal transduction systems and their environmental cues influencing *agfD* transcription. OmpR-P binding at D1 is absolutely required for activation and at low OmpR levels, OmpR-P binds to a high-affinity site and stimulates *agfD* transcription, at high OmpR levels, OmpR-P binds to additional low-affinity sites that shut off *agfD* transcription. In microaerophilic conditions, IHF out competes with OmpR-P for binding at sites D3-6 leading to *agfD* activation. H-NS activates *agfD* transcription. MlrA acts directly or indirectly on the *rpoS*-dependent *agfD* promoter. Growth phase dependent RpoS is absolutely required for *agfD* transcription and Crl, a thermosensor that accumulates at 30°C, interacts directly with RpoS-holoenzyme and promotes its binding with RNAP and subsequent transcription of *agfBAC* (Bougdour *et al.*, 2004). Cells can avoid RpoS dependency with an *agfD* promoter mutation that could enhance OmpR-P binding to the high affinity site. CpxR-P represses *agfD* transcription in high salt and in *E. coli*; high sucrose represses *agfD* activation through H-NS and RcsB (Rcs system not shown; Jubelin *et al.*, 2005). Curved gray lines; protein interactions, arrows; activation, circle arrows; repression, lines; unknown, dotted arrows; hypothetical pathways. Reprinted from Gestel and Romling (2003), with permission from Elsevier.

## 2.0 HYPOTHESES, OBJECTIVES, SIGNIFICANCE AND SPECIFIC AIMS

### 2.1 Hypotheses

- (i) Tafi assembly is more complex than the proposed self-assembling extracellular mechanism.
- (ii) Tafi is associated with extracellular polysaccharides that are important for biofilm formation and environmental persistence.

### 2.2 Objectives and significance

The long-term goals of this research were: (i) to reveal fundamental principles governing the assembly of thin aggregative fimbriae (Tafi); the assembly pathway has not been elucidated but evidence indicates it is unique and it has been proposed as a model for the assembly of fibers that cause debilitating human conditions such prion related diseases and (ii) to identify and characterize Tafi associated exopolysaccharides (EPS); the EPS was anticipated to contribute to bacterial environmental and/or host persistence and could ultimately serve as targets for prevention of biofilm formation in medical settings and food industry.

### 2.3 Specific aims

The research agenda for this thesis included investigations focused on understanding the mechanisms by which Tafi are assembled by investigating the functional roles of minor Tafi components and assessing the validity of the ENP pathway of Tafi assembly by confirming the direction of fiber polymerization. Investigations were equally focused on structural, biochemical, genetic, regulatory and functional characterization of the novel *Salmonella* exopolysaccharides associated with Tafi.

### **3.0 AgfC and AgfE of the *Salmonella enterica* Enteritidis thin aggregative fimbriae operon facilitate fimbrial assembly.**

#### **3.1 Introduction**

Fimbriae are cell-surface protein polymers that mediate adhesive interactions important for a variety of fundamental microbial functions: host and environmental persistence, development of biofilms, motility, colonization and invasion of cells and conjugation (Duguid *et al.*, 1966; Low, 1996). The assembly pathways of different fimbriae are divided into four mechanisms: chaperone-usher, alternate chaperone, general secretion and extracellular nucleation-precipitation (ENP) (Soto & Hultgren, 1999). In the first three pathways, fiber growth occurs intracellularly and subunits cross the periplasm: in the case of the chaperone dependent pathways, subunits interact with a chaperone protein that caps interactive surfaces thus preventing aggregation, or in the case of the general secretion pathway, subunits pass through a putative cell envelope spanning scaffold (Burrows, 2005). The ENP pathway deviates from these systems in that fiber growth is proposed to occur extracellularly (Hammar *et al.*, 1996). It is unknown how the subunits cross the periplasm.

Thin aggregative fimbriae (Tafi) are the only fimbrial system dependent on the ENP pathway. Tafi are known as curli because in the absence of extracellular polysaccharides their morphology are curled; however, when co-expressed with extracellular polysaccharides (EPS) such as cellulose and O-Antigen capsule their morphology appears as a tangled amorphous matrix (White *et al.*, 2003). Tafi and EPS form the extracellular matrix that produces a colony morphotype that appears red, dry and rough (rdar) on Congo red agar (Romling *et al.*, 2000; White *et al.*, 2003) and is highly conserved in most *Salmonella* and *E. coli* strains as well as other members of the *Enterobacteriaceae* family (Arnqvist *et al.*, 1992; Collinson *et al.*, 1991; Collinson *et al.*, 1992; Doran *et al.*, 1993; Gerstel & Romling, 2003; White *et al.*, 2006; Zogaj *et al.*, 2003). Tafi are essential for the formation of the extracellular matrix (White *et al.*,

2006) which is involved in multicellular aggregation (Romling *et al.*, 2000), pellicle formation (Collinson *et al.*, 1993), biofilm formation (Austin *et al.*, 1998; Gerstel & Romling, 2003; Prigent-Combaret *et al.*, 2000; Vidal *et al.*, 1998), environmental persistence (White *et al.*, 2006) and the binding of plant tissues (Barak *et al.*, 2005). Tafi also have pathogenesis-related roles in that they accelerate amyloidosis in mice (Lundmark *et al.*, 2005), bind fibronectin (Arnqvist *et al.*, 1992; Collinson *et al.*, 1993; Olsen *et al.*, 2002), are proinflammatory (Bian *et al.*, 2000; Bian *et al.*, 2001; Persson *et al.*, 2003; Tukul *et al.*, 2005), enhance adherence to eukaryotic cells (Dibb-Fuller *et al.*, 1999; Kim & Kim, 2004; La Ragione *et al.*, 2000; Sukupolvi *et al.*, 1997), and mediate invasion of eukaryotic cells (Gophna *et al.*, 2001; Gophna *et al.*, 2002; Uhlich *et al.*, 2002).

The genes involved in Tafi production are organized into two adjacent divergently transcribed operons, *agfBAC* and *agfDEFG* (Collinson *et al.*, 1996). Both operons are required for biosynthesis and assembly (Collinson *et al.*, 1993). *agfA* encodes the major fimbrial subunit (Collinson *et al.*, 1991). *agfB* encodes the minor fimbrial subunit which is cell associated and required to nucleate polymerization of AgfA into insoluble Tafi fibers (Bian & Normark, 1997; White *et al.*, 2001). *agfD* encodes a transcriptional regulator that positively regulates the expression of Tafi (Romling *et al.*, 1998), cellulose (Romling *et al.*, 2000) serine hydroxymethyltransferase (Chirwa & Herrington, 2003) and O-Antigen capsule (Gibson, *et al.*, submitted) and negatively regulates factors that inhibit biofilm formation (Brombacher *et al.*, 2003). The *agfG,F,E* and *C* gene products have not been previously studied in *Salmonella* species but are homologous to *csgG,F E* and *C* in *E. coli*. *csgG* encodes an outer membrane lipoprotein required for fimbrin secretion and stabilization (Chapman *et al.*, 2002; Loferer *et al.*, 1997) and assembles into an oligomeric complex with a central pore (Robinson *et al.*, 2006). *csgEF* encode proteins that interact with the CsgG complex (Robinson *et al.*, 2006) yet have uncharacterized roles in assembly where CsgE has been called chaperone-like (Chapman *et al.*, 2002). The *agfC* (*csgC*) gene had not been

previously characterized and has been referred to as *orfC* due to the lack of evidence for transcription or a distinct phenotype accompanying inactivation (Collinson *et al.*, 1996; Hammar *et al.*, 1995).

The unique ENP pathway leading to Tafi formation is anticipated to be used as a model for the assembly of amyloid fibers (Chapman *et al.*, 2002) and therefore any contribution to their assembly details may prove helpful in the understanding of the associated disorders such as prion-related and Alzheimer's diseases. In this model, surface localized AgfB subunits nucleate the polymerization of AgfA subunits into insoluble surface fibers, which are proposed to spontaneously elongate from the free distal end (Hammar *et al.*, 1996). The evidence in support of this model is based on intercellular complementation between donor and recipient cells in LPS O-polysaccharide deficient *E. coli* K12 (Hammar *et al.*, 1996). In *Salmonella* intercellular complementation has also been demonstrated but only in LPS O-polysaccharide deficient mutants (White *et al.*, 2003). Therefore the significance of intercellular complementation in Tafi expressing strains with *wt* LPS and other extracellular polysaccharides seems uncertain (White *et al.*, 2003).

The purpose of this study was to investigate Tafi assembly through examination of the minor assembly factors led by the discovery of an active *agfC* gene. The conservation of *agfC* with *agfBA* throughout *Salmonella*, *E. coli* and even in *Shigella*, where the operon has been inactivated (Sakellaris *et al.*, 2000) suggests that *agfC* is functionally important. However, detection of *agfC* (*csgC*) transcripts (Collinson *et al.*, 1996; Hammar *et al.*, 1995) or assignment of a biological role to the product of *agfC* has not been reported previously. The predicted amino acid sequence of AgfC has no known motifs, domains, or homologues. In this study, we have shown *agfC* to be co-transcribed with *agfBA* and found that AgfC plays a role in the extracellular assembly of AgfA subunits into Tafi fibers.

## 3.2 Material and methods

### 3.2.1 Bacterial strains, growth conditions and culture media

*Salmonella* Enteritidis 27655-3b (Feutrier *et al.*, 1986) was routinely grown at 37°C for 24 h on T agar (Collinson *et al.*, 1991) unless stated otherwise. *E. coli* BL21(DE3) (Stratagene) harboring pET44(c) (Novagen) carrying an inserted sequence of *agfC* fused to the plasmid encoded C-terminal hexa-histidine tag (*agfC-his*) was grown at 37°C for 18 h with agitation in 1.2% tryptone, 2.4% bacto-yeast extract and 0.4% glycerol broth supplemented with 100 µg/mL ampicillin. *Salmonella* Enteritidis 3b  $\Delta$ *agfC* harboring pARA-A5 (Mayer, 1995) carrying *agfC-his* was grown at 37°C for 18 h with agitation in 1% tryptone broth (pH 7.2) for cell localizations or T agar for TEM experiments. Media were supplemented with 100 µg/mL ampicillin and 0.5% L-arabinose. *E. coli* XL-1 Blue (Stratagene) harboring pBCKS (Stratagene), pHAG (Collinson *et al.*, 1996), pGEM® - T Easy (Promega) or pHSG415 (Hashimoto-Gotoh *et al.*, 1981) was grown at 28 or 37°C for 24 or 48 h with agitation in LB broth supplemented with 50 µg/mL chloramphenicol or 100 µg/mL ampicillin and 40 mg/mL X-gal in addition to 1 mM IPTG when required.

### 3.2.2 RT-PCR

Cellular RNA was stabilized with RNA Protect Bacteria Reagent™ (Qiagen). Cells were incubated with 0.4 mg/mL lysozyme in the presence of Superase™ (Invitrogen) and lysed using a Qias shredder™. RNA was extracted using a RNA extraction kit™ (Qiagen) and contaminating DNA was digested with DNase I (Qiagen) by the method of Wang *et al.* (2002a). RT-PCR was carried out using Sensiscript™ reverse transcriptase (Qiagen) as recommended by the manufacturer. The RT-PCR primers used are listed in Table 3.II.

### 3.2.3 Expression and purification of hexa-histidine tagged AgfC

*agfC* was PCR-amplified from *Salmonella* Enteritidis 3b chromosomal DNA using primers listed in Table 3.II and fused to a C-terminal hexa-histidine tag by cloning into pET44(c). *E.coli* BL21(DE3) cells harboring pET44(c)::*agfC-his* were grown to  $A_{600}$  0.6 and induced to produce soluble protein by the addition of 0.1 mM IPTG at 30°C for 18 h with agitation or insoluble protein by addition of 1 mM IPTG at 37°C for 4 h with agitation. Protein was recovered from cells and purified using a 50% Ni-NTA slurry (Qiagen) as recommended by the manufacturer.

### 3.2.4 Production of polyclonal serum against hexa-histidine tagged AgfC

Recombinant hexa-histidine tagged AgfC (AgfC-his) was gel purified and used to immunize a New Zealand white rabbit. Subcutaneous and intramuscular injections of 200 µg of AgfC-his prepared in Emulsigen™ adjuvant (MPV Laboratories) were performed three times at two-week intervals with a final boost of 100 µg of AgfC-his in adjuvant. Three weeks following the final booster injection, serum was collected and the antibody titre was determined by ELISA using affinity-purified recombinant AgfC-his antigen.

### 3.2.5 Cellular localization

*Salmonella* Enteritidis 3b  $\Delta$ *agfC* harboring pARA::*agfC-his* was grown to  $A_{600}$  0.6 and gene expression was induced at 37°C for 3 h with agitation. Cells were subjected to osmotic shock according to the method of Sweet *et al* (1979). Cellular fractions were separated following protocols from Sambrook and Russell (Sambrook, 2001). Samples were analyzed by SDS-PAGE and western blotting using serum generated against AgfC-his or against cytoplasmic isoleucine t-RNA synthase (control).

### 3.2.6 N-terminal sequencing of AgfC-his

Proteins in periplasmic osmotic shock samples were precipitated in 10% trichloroacetic acid / acetone mixture overnight at 4°C, washed in ice cold acetone, centrifuged (16000 X g, 10 min) and allowed to air dry at RT for 1 h. The sample was solubilized in 90% FA, lyophilized, resuspended in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to a PVDF membrane (Biorad) and stained with Sypro™ Ruby protein blot stain (Molecular Probes). Approximately 100 ng of AgfC was cut out and 8 cycles of Edman degradation were performed.

### 3.2.7 Cellulose binding assay

Avicel micro-crystalline cellulose (MCC) was obtained from Sigma-Aldrich and non-crystalline cellulose (NC) was synthesized following the procedure of Boraston *et al.* (Boraston *et al.*, 2001). The cellulose-binding assay utilized 100 µg protein and 100 mg MCC or NC in 1 mL of PBS. The family 17 cellulose binding module (CBM17) from *Clostridium cellulovorans* cellulose 5A (Boraston *et al.*, 2000) was used as a positive control for cellulose binding and bovine serum albumin (BSA; Sigma-Aldrich) was used as a negative control. The cellulose and protein slurry was mixed at 4°C for 1.5 h using a rotary shaker before transferring to a 1 mL T-1000 pipette tip (Axygen) blocked with glass wool. The flow through fraction was collected. The column was washed three times with 1 mL PBS and each fraction was collected. Bound protein was eluted with SDS-PAGE sample buffer. All fractions were resolved by SDS-PAGE and analyzed by Gelcode™ protein staining.

### 3.2.8 Generation of *Salmonella* Enteritidis 3b mutant strains

Overlap-extension PCR (Horton *et al.*, 1989) was used to generate deletion constructs for *agfC* (72 bp deletion), *agfE* (72bp deletion) and *agfF* (101 bp deletion) containing six-frame translational stop codons at the beginning of the gene and an *agfBAC* construct with the putative stem-loop structure between *agfBA* and *agfC*

replaced with random sequence (*agf-SL*). Gene products were either cloned directly into pHSG415 (*EcoRI* and *HindIII*) or subcloned from pGEM® - T Easy (*PstI* and *HindIII*). Wild type *agfC*, *agfE*, *agfF* or *agfSL* in *Salmonella* Enteritidis 3b or *Salmonella* Enteritidis 3b  $\Delta$ *bcsA* (White *et al.*, 2003) was replaced with the appropriate deletion construct according to the method of White *et al.* (1999). PCR was used to screen for deletion strains. Genotypes in final strains were confirmed by sequencing DNA regions of interest that were PCR-amplified from the chromosome. The PCR primers used are listed in Table 3.II. *Salmonella* Enteritidis 3b *fliC::Tn10dCm* is described elsewhere (Gibson, submitted).

### 3.2.9 Recombinant DNA Techniques

Purified plasmids were electroporated into *Salmonella* Enteritidis 3b or *E. coli* strains using standard techniques (Gene pulser electroprotocol, BioRad). Recombinant plasmids were purified using QIAprep™ spin kits (Qiagen). Restriction enzyme digestions (New England Biolabs) and ligation reactions (Gibco-BRL) were carried out as recommended by the manufacturers. DNA fragments were purified from agarose gels using Qiaquick Gel Extraction™ Kits (Qiagen). PCR were carried out using Taq DNA polymerase (Boehringer Mannheim) or Proofstart® DNA Polymerase (Qiagen) in buffer supplied by the manufacturer. Thermocycling was carried out in a PTC-100™ Programmable Thermal Controller (MJ Research Inc.). All RT and PCR primers were purchased from Alpha DNA ([www.alphadna.com](http://www.alphadna.com)).

### 3.2.10 Transmission Electron Microscopy

Cells were resuspended in 10 mM Tris-HCl (pH 8.0), placed onto 0.3% Formvar-coated 200 mesh copper grids and negatively stained with 2% UA (pH 7) for 15 s. Samples were visualized with a Hitachi H7600 TEM under HC-zoom mode at 100 kV.

### 3.2.11 Purification of aberrant Tafi from *Salmonella* Enteritidis 3b $\Delta bcsA\Delta agfC$

Fimbriae from *Salmonella* Enteritidis 3b  $\Delta bcsA\Delta agfC$  and *Salmonella* Enteritidis 3b  $\Delta bcsA$  strains were isolated and purified as previously described (Collinson *et al.*, 1991). Briefly, cells were resuspended in 10 mM Tris-HCl (pH 7.2), vortexed (3 X 1 min) at medium speed and harvested by centrifugation (6000 X g, 10 min). The supernatant samples were lyophilized, resuspended in 750  $\mu$ l SDS-PAGE sample buffer and boiled for 10 min before electrophoresis (5 hrs at 150 V). The SDS-insoluble material that did not enter the stacking gel was recovered from the well, washed in dH<sub>2</sub>O, resuspended in 90% FA and lyophilized. This material was resuspended in 750  $\mu$ l SDS-PAGE sample buffer and electrophoresed a second time. The FA-insoluble material that did not enter the stacking gel was recovered from the well, resuspended in 6N HCl, microwaved for 5 s at high power (900 W), resuspended in SDS-PAGE sample buffer and electrophoresed a third time as described below.

### 3.2.12 Hydrophobicity assay

The protocol was carried out according to the method of Rosenberg (Rosenberg, 1980). Cells were washed, resuspended and adjusted to A<sub>400</sub> 0.6 in 13.6 mM sodium triphosphate buffer (pH 7.0). Cells (4 mL) were aliquoted into acid washed test tubes and overlaid with 1 mL of n-octane. The suspension was mixed vigorously for 1 min by vortexing. The immiscible phases were allowed to separate for 15 min, the aqueous phase was removed and the A<sub>400</sub> was measured. Taking the decrease in A<sub>400</sub> of the aqueous phase and dividing by the initial A<sub>400</sub> calculated the percent hydrophobicity.

### 3.2.13 Antibody capture of Tafi subunits in *Salmonella* Enteritidis 3b $\Delta bcsA$ strains

*Salmonella* Enteritidis 3b  $\Delta bcsA$  strains were grown at 37°C for 20 h with agitation in media supplemented with 1% tryptone (Difco) at pH 7.2 and 30% polyclonal rabbit serum specific to AgfA (Collinson *et al.*, 1991), AgfB (White *et al.*, 2001) or fetal calf serum (negative control). For SDS-PAGE and immunoblotting, cultures were

adjusted to  $A_{600}$  1 in binding buffer (20 mM  $\text{Na}_2\text{HPO}_4$ , 50 mM Tris-HCl at pH 7.2), centrifuged (6000 X g, 10 min), resuspended in 250  $\mu\text{l}$  of 90% FA or  $\text{dH}_2\text{O}$  and lyophilized. The supernatant was incubated with 100  $\mu\text{l}$  of Protein A Sepharose™ 4 Fast Flow 50% slurry (Amersham Biosciences) at 4°C for 1 h on a rotary shaker. Protein A beads were recovered by centrifugation (16000 X g, 10 min) and remaining supernatant proteins were precipitated by addition of acetone (Pohl, 1990). FA-treated samples were loaded directly onto SDS-PAGE whereas all other samples were boiled for 5 min before loading. ELISA experiments were performed according to the method of Engvall (1976) in high-binding, flat bottom, polystyrene 96-well plates (Costar). Cells were harvested (6000 X g, 10 min), washed twice in 1 mL 100 mM glycine (pH 2.5), washed twice in 1 mL phosphate buffered saline (pH 7.4) (PBS), adjusted to  $A_{600}$  0.1 in each well and heat fixed at 90°C for 4 h. AgfA or AgfB were detected using AgfA-specific monoclonal antibody ascites or AgfB-specific polyclonal serum followed by goat-anti-mouse or rabbit immunoglobulin G-alkaline phosphatase conjugates (Cedarlane Laboratories Ltd.).

### 3.2.14 SDS-PAGE, protein staining and immunoblotting

Cells were resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0) and adjusted to  $A_{600}$  0.1 (for protein staining) or  $A_{600}$  1 (for immunoblotting) and 1 mL aliquots were vortexed (3 X 1 min) to shear off cell surface material. The cells were harvested by centrifugation (6000 X g, 10 min), resuspended in SDS-PAGE sample buffer and boiled for 10 min before electrophoresis. Proteins in supernatant fractions were precipitated with acetone and resuspended in SDS-PAGE sample buffer. Tafi fimbrial protein samples were prepared as previously described (White *et al.*, 2003). SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) with a 5% stacking gel and 12% resolving gel. Proteins separated by SDS-PAGE were either protein stained with Gelcode™ (Bio-Rad) or electrophoretically transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell™ (Bio-Rad Laboratories) in buffer

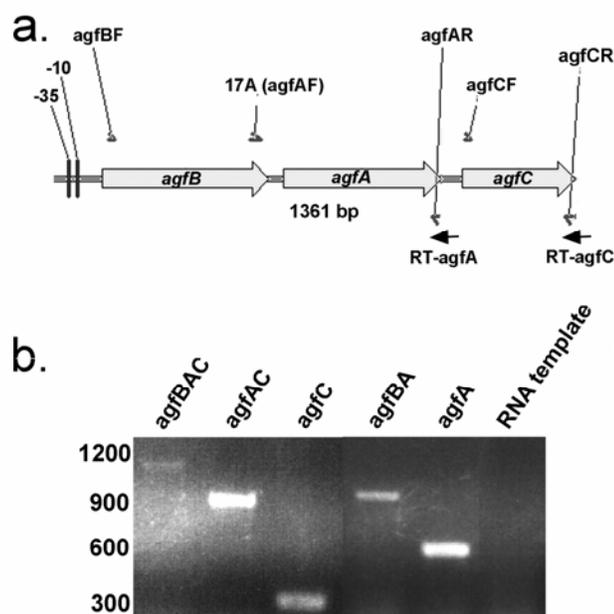
recommended by the manufacturer. Proteins were detected using immune serum specific to AgfA, AgfB, flagella (*Salmonella* H Antisera, Difco), or AgfC-his followed by goat-anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR, Ltd.). Immunoreactive material was visualized using an Osysey™ Biosciences scanner (LI-COR, Ltd.).

### 3.3 Results

#### 3.3.1 *agfC* is co-transcribed with *agfBA*

To detect *agfC* transcripts, RT-PCR was performed using *agf*-specific primers (Fig. 1a) and Sensiscript™ reverse transcriptase (Qiagen). PCR products corresponding to *agfBAC*, *agfAC* and *agfC* were amplified using gene-specific forward primers and an *agfC* reverse primer (Fig. 3.1b). The results indicated that *agfC* is co-transcribed with *agfBA* as a polycistronic transcript. We believe that *agfC* is transcribed in low amounts because 1) transcripts were detected using Sensiscript™, a reverse transcriptase preparation that can detect as few as 1-4 transcripts/cell, 2) transcripts were not detected using a less sensitive reverse transcriptase (Omniscript™, Qiagen; data not shown) and 3) previous studies did not detect *agfC* transcripts by Northern blotting (Collinson *et al.*, 1996; Hammar *et al.*, 1995).

To investigate whether expression of AgfC was inhibited by a RNA stem-loop structure immediately upstream of the *agfC* start codon (Collinson *et al.*, 1996), the stem-loop was replaced in phase with a random sequence. We still could not detect AgfC within the different cell fractions, suggesting that there wasn't a large increase in expression. Removal of the stem-loop did, however, lead to decreased immunological levels of AgfA and a decrease in Congo red binding to cells (data not shown). This indicated that the stem-loop structure most likely plays a role in stabilizing *agfA* mRNA and does not interfere with *agfC* transcription.



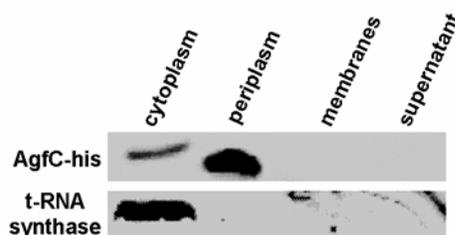
### Figure 3.1. Reverse transcriptase (RT)-PCR of *agfC*.

(a) Schematic of primer binding sites in the *agfBAC* operon. The -10 and -35 promoter regions for *agfB* are shown; R = reverse primers used in PCR; F = forward primers used in PCR; RT = reverse transcription primers used to generate cDNA. (b) PCR amplifications of *agfBAC* cDNA were performed using primers agfBF (*agfBAC*), agfAF (*agfAC*) or agfCF (*agfC*) with agfCR, agfBF and agfAR (*agfBA*), agfAF and agfAR (*agfA*). RNA template after DNase digestion was used for negative control PCR reactions. Size of DNA bands (in bp) are indicated on the left of the 2% agarose gel.

#### 3.3.2 Cell localization of AgfC

Native AgfC could not be detected in *wt* cell fractions using high titre polyclonal immune serum raised against AgfC-his. However, when *agfC-his* was expressed *in trans* in an  $\Delta agfC$  strain, AgfC-his was localized to the periplasm (Fig. 3.2). In addition, minor amounts of AgfC-his were detected in the cytoplasm presumably due to over-expression. Periplasmic AgfC-his had an apparent molecular mass of 13.4 kDa, whereas cytoplasmic AgfC-his had an apparent molecular mass of 14.4 kDa. N-

terminal sequencing of the periplasmic form of AgfC-his confirmed that it was cleaved between residue 8 and 9 generating a mature 100 amino acid protein. This indicated that AgfC could be processed and translocated into the periplasm, suggesting this was the normal cellular location. In addition, these results suggested that native AgfC was only present in small amounts.



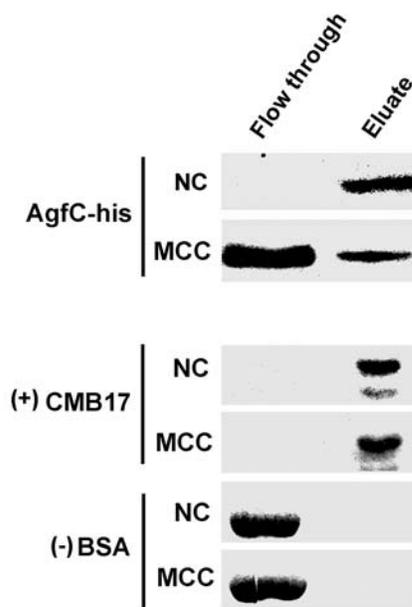
**Figure 3.2. Immunoblot analysis of AgfC in cellular fractions.**

*Salmonella* Enteritidis 3b  $\Delta agfC$  with AgfC-his expressed *in trans* was subjected to osmotic shock and cellular fractions were separated. Proteins were electrophoresed in SDS and analyzed by immunoblotting using polyclonal serum generated against AgfC-his or t-RNA synthase (control).

### 3.3.3 Purified histidine tagged AgfC binds non-crystalline cellulose

To assess the functional role of AgfC, a hexa-histidine tagged AgfC (AgfC-his) construct was expressed in an *E. coli* host and purified by affinity chromatography. At one step during the purification procedure, the AgfC-his protein was found to bind to a regenerated cellulose column. Since Tafi is co-expressed with cellulose under normal physiological conditions, we wanted to determine if cellulose binding of AgfC was specific. Importantly, the hexa-histidine tag does not affect cellulose binding (McLean

*et al.*, 2000). The ability of AgfC to bind either NC or Avicel™ MCC was compared to CBM17, a protein module known to bind cellulose (Boraston *et al.*, 2003). CBM17 was detected in the eluate of both NC and MCC, whereas AgfC was mostly detected in the NC column eluate (Fig. 3.3). This indicated that AgfC had binding affinity for the non-crystalline regions of cellulose. Bovine serum albumin was used as a negative control and did not bind to either cellulose column.



**Figure 3.3. SDS-PAGE analysis of a cellulose binding assay of AgfC-his.**

Purified AgfC-his was incubated with NC or MCC. The cellulose protein slurry was immobilized on a column, washed and bound protein was eluted with SDS-PAGE sample buffer. Fractions were analyzed by SDS-PAGE and protein staining. Results are representative from least three independent expts. CBM17, which has specific affinity to the non-crystalline regions of cellulose, was used as a positive control. BSA was used as a negative control.

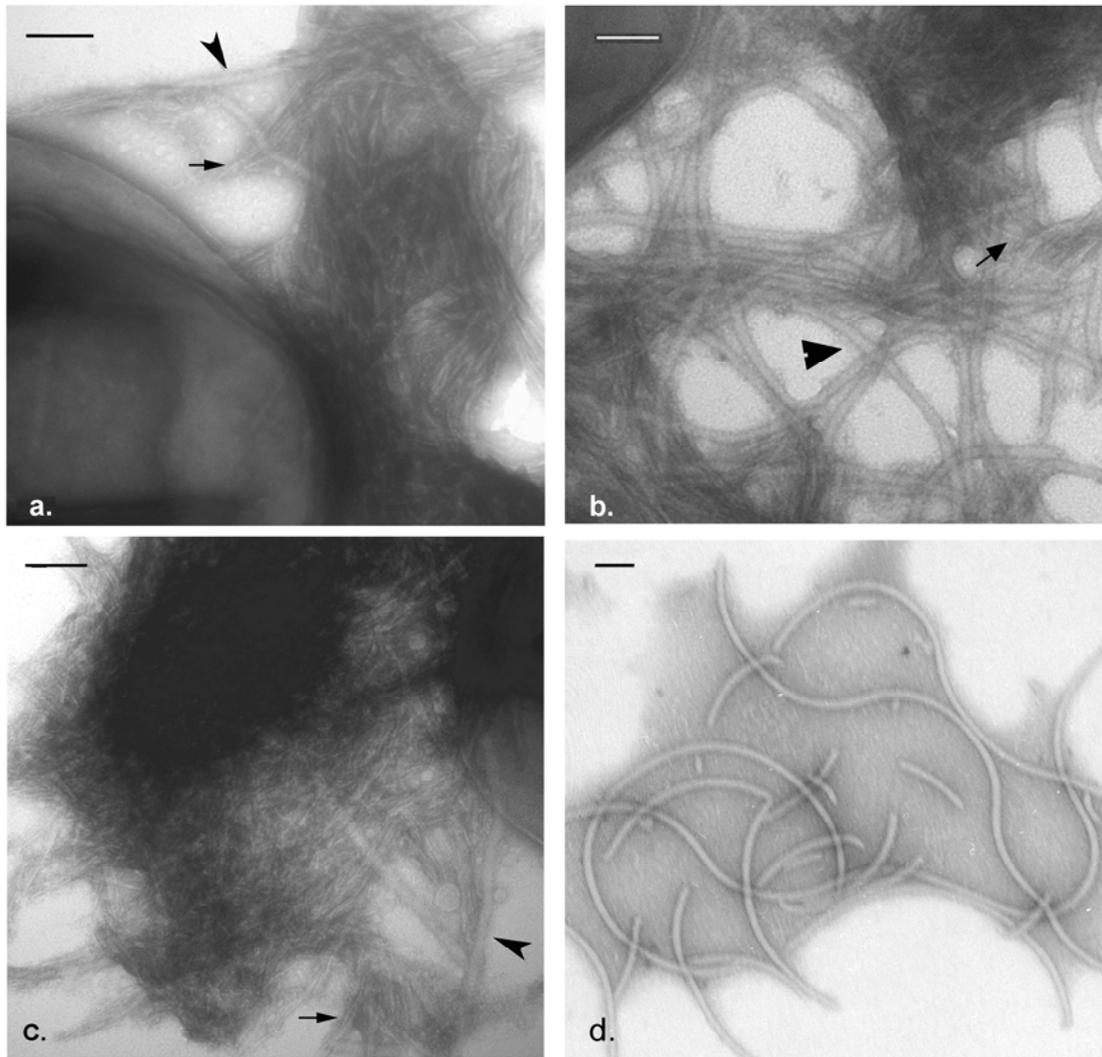
### 3.3.4 The *Salmonella* Enteritidis 3b $\Delta agfC$ mutant expressed an aberrant form of Tafi

When *wt* and  $\Delta agfC$  strains were compared by transmission electron microscopy (TEM), large, 20 nm fibers were consistently more abundant on the cell surface of the  $\Delta agfC$  mutant (Fig. 3. 4a, b). In contrast to normal Tafi (5-7 nm), the 20 nm fibers were not recognized by an AgfA-specific monoclonal antibody (data not shown). To confirm that the appearance of 20 nm fibers was due to the lack of AgfC expression, *agfC* was expressed *in trans* in the  $\Delta agfC$  strain. TEM examinations of the complemented strain (Fig. 3. 4c) appeared similar to *wt* where there were far fewer 20 nm fibers on the cell surface.

To eliminate the possibility of the 20 nm fibers as flagella fragments, supernatant samples containing sheared off surface components from the *wt* and the  $\Delta agfC$  mutant were compared by SDS-PAGE and immunoblotting (Fig. 3. 4a). Comparisons of these samples did not reveal any differences in amounts of flagellin between the strains. Furthermore, comparison of protein profiles from whole cell lysates of the *wt* and the  $\Delta agfC$  mutant did not reveal any differences between the strains (data not shown). Morphological appearance of the 20 nm fibers was similar in both cellulose<sup>+</sup> ( $\Delta agfC$ ) and cellulose<sup>-</sup> ( $\Delta bcsA\Delta agfC$ ) strains by TEM examinations (data not shown). To determine if the 20 nm fibers were related to Tafi, we purified them from the cell surface of cellulose-deficient ( $\Delta bcsA$ ) *wt* and  $\Delta agfC$  strains by the method of Collinson *et al.* (Collinson *et al.*, 1991). After SDS-PAGE, SDS-insoluble material that did not enter the gel was recovered from the wells of both the *wt* and  $\Delta agfC$  samples. The material recovered from the  $\Delta agfC$  mutant was enriched with the 20 nm fibers (Fig. 3. 3d). When the SDS-insoluble material was treated with 90 % formic acid (FA), Tafi were completely depolymerized and AgfA could be resolved and detected after electrophoresis (Fig. 3. 4b, +FA). However, in samples from the  $\Delta agfC$  mutant, a greater amount of FA-insoluble material, relative to the *wt*, remained in the well. This material was recovered and solubilized in 6 N HCl by microwave treatment for five seconds. Subsequent SDS-PAGE and immunoblot analysis revealed a 16.5 kDa

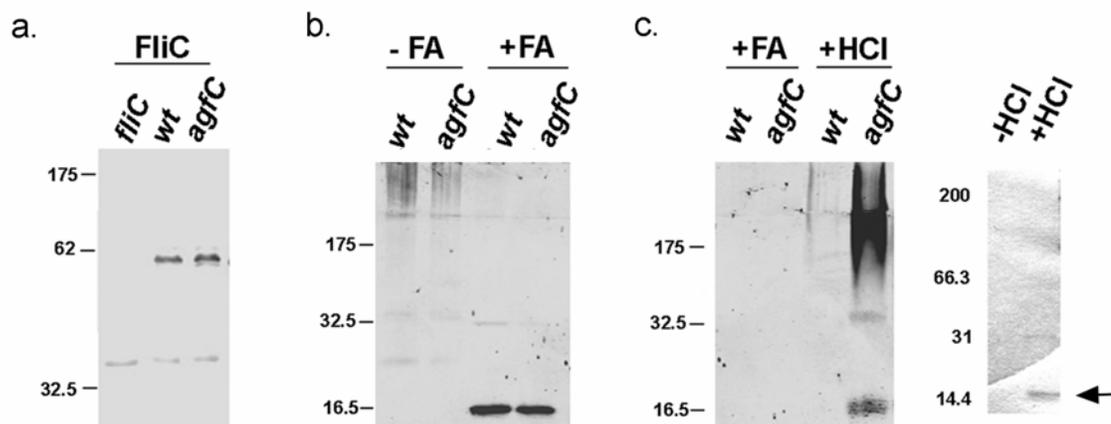
protein band and some higher molecular oligomers that were recognized by polyclonal serum generated to whole Tafi (Fig. 3. 4c, + HCl). No corresponding bands were detected in samples from the *wt* strain (Fig. 3. 4c) indicating that the 20 nm fibers observed on *wt* cells (Fig. 3. 3a) probably represented flagella.

Analysis of the 16.5 kDa protein band from  $\Delta agfC$  (Fig. 3. 4c, +HCl) by MALDI-TOF mass spectrometry yielded only peptides that matched AgfA sequence. This indicated that the SDS- and FA-insoluble 20 nm fibers were comprised only of AgfA. We named them aberrant Tafi fibers, since regular Tafi are only 5-7 nm in diameter (Collinson *et al.*, 1991). AgfA monomers derived from the aberrant Tafi fibers were no longer recognized on immunoblots using an AgfA-specific Mab that recognizes a conformational epitope (data not shown). This suggested that the AgfA subunits from the 20 nm fibers were structurally altered. Alternatively, the epitope may have been destroyed by the HCl treatment. AgfB was not detected by MALDI-TOF mass spectrometry or western blotting with AgfB-specific polyclonal serum in the HCl-treated material indicating that AgfA was likely the sole subunit of the 20 nm fibers (data not shown). These results indicate that the  $\Delta agfC$  mutant produces an aberrant population of unusually stable Tafi comprised of only AgfA.



**Figure 3. 4. Transmission electron micrographs of *Salmonella* Enteritidis 3b cell surface fibers.**

Cell preparations of *wt* (a),  $\Delta agfC$  mutant (b),  $\Delta agfC$  mutant with AgfC-his expressed *in trans* (c), and semi-purified 20 nm fibers from the  $\Delta agfC$  mutant (d) were negatively stained with uranyl acetate. Arrowheads indicate 20 nm fibers and arrows indicate 5 - 7 nm Tafi fibers. (Bars represent 100 nm). At least 5 independent experiments with at least 10 images of each strain were generated. Representative images are shown.



**Figure 3.5. SDS-PAGE and immunoblot analysis comparing cellular proteins in *Salmonella* Enteritidis 3b and the  $\Delta agfC$  mutant**

Supernatant samples containing sheared off surface components were subjected to electrophoresis and analyzed by protein staining or immunoblotting. Molecular mass markers (in kDa) are indicated on the left of each gel or immunoblot.

(a) The relative levels of flagellin were compared by immunoblotting using polyclonal serum generated against FliC (Difco). A *fliC* mutant was included as a control.

(b) SDS-insoluble material (SDS-IM) that did not enter the polyacrylamide gel was recovered from the well after electrophoresis and loaded onto a new polyacrylamide gel and electrophoresed a second time (-FA) or pre-treated with 90% formic acid to depolymerize Tafi (+FA) prior to electrophoresis. AgfA was detected on corresponding immunoblots using polyclonal serum generated against whole Tafi.

(c) Formic acid-insoluble material (FA-IM) from the  $\Delta agfC$  mutant recovered from the well after the second electrophoresis (b; +FA) was heated in 6 N HCl, electrophoresed a third time and analyzed by immunoblotting using polyclonal serum generated against AgfA (left panel) or protein staining (right panel). The arrow indicates the protein band that was excised and treated with trypsin to generate peptides for analysis by MALDI-TOF mass spectrometry.

### 3.3.5 The cell surface of *Salmonella* Enteritidis 3b $\Delta agfC$ is more hydrophobic relative to the *wt*

The consequence of aberrant Tafi expression in the  $\Delta agfC$  mutant was examined by comparing the cell hydrophobicity of the *wt* and the  $\Delta agfC$  mutant using the BATH

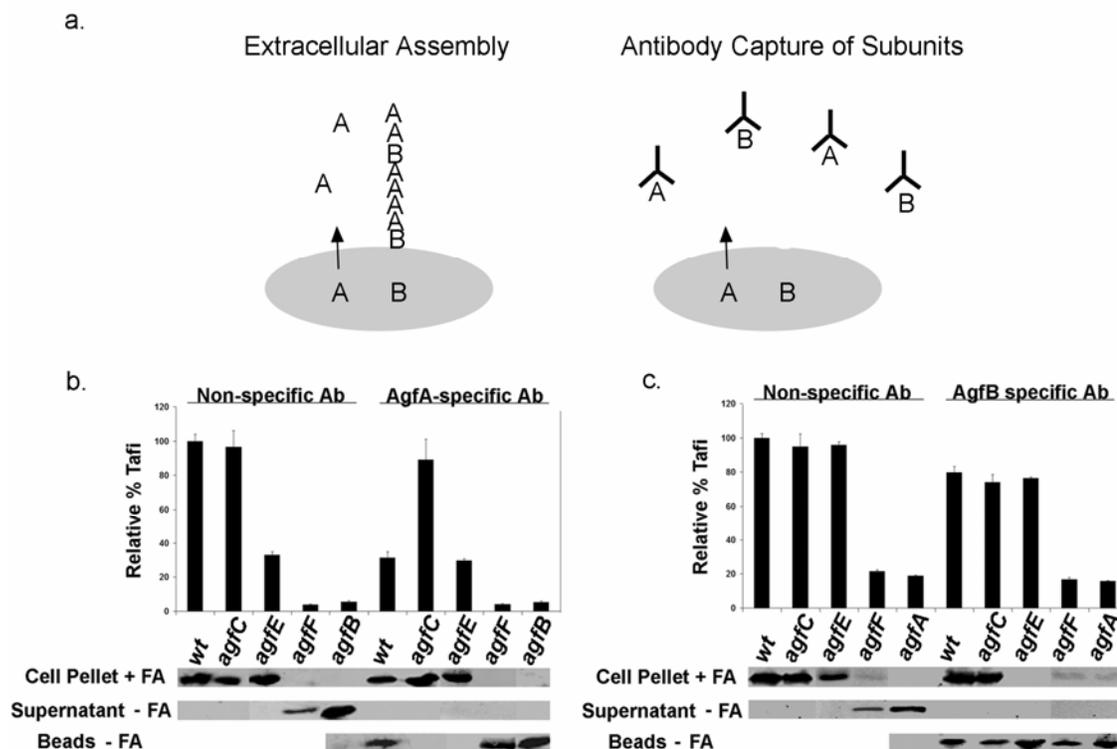
test (Rosenberg, 1980). This test is based on the degree of general affinity of the cells for a liquid hydrocarbon, n-octane. The averaged value generated from three independent experiments for the  $\Delta agfC$  mutant was  $65.3\% + 1.8/- 2.3$  whereas the *wt* was  $46.2\% + 4.3/- 3.2$ . This demonstrated that the cell surface of the  $\Delta agfC$  mutant was  $\sim 20\%$  more hydrophobic than that of the *wt*, presumably due to the altered morphology of Tafi.

### 3.3.6 AgfC and AgfE are Tafi assembly factors

Assembly of Tafi has been proposed to occur at the cell surface by addition of AgfA and AgfB monomers to the distal end of each filament. Since aberrant Tafi fibers were produced in the  $\Delta agfC$  mutant, this suggested the assembly process was altered in some way and we therefore wanted to investigate Tafi assembly in more detail. Strains with in-frame deletions in *agfE* and *agfF*, two proposed Tafi assembly factors (Chapman *et al.*, 2002), were generated to facilitate comparisons with the  $\Delta agfC$  mutant. The  $\Delta agfB$  and  $\Delta agfA$  mutants have been described previously (White *et al.*, 2001). To determine if subunits were accessible to specific antibodies, *wt* and mutant strains were grown in the presence of AgfA- or AgfB-specific immune serum. If the subunits are secreted as soluble monomers as proposed in the ENP pathway, we reasoned that specific antibodies would capture a proportion of the subunits before fiber polymerization on the cell surface (Fig 5a). After growth, antibody complexes were recovered from the supernatant using a pull-down assay with Protein A-Sepharose™ and analyzed by immunoblotting. The amounts of AgfA or AgfB subunits not captured by antibodies but incorporated into polymerized Tafi at the cell surface were measured by ELISA and quantified.

In the *wt* strain grown without serum, AgfA and AgfB were only detected in the cell-associated, polymerized form and required treatment with FA to depolymerize so as to enter SDS-PAGE as expected (Fig. 3. 5, Cell Pellet + FA). In the  $\Delta agfB$  and  $\Delta agfA$  control strains, AgfA and AgfB, respectively, were detected as soluble proteins in the

supernatants (Fig. 3. 5, Supernatant – FA). When *wt* cells were grown in the presence of AgfA- or AgfB-specific serum a proportion of soluble AgfA or AgfB was captured (Fig. 3. 5, Beads – FA). This indicated that during normal Tafi assembly AgfA and AgfB were accessible to antibodies outside of the cell in agreement with the ENP proposal. The presence of AgfA-specific immune serum caused a 69 % reduction in the amount of polymerized Tafi on the *wt* cell surface (Fig. 3. 5b) indicating the antibodies were competing for polymerization competent subunits. In the  $\Delta agfC$  mutant, levels of polymerized Tafi on the cell surface did not change significantly, but soluble AgfA monomers were not captured indicating that Tafi assembly was not proceeding through soluble/accessible intermediates. For the  $\Delta agfE$  mutant, initial levels of cell-surface, polymerized Tafi were  $\sim 67$  % less than *wt*. However, in the presence of AgfA-specific antibodies, levels of polymerized Tafi on the cell surface did not change significantly, like the  $\Delta agfC$  mutant, and antibodies did not capture soluble AgfA. As expected, the  $\Delta agfF$  mutant did not produce polymerized Tafi on the cell surface and soluble AgfA was detected outside of the cell (Chapman *et al.*, 2002). These results were similar to the  $\Delta agfB$  control mutant. Parallel experiments using AgfB-specific antibodies were in contrast; soluble AgfB was captured in all five strains (Fig. 3. 5c, Beads - FA). There was also a corresponding decrease in the amount of polymerized AgfB for these strains, detected by ELISA (Fig. 3. 5b). These results indicate AgfC and AgfE affect the antibody accessibility of AgfA but not of AgfB suggesting they may influence the structure of assembly competent AgfA.



**Figure 3. 6. Antibody capture of AgfA and AgfB subunits in *Salmonella* Enteritidis 3b strains.**

(a) A cartoon depicting the extracellular polymerization of Tafi subunits and the capture of Tafi subunits in the presence of specific antibodies preventing Tafi polymerization. (b-c) Cells were grown in the presence of AgfA- or AgfB-specific polyclonal immune serum. Non-specific immune serum raised against fetal calf serum was used as a control. Bar graphs represent the relative amounts of AgfA (b) or AgfB (c) in cell-associated Tafi as determined by ELISA. Bars represent the averaged percent values (compared to *wt* at 100%) from three independent experiments performed in triplicate; error bars represent the standard deviations. Insets below the bar graphs show immunoblot analysis of antibody-bound AgfA (b) or AgfB (c) subunits isolated from cell pellet or supernatant samples. Antibody-protein complexes were captured using protein A-coated polystyrene beads. The results shown are representative of five independent experiments. Formic acid treatment was used to differentiate between subunits incorporated into insoluble Tafi (+FA) or present as soluble monomers (-FA).

Table 3.I. Immunological comparison of AgfA and AgfB from *Salmonella* Enteritidis 3b and isogenic *agf* mutant strains.

Strain	3b		$\Delta$ agfA		$\Delta$ agfB		$\Delta$ agfC		$\Delta$ agfE		$\Delta$ agfF	
	A	B	A	B	A	B	A	B	A	B	A	B
<b>Agf</b>												
<b>Subunit<sup>a</sup></b>												
<b>CD +FA</b>	+++	+++	-	+++	-	-	+++	+++	+++	+++	-	+
<b>CD -FA</b>	-	-	-	++	-	-	-	-	-	-	-	+
<b>AP +FA</b>	-	-	-	+++	++	-	-	-	+	-	+++	+
<b>AP -FA</b>	-	-	-	++	++	-	-	-	-	-	+	+
<b>Soluble</b>	-	-		+	+		-	-	-	-	+	+
<b>Ab cap.</b>	+	+		+	+		-	+	-	+	+	+

<sup>a</sup>The intensities of AgfA and AgfB bands on immunoblots were scored as follows: +++; strong, ++; moderate; +; weak, -; none detected; CD, cell debris; AP, agar plug; Ab cap, captured by specific antibody.

### 3.4 Discussion

This work implicates AgfC as an accessory protein that facilitates polymerization of AgfA subunits into regular Tafi fibers. In the past, the *agfC* gene was labeled *orfC* with no ascribed function. However, we have demonstrated that *agfC* is co-transcribed as part of the *agfBAC* operon and influences normal Tafi morphology. Both AgfC and AgfE were found to play a role in extracellular Tafi assembly.

*agfC* mRNA was detected by RT-PCR using Sensiscript™ (Qiagen). Although this technique is not quantitative, we believe that *agfC* is transcribed in low levels since previous transcription studies using less sensitive techniques, could not detect *agfC* (*csgC*) transcripts (Collinson *et al.*, 1996; Hammar *et al.*, 1995). It was previously hypothesized that the RNA stem-loop structure immediately preceding the *agfC* start codon inhibited transcription (Collinson *et al.*, 1996). However, in-frame replacement of the stem-loop structure did not increase AgfC expression, but rather appeared to

reduce *agfA* expression. This suggests that the stem-loop structure between *agfA* and *agfC* has an mRNA stabilizing function that likely ensures that sufficient amounts of Tafi fimbrial components are produced. The low level of *agfC* transcription could be due to an unstable *agfC* mRNA.

AgfC protein was also produced in minute amounts; it was not detected in cell extracts. Similarly, other fimbrial systems have minor proteins that are present in minute quantities making clear definitions hard to assign because of the difficulty to detect (Craig *et al.*, 2003). Although artificial, we were able to detect AgfC when over-expressed *in trans*. Using this method, we found AgfC was primarily located in the periplasm, and N-terminal sequencing showed the mature length to be 100 amino acids. Therefore, we hypothesize the periplasm is the normal cellular compartment for native AgfC.

A serendipitous finding was that AgfC-his specifically binds to the non-crystalline regions of cellulose. This observation may be biologically relevant considering Tafi and cellulose are co-regulated (Gerstel and Romling, 2003; Romling *et al.*, 2000) and intimately associated in the extracellular matrix (White *et al.*, 2003). Since AgfC could not be detected extracellularly, even in the complemented strain, it is not likely that AgfC links Tafi to cellulose outside of the cell. Tafi and cellulose assembly may be coordinated in the periplasm somehow but there is currently no evidence for this type of interaction. Furthermore, cellulose expression appeared to be comparable to *wt* levels in the  $\Delta agfC$  mutant as determined by Calcofluor staining (data not shown). This suggests that AgfC is not involved in the regulation, transport or assembly of cellulose. We presently do not understand the exact role AgfC has in binding cellulose.

Deletion of AgfC caused measurable changes in *Salmonella* Enteritidis 3b cell surface properties. The  $\Delta agfC$  mutant cells produced aberrant Tafi with an increased width of 20 nm, showed increased hydrophobicity and were more susceptible to complement killing (data not shown). Although not directly demonstrated, these

characteristics could stem from the abundant proliferation of aberrant Tafi on the cell surface. An AgfA-specific monoclonal antibody only weakly recognized, if at all, aberrant Tafi suggesting they were structurally distinct from native Tafi. Furthermore, aberrant Tafi did not depolymerize upon treatment with FA as do normal Tafi but required the presence of HCl and heat treatment. After depolymerization, AgfB was not detected, either as protein fragments or immunologically, indicating that the aberrant Tafi were comprised solely of AgfA. This is unlike normal Tafi which are comprised of both AgfA and AgfB at a ratio of  $\sim 20:1$  (White *et al.*, 2001). We therefore hypothesize that the AgfA subunits, as presented in aberrant Tafi, have adopted an alternate conformation leading to polymerization of thicker filaments and that this conformation had not conscripted AgfB subunits as in normal Tafi. Formation of this alternate conformation is somehow prevented by AgfC perhaps by complexing (chaperoning) with preassembly AgfA although we have no direct evidence to show this. Consequently, AgfC biases AgfA assembly toward normal Tafi structure. However, since normal Tafi were still present on the  $\Delta agfC$  mutant cell surface the requirement for AgfC is apparently not absolute suggesting that an equilibrium exists between two alternate AgfA preassembly conformers, one leading to normally structured Tafi and the other to aberrant Tafi. Morphologically similar fibers such as those in the  $\Delta agfC$  mutant have been observed in *E. coli* where over-expression of CsgB (AgfB) fused to a maltose-binding protein resulted in the production of morphologically altered, highly ordered CsgA (AgfA) assemblages that, although uncharacterized, appeared curved, loosely aggregated, 10–15 nm in diameter and did not incorporate CsgB (AgfB) (Bian & Normark, 1997). Additionally, others have observed that in the absence of CsgE (AgfE), curli (Tafi) fibers are morphologically altered (Chapman *et al.*, 2002). This indicates that AgfA has the ability to form alternate tertiary and hence quaternary structures when normal assembly factors are disturbed.

The role of AgfC in maintaining conformational integrity of AgfA prompted a further investigation into Tafi chaperones and assembly factors. The proposed ENP

pathway for *E. coli* K12 curli largely rests on growth complementation of *E. coli* K12 CsgA donor (*csgB*<sup>-</sup> or *csgF*<sup>-</sup>) and acceptor (*csgB*<sup>+</sup>) strains and Congo red detection of polymerizing curli (Bian and Normark, 1997). Since this type of analysis is not feasible with *wt Salmonella* sp. (White *et al.*, 2003) we devised a quantitative analytical test of this system. When *Salmonella* Enteritidis 3b was grown in the presence of AgfA- or AgfB- specific antiserum, soluble subunits were captured and Tafi polymerization was partially inhibited. From these results, it is evident that both AgfA and AgfB monomers are transiently soluble outside the cell before polymerization on the cell surface. This supports the unique ENP pathway of *Salmonella* Tafi as originally proposed for *E. coli* curli (Hammar *et al.*, 1996) and shows that competition for soluble intermediates interferes with the degree of polymerization. It is also possible that these results are the effect of the disassembly of Tafi. In contrast, soluble AgfA subunits could not be captured in the  $\Delta$ *agfC* or  $\Delta$ *agfE* mutants even though Tafi polymerization was still occurring. This corroborates previous studies where a  $\Delta$ *csgE* ( $\Delta$ *agfE*) mutant was unable to donate CsgA (AgfA) subunits when cross-streaked against the CsgB<sup>+</sup> recipient indicating that in the absence of CsgE, AgfA was no longer secreted even though Tafi was still polymerized on the cell surface (Chapman *et al.*, 2002). This indicates Tafi assembly can still occur without proceeding through the extracellular antibody accessible pathway. This is an important distinction from the ENP pathway and demonstrates that extracellular Tafi assembly is not obligatory in these mutants. In *wt*, the ENP pathway accounts for ~75% Tafi polymerization when observed this way. It is possible that the remaining Tafi subunits were not captured by limiting amounts of antibody when using 30% serum; however the results were similar when using up to 70% serum. We therefore interpret these results to indicate that Tafi can assemble through an intracellular pathway in the *wt* strain and in the absence of AgfC or AgfE the extracellular pathway is lost. Alternatively, in the absence of AgfC or AgfE, the antibody is no longer able to recognize and capture the conformationally altered AgfA subunit.

Unlike AgfA, AgfB could be captured by AgfB-specific antibody in the *wt* and the individual deletion strains before polymerization. Therefore, AgfC and AgfE specifically affect AgfA but not AgfB. This is not surprising since from a comparison of the 3D models of AgfA and AgfB, although paralogous, the predicted surface chemistries are distinct (White *et al.*, 2001). Thus AgfB monomers proceed to the cell surface by a distinct system from AgfA.

These data indicate that the roles of AgfC and AgfE involve maintaining an AgfA conformation appropriate for extracellular polymerization into Tafi. This would be necessary since AgfA is a self-polymerizing molecule comprised of tandem arrays of repeated structural motifs (Collinson *et al.*, 1999). Tandem arrays of repeats spontaneously stack to form elongated structures usually stabilized by hydrophobic interactions, both within a repeat and between adjacent repeats (Main *et al.*, 2005). Evidence indicates that the soluble form of AgfA (CsgA) is primarily  $\alpha$ -helical and has not folded into the parallel  $\beta$ -sheet predicted for AgfA in Tafi (Collinson *et al.*, 1999), but will slowly assemble spontaneously when purified (Chapman *et al.*, 2002). Cherny *et al.*, (2005) demonstrated that specific peptides corresponding to the AgfA oligopeptide repeats will self-assemble *in vitro* and even a 24 aa N-terminus peptide of curli (Tafi) has the propensity to oligomerize and form a thermally stable  $\alpha$ -structure (Olsen *et al.*, 2002). Presumably the bacterial assembly apparatus (AgfBCEFG) provides the nucleation/assembly platform to prevent premature subunit polymerization while crossing the periplasm, facilitate folding and to accelerate the process. The results here suggest that AgfC and AgfE are important for maintaining AgfA in a suitable conformation for extracellular polymerization.

The formation of the highly stable, 20 nm thick aberrant fimbriae in the absence of AgfC reveals that AgfA, can assume an alternate highly ordered structure. Three possible structures ( $\beta$ -barrel,  $\beta$ -prism and parallel  $\beta$ -helix), each containing considerable  $\beta$ -sheet structure, were originally predicted for AgfA (Collinson *et al.*, 1999) indicating potentially alternate folding pathways. Which conformations are assumed in normal

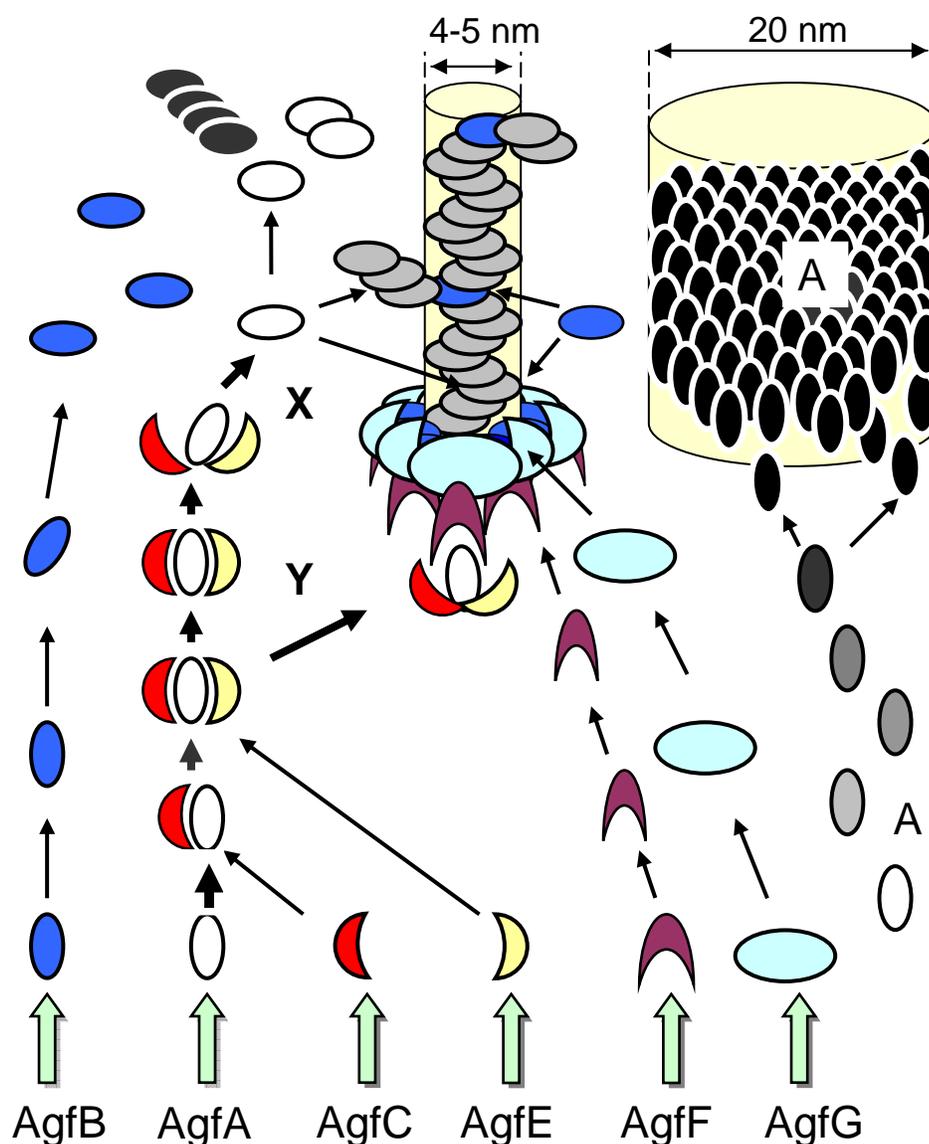
Tafi and aberrant Tafi are unknown as both structures are inherently stable, although the latter considerably more so. The parallel  $\alpha$ -helix structure, being the most stable, was predicted to have the lowest free energy ( $\Delta G = -64 \text{ kcal mole}^{-1}$ ). This may represent the exceptionally stable form encountered in aberrant Tafi.

The question naturally arises as to how such a highly ordered structure as aberrant Tafi assembles. Although unknown, spontaneous formation of large cylindrical assemblages of monomers is known to occur for other proteins or peptides (Lehn, 2002; Rajagopal and Schneider, 2004). In the chaperone-usher pathway of fimbrial assembly, premature polymerization of fimbrin subunits in the periplasm is inhibited by a chaperone protein that facilitates proper folding of the subunit and caps its interactive surfaces priming it for polymerization (Sauer *et al.*, 2004). The results here suggest that AgfC and AgfE may be involved in facilitating Tafi assembly by influencing AgfA export and/or conformation thus facilitating extracellular polymerization. This role is similar to other chaperones but since there is no evidence that these proteins interact directly like in the chaperone-usher fimbriae they are considered atypical.

This is the first report revealing experimental observations of AgfC in Tafi assembly. Experimental observations have been made for CsgE (AgfE) and CsgF (AgfF; Chapman *et al.*, 2002) but due to the insoluble nature of these fimbriae and their unique assembly pathway, mechanisms involving Tafi assembly machinery have still not been experimentally defined. AgfC, E and F are all small with no known homologies, other than with *E. coli*, in the databases. Thus, we attempted to bring together these observations and previous results in to a comprehensive model describing the events effecting Tafi formation (Fig. 3. 7). In this model AgfB is seen to exit the cell by an undefined mechanism different from AgfA chaperone mediated events. AgfA has three possible fates: 1) proceeding through the ENP pathway chaperoned by AgfC and AgfE and polymerizing into Tafi at the cell surface, presumably at the AgfBFG assembly complex and possibly even at branch points defined by AgfB subunits; 2) proceeding

through an intracellular pathway also forming Tafi at the assembly complex; 3) unchaperoned by AgfC and AgfE, and in the case of AgfC absence, spontaneously forming aberrant Tafi. This model suggests that Tafi fiber growth occurs both by an extracellular assembly process predicting distal polymerization and by an intracellular assembly process predicting proximal polymerization.

The detection of the *agfC* transcript in *Salmonella* Enteritidis 3b, the finding that AgfC and AgfF direct AgfA assembly traffic, the elucidation of an alternate Tafi assembly pathway and the discovery that AgfC binds cellulose should prove instructive for further studies on Tafi (curli) assembly. These studies may possibly provide insight into other pathways of aggregative fiber assembly such as in prion-related diseases and also shed light on the critical process of the extracellular matrix formation leading to bacterial environmental persistence.



**Figure 3.7. Model for the growth of normal and aberrant Tafi fimbriae in *Salmonella*.**

The model shows the posttranslational events leading to fiber assembly. On the left, AgfB subunits migrate through the periplasm and through the OM unchaperoned to the cell surface and assembly complex. AgfA chaperoned by AgfC and AgfE migrates to the cell surface primarily by the extracellular nucleation-precipitation pathway (X) or directly to the assembly complex at the cell surface by an intracellular pathway (Y), where after shedding its chaperones engages polymerization into Tafi fibers. The Tafi assembly complex comprised of AgfBF and the lipoprotein AgfG is hypothetically

presented as an ordered assembly scaffold in the OM. Tafi are seen to grow from the complex by polymerization of AgfA subunits occasionally interspersed with AgfB subunits, possibly at polymerization branch points. On the right, AgfA monomers in the absence of AgfC and AgfE assume altered conformation(s) represented by shades of grey. In the absence of AgfC a highly ordered and stable conformation causes the formation of unusually large and stable, 20 nm supramolecular AgfA assemblages (A).

Table 3.II. Primers used in this study.

Product	Reaction	Primer Sequence (5'-3')
cDNA	RT- <i>agfA</i>	TTAATACTGGTTAGCCGTGG
	RT- <i>agfC</i>	CTACTGTGCAGAAGGCGGCC
RT-PCR	<i>agfBA</i>	Forward: <i>agfBF</i> (AGGGTGACAGCATGAAAAAC) Reverse: <i>agfAR</i> (GTTAGCCGTGGCGTTGTTG)
	<i>agfA</i>	Forward: 17-A ( <i>agfAF</i> ) (GCAGAATTCAGCAGTTGTAGTG CAGAAACAGTCGCATAT) Reverse: <i>agfAR</i> (GTTAGCCGTGGCGTTGTTG)
	<i>agfBAC</i>	Forward: <i>agfBF</i> Reverse: <i>agfCR</i> (GAAGGCGGCCATTGTTGTGA)
	<i>agfAC</i>	Forward: 17-A Reverse: <i>agfCR</i> (GAAGGCGGCCATTGTTGTGA)
	<i>agfC</i>	Forward: <i>agfCF</i> (CTTTATTGCTCCTTGCCGC) Reverse: <i>agfCR</i> (GAAGGCGGCCATTGTTGTGA)
	PCR 1	Forward: 17-A Reverse: <i>agfC-C</i> (CATACTTTATTGCTCCGTTAATTAATT AACGTCACATTAACGAACCCTGCGTCTGTCAG)

<i>ΔagfC</i>	PCR 2	Forward: agfC-B (TTCGTTAATGTGACGTTAATTAATT AACGGAGCAATAAAGTATGCATAATTCCTCCCG) Reverse: 17-D (AGACGCAAGCTTCGTTAATGTGACCT GAGGGATCACCG)
	Screening	Forward: 17-A Reverse: agfC2 <sup>a</sup> (CCGCAAGCTTCAAAACTACTGTGCA GAAGGCGG)
<i>ΔagfE</i>	PCR 1	Forward: AgfE/F-A (TACTGGAATTCACCGAGGAAAGG ATCTC) Reverse: AgfE-B (AAACGCTATCTGACCTGTTAATTAAT TAACAACAGAGGAAGCATTAATCGCCGACAAAT)
	PCR 2	Forward: AgfE-C (AATGCTTCCTCTGTTGTTAATTAATT AACAGGTCAGATAGCGTTTCATGGCCTTACCGCC) Reverse: AgfE/F-D (ATAAGAAGCTTATCCACTATTGGC AGGA)
	Screening	Forward: defg13 <sup>a</sup> (CAGCGAGAATACGGTTAAG) Reverse: defg8 <sup>a</sup> (CGCCTGGGCGCTATTCAAT)
<i>ΔagfF</i>	PCR 1	Forward: AgfE/F-A Reverse: AgfF-B (CGTGTTAAACATGCAGGTTAATTAAT TAACTCGCGACGGACAGCTCCAGCTCAACGT CAC)
	PCR 2	Forward: AgfF-C (AGCTGTCCGTCGCGAGTTAATTAATT AACCTGCATG TTTAACACGCATGACAGCCTCCGG) Reverse: AgfE/F-D
	Screening	Forward: defg9 <sup>a</sup> (CCGTCTGCTTCGCATTAGC) Reverse: defg1 <sup>a</sup> (GGTTTCGCAGCTTGTTTCGG)

<i>agfSL</i>	PCR 1	Forward: 17-A Reverse: SL-B (AATTTCTCCCGCATCCTTCAGTTTCTT ATGCCTTGCTTTTCGCATTAGCGCAGACGCTAAATTAA TACTG)
	PCR 2	Forward: SL-C (GTCTGCGCTAATGCGAAAGCAAGGCAT AAGAAACTGAAGGATGCGGGAGGAAATTATGCATAC TTTATTG) Reverse: AgfC2 (CCGCAAGCTTCAAAACTACTGTGCA GAAGGCGG)
	Screening	Forward: 17-A Reverse: SLdel <sup>b</sup> (CATCCTTCAGTTTCT)
<i>agfC</i> ·his	<i>agfC</i> ·his	Forward: FagfC (GGAACATATGCATACTTTATTGCTC) Reverse: RagfC (CAATCCATCTCGAGCTTGTCGTCGTC ATCCTGTGCAGGAAGGCGGCCA)

<sup>a</sup> primer designed to amplify the region around the deleted sequence

<sup>b</sup> primer designed to amplify the region around the substituted sequence

#### **4.0 *Salmonella* produces an O-Antigen capsule translocated by the *emc* operons, regulated by AgfD and important for environmental persistence.**

##### **4.1 Introduction**

Extracellular polysaccharides (EPS), together with proteins and nucleic acids, form an amorphous matrix encasing multicellular microbial communities and mediate direct interactions between the bacteria and the environment. Generally, EPS is considered capsular when covalently attached to lipids, whereas secreted EPS molecules or slime are easily sloughed from the cell surface with no visible means of attachment (Roberts, 1996). Proposed functions of bacterial EPS include: provision of a stable matrix resulting in environmental or host persistence, prevention of microbial desiccation via formation of a hydrated gel, facilitation of biofilm maturation, prevention of phagocytosis and immune system evasion, and providing hydration for swarming (Gygi *et al.*, 1995; Parsek and Fuqua, 2004). There is mounting literature indicating that EPS embedded microbial populations are culpable for chronic infections (Conway *et al.*, 2004; Vuong *et al.*, 2004).

*E. coli* strains produce more than 80 chemically and serologically distinct capsules which have been separated into four groups, in addition to cellulose and colanic acid EPS (Roberts, 1996; Whitfield and Roberts, 1999). Group 4 capsules, formerly group 1, comprise structurally identical lipopolysaccharide O-polysaccharides (LPS) and are thus known as 'O-Antigen (O-Ag) capsules' (Whitfield and Roberts, 1999). Similar to the heteropolymeric LPS O-Ags, the repeat units of group 1 capsules are known to be

assembled on an undecaprenol phosphate carrier lipid at the cytoplasmic face of the inner membrane, transferred across the inner membrane by a "flippase", Wzx, and polymerized by the O-Ag polymerase, Wxy, resulting in high molecular weight (HMW) capsular polysaccharides that are translocated across the outer membrane for final assembly at the cell surface (Whitfield and Roberts, 1999). Recently a seven gene operon (*ymcDCBA*, *yccZ*, *etp*, *etk*) involved in the surface translocation of the group 4 O-Ag capsule from enteropathogenic *E. coli* (EPEC) was identified (Peleg *et al.*, 2005). The operon is present in some *E. coli* strains but not in *Salmonella*. Each gene is required for surface translocation in EPEC; mutation of any single gene results in the loss of capsule expression. YccZ, Etp, and Etk, are predicted Wza, Wzb and Wzc *E. coli* homologues respectively, involved in the export of group 1 capsules and colanic acid (Peleg *et al.*, 2005). Multimeric complexes of the outer membrane  $\beta$ -barrel lipoprotein, Wza, provide a channel for polymer export and associates with Wzc, an inner membrane tyrosine kinase; Wzb is an acid phosphatase (Beis *et al.*, 2004; Nesper *et al.*, 2003; Reid and Whitfield, 2005).

*S. enterica* serovar Enteritidis, is a persistent food-borne enteric human pathogen capable of forming biofilms on abiotic surfaces (Austin *et al.*, 1998). This has major economic and health consequences (Kennedy *et al.*, 2004; Pang *et al.*, 1995). Biofilm formation in *Salmonella* is associated with the multicellular and aggregative behaviour variously described as rdar (Romling *et al.*, 1998), rugose (Anriany *et al.*, 2001) or lacy (Guard-Petter *et al.*, 1996). This multicellular behaviour is highly conserved amongst the

*Salmonellae* (White *et al.*, 2006) and is characterized by the elaboration of thin aggregative fimbriae (Tafi, curli; Collinson *et al.*, 1993), cellulose (Zogaj *et al.*, 2001) and other uncharacterized EPS (White *et al.*, 2003). Together these components form the extracellular matrix that confers resistance to acid and bleach and facilitates environmental persistence (Anriany *et al.*, 2001; Ryu and Beuchat, 2005; Scher *et al.*, 2005; Solano *et al.*, 2002, White *et al.*, 2006).

AgfD (CsgD) from the Tafi (Curli) operon positively regulates elements that promote biofilm formation: Tafi (Romling *et al.*, 1998), cellulose (Romling *et al.*, 2000), serine hydroxymethyltransferase (Chirwa and Herrington, 2003) and a biofilm associated protein, BapA (Latasa *et al.*, 2005), and negatively regulates factors that inhibit biofilm formation (Brombacher *et al.*, 2003). Thus AgfD is a biofilm control point (Gerstel *et al.*, 2003). Activation of the *agfD* promoter is regulated by RpoS, OmpR, MlrA, CpxR, H-NS and IHF (Gerstel *et al.*, 2003; Jubelin *et al.*, 2005). Transcription of *agfD* is maximal during the stationary phase of growth in media of low osmolarity and 30°C (Gerstel *et al.*, 2003). A single point mutation in the promoter region of *agfD* in *Salmonella* Enteritidis 27655-3b (SE 3b), the strain studied here, renders Tafi expression RpoS and temperature independent (Romling *et al.*, 1998).

In this study, we have identified and characterized an O-Ag capsule associated with the *Salmonella* extracellular matrix. Previously, we and others had observed EPS in *Salmonella* that was distinct from the known EPS: colanic acid, cellulose and the Vi-Antigen (Prouty and Gunn, 2003; Solano *et al.*, 2002; White *et al.*, 2003). Although

*Escherichia coli* O-Ag capsules have been characterized (Whitfield and Roberts, 1999), this is the first published report of a *Salmonella* O-Ag capsule. We have identified two operons, *yihU-OyshA* and *yihVW*, to be important for capsule surface assembly and translocation; thus we have renamed them *emcA-H* and *emcIJ* for extracellular matrix capsule. EmcE and EmcG were predicted to be homologues of Wza and Wzc and we demonstrated that they are important for capsule assembly and translocation. We show that the *emc* operons are differentially regulated by AgfD, in coordination with other extracellular matrix components. The O-Ag capsule plays a fundamental role in protection of cells against desiccation stress but does not appear to affect the formation of the extracellular network between cells. The *emc* genes are conserved throughout *Salmonellae*. Thus, the *Salmonella* O-Ag capsule represents a conserved component of the extracellular matrix that is important for environmental persistence.

## 4.2 Material and methods

### 4.2.1 Bacterial strains, culture media and growth conditions

*Salmonella enterica* serovar Enteritidis 27655 3b (Feutrier *et al.*, 1986) was routinely grown at 37°C for 24 h 1% tryptone (T) agar (Collinson *et al.*, 1991) unless indicated. For capsule purification experiments, *Salmonella* Enteritidis 3b  $\Delta bcsA$  (White *et al.*, 2003) was grown on agar supplemented with 0.05% yeast extract, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% NH<sub>4</sub>Cl, 0.3% KH<sub>2</sub>PO<sub>4</sub> and 1% glucose at 28°C for 5 days. For cloning, *E. coli* XL-1 Blue (Stratagene) harboring pBCKS (Stratagene), pHAG (Collinson *et al.*, 1996), pGEM® - T Easy (Promega) or pHSG415 (Hashimoto-Gotoh *et al.*, 1981) was grown at 28 or 37°C for 24 or 48 h with agitation in LB broth

supplemented with 50  $\mu\text{g}/\text{mL}$  chloramphenicol, 100  $\mu\text{g}/\text{mL}$  ampicillin, 40  $\mu\text{g}/\text{mL}$  X-Gal and 1 mM IPTG as required. For transposon mutagenesis, *Salmonella* Enteritidis 3b transduction mixtures and  $\Delta bcsA$  Tn10dCm mutants were incubated at 37°C overnight on LB medium or broth supplemented with 50  $\mu\text{g}/\text{ml}$  kanamycin, 12  $\mu\text{g}/\text{mL}$  chloramphenicol, and 10 mM EGTA. For *lux* assays, overnight cultures were diluted 1 in 600 in 1% tryptone, pH 7.2 supplemented with 50  $\mu\text{g}/\text{ml}$  kanamycin to a final volume of 150  $\mu\text{l}$  in 96-well clear-bottom black plates (9520 Costar; Corning Inc.) and overlaid with 50  $\mu\text{l}$  of mineral oil. Cultures were assayed for luminescence (0.1s) and absorbance (620 nm, 0.1s) every 30 min in a Wallac Victor (Perkin-Elmer Life Sciences, Boston, Mass.) during growth at 28°C with agitation for 48 h (three 90 s shaking periods with 10 min spacing). For whole cell ELISA assays and desiccation experiments, strains were inoculated onto T agar and grown at 28°C for six days.

#### 4.2.2 EPS purification

Cells scraped off agar surfaces were resuspended in 1% phenol, mixed vigorously by vortexing and incubated at RT for 30 min. Cellular debris was pelleted by centrifugation (16000 X g, 4°C, 5 h). The aqueous phase of the supernatant was removed and four volumes of ice-cold acetone were added on ice while constantly stirring with a glass rod for at least 10 min. Precipitated material was spooled, washed with acetone and air-dried at RT overnight. The material was solubilized in dH<sub>2</sub>O with heat and gentle agitation, dialyzed (MW 6-8; Spectrum®) overnight in dH<sub>2</sub>O and lyophilized. Approximately one agar plate of cells (d=140 mm; Fischer) yielded 4 mg of crudely purified EPS. The EPS (10 mg/1mL) was separated on a Superose 6 column (Pharmacia) that had been equilibrated in phosphate buffered saline, pH 7.4 (PBS). PBS was flowed through the column at a rate of 0.4 mL/min and fifty 1 mL fractions were collected every 2 min. Fractions with a high A<sub>220</sub> were collected and analyzed by immunoblotting, silver and protein staining.

#### 4.2.3 Generation of polyclonal serum against EPS and capsule

Crudely purified EPS was subjected to SDS-PAGE and resolved by electrophoresis. The stacking gel containing the EPS material was excised and broken up using a tissue homogenizer. The EPS material was conjugated to KLH (PIERCE) and BSA (SIGMA) following procedures from Conlan *et al* (Conlan *et al.*, 1999). The conjugation was confirmed by SDS-PAGE and staining for proteins and immunoblot analysis using serum generated against whole Tafi (Collinson *et al.*, 1991). The EPS-KLH conjugate prepared in Emulsigen™ adjuvant (MPV Laboratories) was used to immunize a New Zealand white rabbit. Subcutaneous and intramuscular injections of 200 µg were performed three times at two-week intervals with a final boost of 100 µg. Three weeks following the final booster injection, serum was collected and the titre was determined by ELISA (Engvall, 1976), using the EPS-BSA conjugate. Capsule specific antiserum was generated by incubating equal volumes of rabbit polyclonal serum generated against purified EPS and *Salmonella* Enteritidis 3b Tn10::galE (White *et al.*, 2003) whole cell suspensions ( $A_{600}$  1) for 1 h at 4°C rotating.

#### 4.2.4 Scanning Electron Microscopy

Glass coverslips that had been overlaid with 1% T agar on 1.5% T agar plates and incubated at 37°C for 24 h were picked off the agar surface, locked in a mount and immersed in 2.5% glutaraldehyde in 0.2 M Millonigs buffer (Millonig, 1961) at room temperature for 24 h. Cells were dehydrated in a series of alcohols followed by incubation on an EMS 850 critical point drier. The coverslip was mounted on a specimen block and gold coated using an Edwards S150B sputter coater and viewed with a Hitachi S-3500N SEM at 15 kV.

#### 4.2.5 Transmission Electron Microscopy

Cells from colonies were resuspended in 10 mM Tris-HCl (pH 8) and placed onto 0.3% Formvar-coated 200 mesh copper grids prior immunostaining with serum generated against purified EPS followed by goat-anti-rabbit serum conjugated to 10 nm

gold (Cedarlane Laboratories) and negatively staining in 2% UA (pH 7) for 15 s. All samples were viewed with a Hitachi H7600 TEM under HC-zoom mode at 100 kV.

#### 4.2.6 Transposon Mutagenesis and screening for capsule-negative mutants

SE 3b harboring pNK (Bender and Kleckner, 1992) was infected with a P22 HT 105/1 int-201 phage (Schmieger, 1972) lysate of *Salmonella* Typhimurium ATCC 14028 containing Tn10dCm on F' (Elliott and Roth, 1988). The resulting transductants were pooled (20,000-30,000 colonies) and a P22 *Salmonella* Enteritidis 3b  $\Delta bcsA$  Tn10dCm transducing fragment library was created using the method of Maloy (Townsend *et al.*, 2001). The resulting stable Tn10dCm mutants were picked into 55 clear flat-bottomed sterile 96-well cell culture plates (Costar®, Corning, N.Y.) and stored in 5% glycerol at -70°C. Tn10dCm mutants were inoculated from glycerol stocks using a sterile 48-pronged metal stamp into 100  $\mu$ l 1% tryptone, pH 7.2 broth in clear flat-bottom sterile 96-well cell culture plates. Growth was measured at A<sub>595</sub> using an ELx 808 Biotek Ultra microplate reader (Biotek) after 24 h growth at 37°C. Whole cells were heat fixed at 85°C in a hybridization oven for 6 h or until all the media had evaporated. ELISAs were performed using the method of Engvall (1976) in flat-bottom high binding 96-well EIA/RIA plates (Costar®, Corning, N.Y.) using rabbit polyclonal serum generated against purified EPS followed by goat anti-rabbit immunoglobulin G-alkaline phosphate (Cedarlane Laboratories Ltd.) and 1 mg/mL p-Nitrophenyl phosphate (SIGMA). The absorbance in each well was detected at A<sub>405</sub> and the final A<sub>405</sub>/A<sub>595</sub> values were calculated and compared to relative percent of capsule on the *wt* surface where *wt* was 100% and background was *Salmonella* Enteritidis 3b Tn10::galE. Mutants with less than 10% capsule on the cell surface were re-screened, streaked onto EBU plates to render the mutants phage-free and re-screened for maintenance of the phenotype. Genomic DNA for PCR, was prepared following the method of Walsh *et al* (Walsh *et al.*, 1991). Sites of Tn10dCm insertion in the

chromosome were amplified by arbitrary primed PCR described by Welsh (Welsh and McClelland, 1990).

#### 4.2.7 Generation of *Salmonella* Enteritidis 3b mutants

Isogenic  $\Delta emcG$  (298 bp deletion),  $\Delta emcE$  (296 bp deletion) and  $\Delta agfD$  (394 bp deletion) mutants containing six-frame translational stop codons at the beginning of the gene and a  $\Delta P_{emc}$  mutant with the putative promoter region replaced with random sequence was generated following the methods of White *et al.* (1999). All primers are listed in Table 4.III.

#### 4.2.8 ELISA

Cells were resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0) and adjusted to  $A_{600}$  0.1 in the wells of flat-bottom high binding 96-well EIA/RIA plates (Costar®, Corning, N.Y.) and ELISAs were performed using the method of Engvall (1976). Cells were treated as described above using capsule specific serum.

#### 4.2.9 Immunofluorescent Microscopy

Cells were scraped off agar surfaces and resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0). Cell suspensions were adjusted so each slide contained 0.05  $A_{600}$ . Cells were incubated for 1 h at room temperature with capsule specific serum diluted in 5% normal mouse serum in PBS. Cells were harvested by centrifugation (6000 X g, 2 min), washed twice with 500  $\mu$ l 5% normal mouse serum in PBS, incubated in goat-anti-rabbit IgG-fluorescein isothiocyanate conjugate (Cedarlane Laboratories Ltd.) for 1 h at RT in the dark and washed again. Cells were viewed under a fluorescent microscope (Nikon Labophot) and imaged digitally.

#### 4.2.10 SDS-PAGE, protein staining and immunoblotting

Cells were resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0) and adjusted to  $A_{600}$  0.1. The cells were harvested by centrifugation (6000 X g, 10 min), resuspended in SDS-PAGE sample buffer and boiled for 10 min prior to electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) with a 5% stacking gel and 12% resolving gel. The EPS was stained with Gelcode™ (Bio-Rad), Alcian blue (SIGMA-ALDRICH), or LPS silver stain (Bio-rad). For immunoblots, EPS material was transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell™ (Bio-Rad Laboratories) in buffer recommended by the manufacturer. Rabbit polyclonal immune serum generated to purified EPS, whole Tafi or specific to capsule followed by goat-anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR, Ltd.) was used for detection. Immunoreactive material was visualized using an Osysey™ Biosciences scanner (LI-COR, Ltd.). Goat polyclonal immune serum generated against *E. coli* Lipid A (BIODESIGN Int.) followed by swine-anti-goat immunoglobulin G-alkaline phosphatase conjugate (Cedarlane Laboratories Ltd.) was used for detection of LPS. Immunoreactive material was visualized by incubating in BCIP and NBT (SIGMA-ALDRICH).

#### 4.2.11 Generation of *lux* reporters

The *emc* intergenic region was PCR amplified from *Salmonella* Enteritidis 3b or *Salmonella* Typhimurium ATCC 14028 chromosomal DNA. PCR products were purified and sequentially digested with *Xho*I and *Bam*HI (Invitrogen Canada Inc.). Following restriction digestion, promoter regions were ligated using T4 DNA ligase (Invitrogen Canada Inc.) into pCS26-Pac (*Xho*I-*Bam*HI) or pU220 (*Bam*HI-*Xho*I) reporter vectors containing the *luxCDABE* operon from *Photobacterium luminescens* (Bjarnason *et al.*, 2003). DNA sequencing was performed by Macrogen (Seoul, South Korea) using primers pZE05 and pZE06 (Bjarnason *et al.*, 2003). The construction of *agfD* and *agfB* reporters has been described elsewhere (White *et al.*, 2006).

#### 4.2.12 Desiccation Experiment

Six individual colonies (d = ~1mm) from *Salmonella* Enteritidis 3b,  $\Delta agfD$ ,  $\Delta agfA$ ,  $\Delta bcsA$ ,  $\Delta agfA\Delta bcsA$ ,  $\Delta emcG$ ,  $\Delta emcE$ ,  $\Delta Pemc$  and *Salmonella* Typhimurium ATCC 14028 were removed from the agar surface and frozen. Three colonies were lyophilized for one week. All colonies were resuspended in 500  $\mu$ l PBS for one hour, vigorously vortexed and homogenized using a sterile applicator stick until even turbidity was reached. Cell mixtures were serially diluted in triplicate and plated in duplicate to determine colony forming unit (CFU) values for each individual colony. The difference between the averaged CFU values for each desiccated colony and the averaged CFU value from initial colonies was statistically analyzed using a one-way ANOVA with Bonferroni's post-test using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA ([www.graphpad.com](http://www.graphpad.com)).

#### 4.2.13 Etest® antibiotic testing

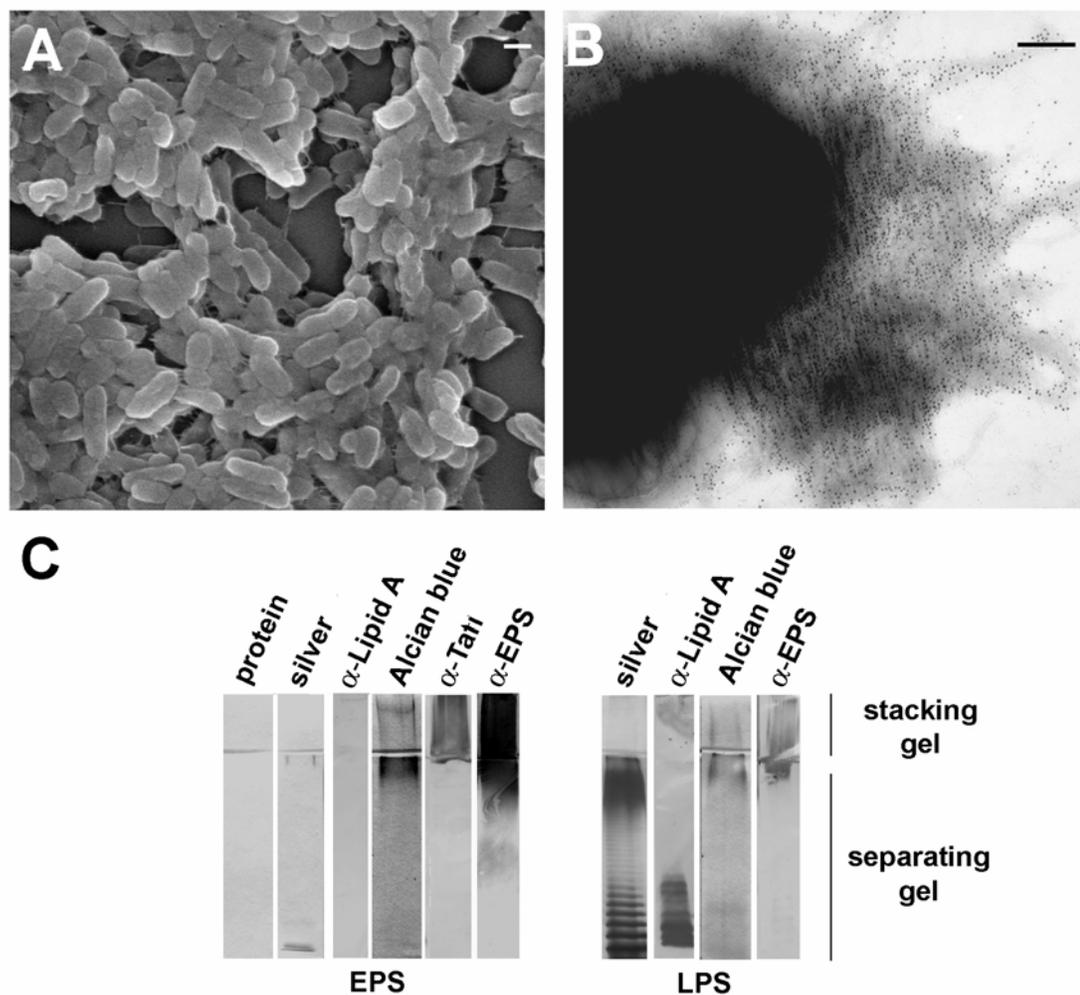
Viable colonies from *Salmonella* Enteritidis 3b,  $\Delta bcsA$  and Tn10dCm::*emcG* $\Delta bcsA$  were homogenized in PBS and adjusted to a 0.5 McFarland standard (NCCLS., 1990). Strains were grown on Mueller-Hinton agar (Becton Dickinson, USA) at 37°C for 18 h in the presence of Etest® strips (a gift from Pamela Kibsey at VIHA, Vancouver Island). MICs were interpreted according to the manufacturer's instructions (AB Biodisk, Sweden).

### 4.3 Results

#### 4.3.1 *Salmonella* EPS

To characterize the EPS associated with the extracellular matrix (Fig. 4. 1A), polysaccharides were purified from a cellulose-deficient background strain ( $\Delta bcsA$ ) of SE 3b. Serum generated against purified EPS was used to immunostain the cell surface

of SE 3b (Fig. 4. 1B) and revealed that the EPS formed an amorphous layer around the cell. To analyze the EPS biochemically, crudely purified fractions were resolved by SDS-PAGE and stained directly or transferred to nitrocellulose for immunoblotting. A diffuse band corresponding to the stacking gel did not react with protein- or LPS-specific stains or serum generated against LPS lipid-A and was stained by the acidic polysaccharide stain, Alcian blue, (Fig. 4. 1C) similar to other EPSs (Junkins and Doyle, 1992; Waldor *et al.*, 1994). The diffuse material was recognized by immune serum generated against whole Tafi or crudely purified EPS (Fig. 4. 1C). These tests indicated that low molecular weight (LMW) LPS was not a significant component of the purified material. However, HMW material from commercially purified LPS was stained by Alcian blue and serum generated against crudely purified EPS (Fig. 4. 1C, blots on right). Thus, commercially purified LPS contained immunologically similar HMW material that was distinct from LMW LPS. These results indicated the fraction of EPS(s) contained immunoreactive, non-proteinaceous polymer(s) of HMW, some of which were acidic.

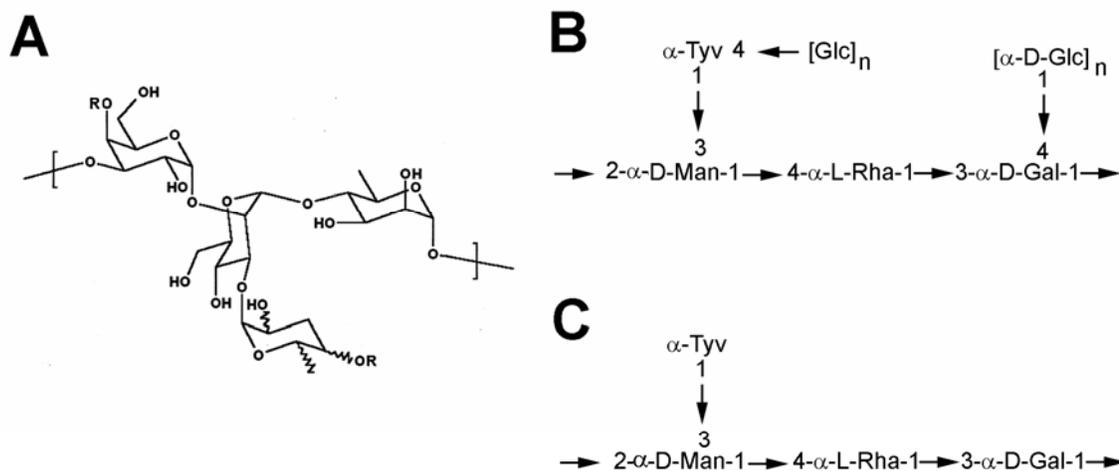


**Figure 4.1. The *Salmonella* EPS associated with the extracellular matrix.**

(A) SEM micrograph of *Salmonella* Enteritidis 3b cells embedded in the extracellular matrix (bar = 1 µm). (B) TEM micrograph of *Salmonella* Enteritidis 3b expressing the extracellular matrix immunostained with serum generated against the purified EPS (bar = 100 nm). (C) SDS-PAGE and immunoblot analysis of 10 µg of purified EPS from *Salmonella* Enteritidis 3b  $\Delta bcsA$  or 10 µg of LPS from *Salmonella* Enteritidis (SIGMA).

#### 4.3.2 *Salmonella* Enteritidis 3b produces an O-Ag capsule

Compositional analysis of the purified EPS fraction indicated the presence of one major, predominant polysaccharide and another minor polysaccharide. Several different fatty acids were also detected, including common LPS components, 3-hydroxytetradecanoic, dodecanoic, tetradecanoic, and hexadecanoic acids, as well as octadecanoic acid and unsaturated fatty acids hexadecenoic acid and octadecadienoic acid which are not normally detected in the LPS of enteric bacteria. The observed levels of fatty acid were consistent with the possibility that they represented a lipid anchor for a glycolipid (Snyder *et al.*, submitted). The repeating oligosaccharide unit for the major polysaccharide (Fig. 4. 2A, B) was similar to the reported LPS O-Ag of *Salmonella* Enteritidis (Hellerqvist *et al.*, 1969) but with some structural differences: the tyvelose residue was partially substituted with a polymeric glucose side chain at the 4 position and the galactose residue was glucosylated in a similar manner as structure II HMW O-Ag (Rahman *et al.*, 1997). Furthermore, the major EPS was composed of >2300 repeat units which is 23 - 143 X that of normal HMW LPS. In *E. coli*, an EPS that is identical to a given strain's O-Ag is called an O-Ag capsule (Whitfield and Roberts, 1999).



**Figure 4. 2. The *Salmonella* Enteritidis 3b capsular polysaccharide structure.** (A) Configuration and (B) linear drawing of the repeating oligosaccharide unit. R; nonstoichiometric glucose substitution (C) The linear drawing of the O-Ag repeating unit from *Salmonella* Enteritidis (Hellerqvist *et al.*, 1969). The mannose residue has also been reported in the  $\beta$ -configuration (Rahman *et al.*, 1997).

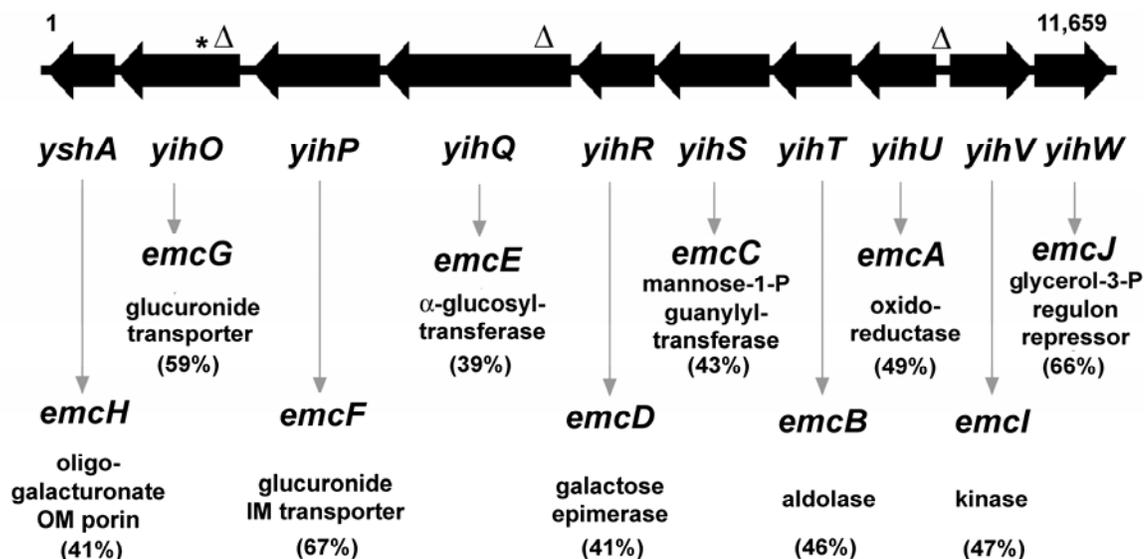
#### 4.3.3 The *emc* operons are important for capsule assembly and translocation

Tn10dCm mutagenesis was used to inactivate and identify prospective genes involved in capsule synthesis, regulation or transport. The serum generated to the whole EPS fraction primarily recognized the HMW capsule, had slight cross-reactivity to the uncharacterized minor EPS (data not shown), but did not strongly recognize LMW LPS (Fig. 4. 1C). This suggested that the serum recognized conformational epitopes formed by extended helices in the HMW capsule, similar to serum generated against other HMW polysaccharides (Brisson *et al.*, 1997). The specificity of the serum allowed us to screen Tn10dCm mutants by ELISA for loss of the capsule. Since the *Salmonella*

capsule repeating unit contained a galactose residue, a *galE::Tn10* reference strain was used as a negative control. We identified nine mutants that were immunologically negative for capsule production (Table 4. I). One of the mutants identified had a *Tn10dCm* insertion in the *yihO* (*emcG*) gene, the seventh gene in a putative eight-gene operon that is adjacent to a divergent putative *yihVW* (*emcIJ*) operon (Fig. 4. 3). The *emc* gene products had homologies and conserved domains to known proteins involved in carbohydrate transport, biosynthesis and regulation (Figure 4. 3; Table 4. II). This led us to speculate that the *emc* operons may be involved in capsule assembly and translocation.

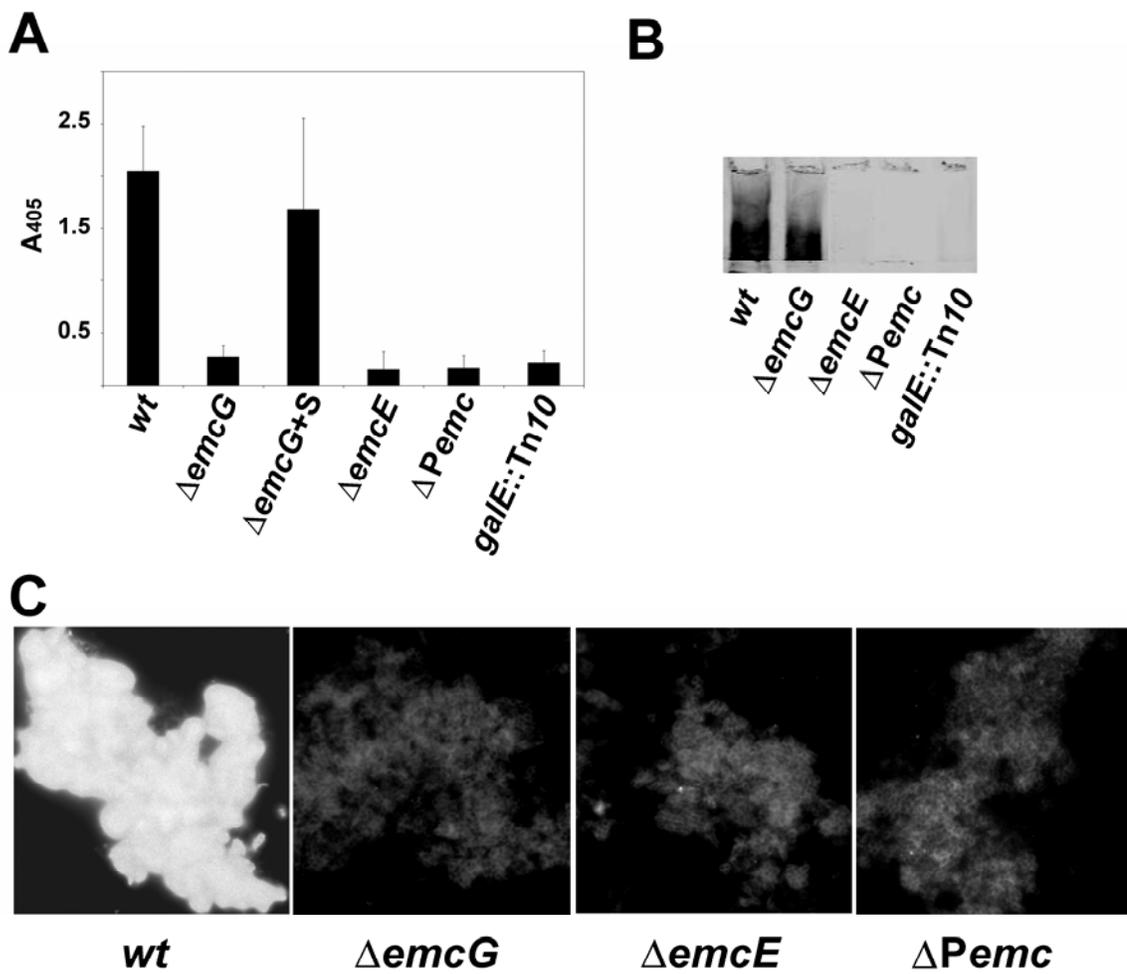
To confirm that the *emc* genes were important in capsule assembly and translocation, in-frame deletion mutants were generated for *emcG*, encoding a putative carbohydrate transporter, and *emcE*, encoding a putative glucosyltransferase (Fig. 4. 3). In addition, the putative promoter region between the divergent *emc* operons was replaced in-frame with a random nucleotide sequence (Fig. 4. 3). Capsule expression in the *emc* mutant strains was measured by ELISA, immunoblotting and immunofluorescence using serum that had been cross-absorbed to the *galE::Tn10* strain (capsule-specific serum). The capsule was not detected on the cell surface of the  $\Delta emcG$  mutant when analyzed by ELISA and immunofluorescence (Fig. 4. 4A). However, when whole cell lysates were subjected to immunoblot analysis, a diffuse band was detected in the stacking gel (Fig. 4. 4B). This indicated that EmcG was required for capsule transport but not for biosynthesis. Sonication of the  $\Delta emcG$  mutant cells prior to ELISA substantiated this because after disruption, the amount of capsule detected was similar to that of the *wt* (Fig. 4. 4A). The capsule could be detected on the surface of the  $\Delta emcG$  cells when they were complemented by *emcG* in *trans* (data not shown). EmcG was predicted to be a lipoprotein (PredictProtein software; [www.predictprotein.org](http://www.predictprotein.org)) containing 12 integral transmembrane spanning regions (TMHMM server; [www.cbs.dtu.dk](http://www.cbs.dtu.dk)). Furthermore, secondary structural predictions using jpred software ([www.compbio.dundee.ac.uk](http://www.compbio.dundee.ac.uk)) indicate that EmcG is 50.1%  $\alpha$ -helical and 16.7%  $\beta$ -

stranded. Thus, it is possible that EmcG is a trans-membrane transport protein structurally analogous to Wza, a membrane lipoprotein also substantially  $\alpha$ -helical that is involved in group 1, group 4 capsule and colanic acid translocation (Whitfield and Roberts, 1999). The capsule was not detected in either  $\Delta emcE$  or the promoter mutant ( $\Delta P_{emc}$ ) when analyzed by ELISA, immunoblotting or immunofluorescence (Fig. 4. 4). EmcE is a glycosidase with homologies to a glucosyltransferase with conserved domains involved in carbohydrate transport. Thus we hypothesize that EmcE is also involved in capsule assembly and translocation (Table 4. II). Lastly, since the  $\Delta P_{emc}$  mutant did not express the capsule, this indicates that the putative promoter region controls the expression of the *emc* genes. Collectively, these results reveal that the *emc* genes are essential for capsule assembly and translocation.



**Figure 4. 3. Structure of the *emc* operons involved in assembly and translocation of the *Salmonella* O-Ag capsule.**

The *yihU-OyshA* and *yihVW* gene clusters, as represented from *S. enterica* serovar Typhimurium LT2 (NC003197), have been renamed *emcABCDEFGH* and *emcIJ* and are represented to scale. The asterisk denotes the insertion site of the Tn10dCm element; insertion marks represent in-frame deletions of the majority of the gene coding sequence or promoter regions performed in SE 3b. Putative functions listed below each of the *emc* genes (NP462896-905) were delineated by comparing Emc sequences to protein sequences encoded in sequenced genomes from the NCBI database. The percentages of similar amino acids for each predicted orthologue are indicated in parenthesis below the gene names: EmcH; KdgM (YP275183) from *Pseudomonas syringae* pv. *phaseolicola* 1448A, EmcG; EmcF; EmcG, UidB (NP416133) from *Bacillus licheniformis* ATCC 14580, EmcE; CtsZ (BAD34980) from *Arthrobacter globiformis*, EmcD; GalM (AF3058) from *Agrobacterium tumefaciens*, EmcC; ManC (E95924) from *Sinorhizobium meliloti* 1021, EmcB; LacD (Q6G7C2) from *Staphylococcus aureus* subsp. *aureus* MSSA476, EmcA; MmsB (YP244045) from *Xanthomonas campestris* pv. *campestris* 8004, EmcI; RbsK (YP177042) from *Bacillus clausii* KSM-K16, and EmcJ; GlpR (NP927560) from *Photobacterium luminescens* subsp. *laumondii* TTO1.

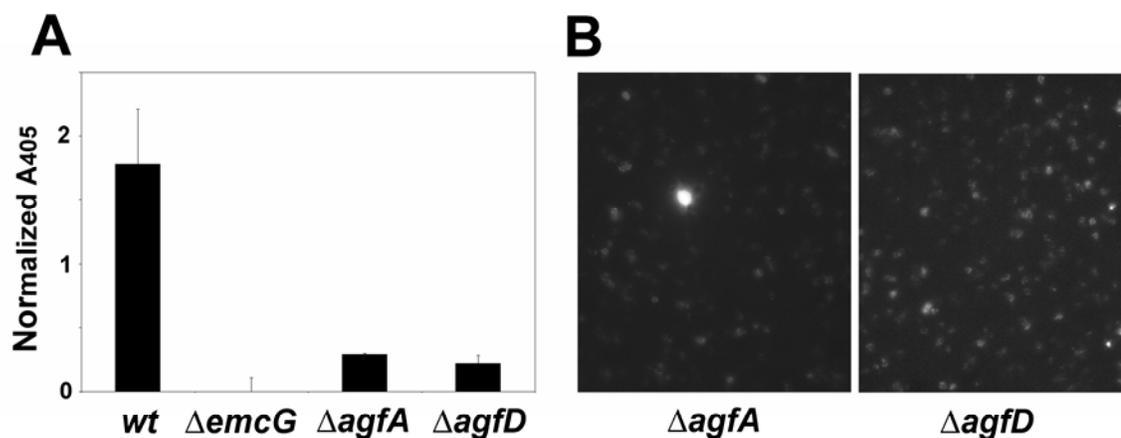


**Figure 4. 4. Immunological analysis of capsule expression on *Salmonella* Enteritidis 3b *emc* mutants.**

*Wt* and *emc* mutants were analyzed using capsule specific serum by (A) whole-cell ELISA, (B) immunoblotting of whole cell lysates, and (C) immunofluorescence. Bars in (A) correspond to averaged values with standard deviations from triplicate samples from three independent experiments.  $\Delta emcG$  cells were sonicated before analysis ( $\Delta emcG+S$ ). In (B), only the region on the immunoblot corresponding to the stacking gel is shown. *Salmonella* Enteritidis 3b *galE::Tn10* was used as a negative control for capsule production.

#### 4.3.4 Capsule expression is synchronized with Tafi and flagella production

Eight additional mutants were identified as having capsule expression at or below the *galE::Tn10* strain background (Table 4.I). Curiously, one mutant had an inactivated *csgG* (*agfG*) gene, the product of which is required for Tafi (curli) subunit secretion and gene mutations result in the degradation of Tafi (Loferer *et al.*, 1997). This suggested that Tafi and capsule expression were somehow linked. To confirm that capsule deficiency in the  $\Delta$ *agfG* mutant was related to a deficiency in Tafi production, capsule expression was measured in in-frame  $\Delta$ *agfA* (White *et al.*, 2001) and  $\Delta$ *agfD* isogenic mutants by ELISA and immunofluorescence (Fig. 4. 5). Capsule expression was not completely negative in either strain, but the surface expression was noticeably decreased compared to the *wt* (~16% and ~13% of *wt* levels, respectively; Fig. 4. 5A). When tested by immunofluorescence microscopy, the Tafi mutants were visibly less aggregative and were mostly negative for the presence of the capsule. This suggests that the capsule expression is linked to the elaboration of Tafi. Some capsular mutants had *Tn10dCm* inactivated genes associated with flagella synthesis or motility. One mutant had the first gene of the *flhBAE* operon inactivated; this operon encodes proteins involved in the export of flagellar subunits (McMurry *et al.*, 2004). Other mutants containing insertions in genes *gidA* and *sopB* were identified; these mutations have known pleiotropic effects on motility (White *et al.*, 2001; Wang *et al.*, 2005). Another mutant had *yedF* inactivated, a gene in the *amyA-fljE* intergenic region which encodes a putative transcriptional regulator. Additionally, STM0652 encodes a putative sigma-54 (RpoN)-dependent transcriptional regulator indicating that capsule synthesis could be influenced by nitrogen metabolism (McCarter, 2004; Merrick, 1993), or other biological activities that include flagellation, chemotaxis, (McCarter, 2004; Merrick, 1993), expression of O-Ag LPS (Bittner *et al.*, 2002), or expression of cell-surface polysaccharides (Boucher *et al.*, 2000). These results suggest there is an unknown relationship between flagella and capsule expression.



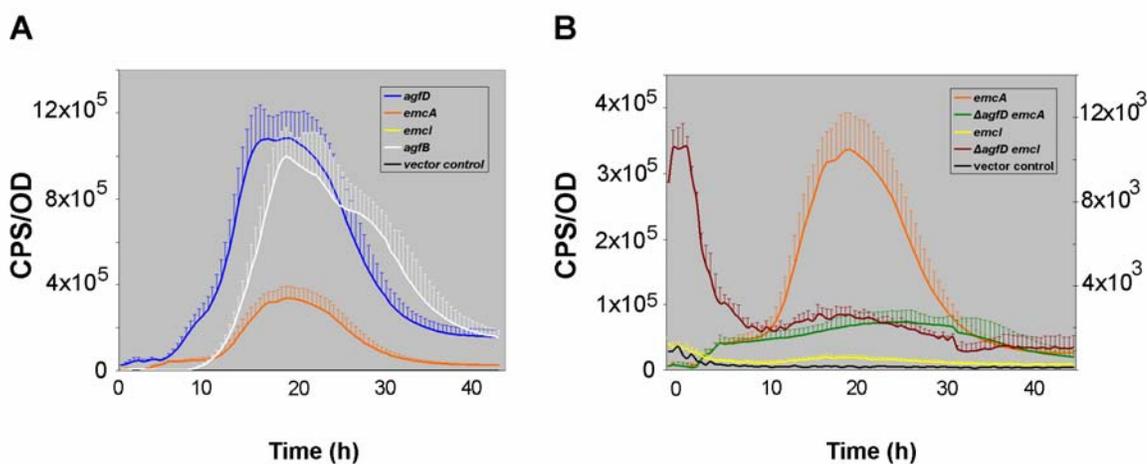
**Figure 4. 5. Immunological analysis of capsule expression on *Salmonella Enteritidis* 3b *agf* mutants.**

(A) ELISA analysis of whole cells incubated with capsule specific serum. The background  $A_{405}$  of the *emcG* strain was subtracted from the  $A_{405}$  of all strains to normalize the data. Standard deviations correspond to averaged values from triplicate samples from three independent experiments. (B) Immunofluorescent images were generated on whole cells incubated with capsule specific serum.

#### 4.3.5 The *emc* genes are differentially regulated via AgfD in coordination with the extracellular matrix genes

The O-Ag capsule was originally detected in association with the AgfD-regulated extracellular matrix (White *et al.*, 2003). Therefore, we wanted to determine if AgfD had a regulatory effect on *emc* expression. *emcA* and *emcI* promoter::*lux* reporter fusions were used to measure the promoter activity of each *emc* operon in SE 3b. During growth in 1% tryptone broth, *emcA* activity was high at  $3.4 \times 10^5$  CPS whereas *emcI* expression was similar to the promoter-less vector control ( $<100$  CPS; Fig 6A). Peak *emcA* expression coincided with peak activity of *agfD* and *agfB* promoter::*lux* fusions suggesting coordinated expression with other extracellular matrix genes. However, when expression was tested in an isogenic  $\Delta agfD$  mutant background, *emcA* activity dropped by  $\sim 80\%$  whereas *emcI* turned on with a peak activity of  $2.6 \times 10^3$  CPS, representing a  $\sim 10$  fold increase in expression (Fig. 4. 6B). This suggested *emcI*

expression was repressed in the presence of AgfD. Increased *emcI* expression coincided with lower *emcA* expression, suggesting that one or both products of the *emcIJ* operon (*emcJ* encodes a putative transcriptional repressor; Table 4. II), may repress *emcA* expression. Therefore we postulate that AgfD positively regulates the expression of the *emcA* operon possibly by repressing the expression of the *emcI* operon. These results suggest that AgfD has a positive regulatory effect on capsule synthesis under the specified conditions.



**Figure 4. 6. The *emc* genes are differentially regulated via AgfD in coordination with the extracellular matrix genes.**

Expression of *emcA*, *emcI*, *agfB* and *agfD* promoter::lux fusions was tested in *Salmonella* Enteritidis 3b (A) and an isogenic  $\Delta$ *agfD* strain (B). Cultures were grown in 1% tryptone at 28 °C for 48 h with agitation and readings were taken every 30 min to monitor luminescence (counts per second; CPS) and cell density ( $OD_{600}$ ). Individual points in each expression curve represent averaged CPS/OD values calculated from triplicate samples from three independent experiments; error bars represent the standard deviations. In (B), CPS/OD measurements for *emcA* are on the left axis; measurements for *emcI* are on the right axis. pU220 is the promoterless vector control (Bjarnason *et al.*, 2003).

Table 4.I. *Salmonella* Enteritidis 3b Tn10dCm mutants deficient in O-Ag capsule production.

Gene flanking Tn10dCm	Function <sup>a</sup>
<i>yihO</i> ( <i>emcG</i> )_NP462897	putative sugar transporter
<i>csgG</i> ( <i>agfG</i> )_NP460110	curli (Tafi) subunit transporter
<i>flhB</i> _NP460871	flagellar biosynthetic protein
<i>yedF</i> _NP460919	putative transcriptional regulator (NP830585_ <i>Bacillus cereus</i> ATCC 14579)
<i>gidA</i> _NP462773	glucose-inhibited division protein
<i>sopB</i> _NP460064	SPI-1 invasion protein
STM0652_NP459644	putative sigma-54 transcriptional regulator (NP744934_ <i>Pseudomonas putida</i> KT2440)
<i>yjeM</i> _NP460034	putative amino-acid transporter (NP561082_ <i>Clostridium perfringens</i> str.13)
STM1060_NP463209	putative Fe-S oxidoreductase (YP206222_ <i>Vibrio fischeri</i> ES114)

<sup>a</sup>, Putative functions were delineated from closest orthologue; locus reference and species listed in parenthesis.

Table 4.II. Conserved Domain Hits of the proteins encoded by the *emc* (*yih*) operons (Marchler-Bauer and Bryant, 2004).

Protein	Conserved Domain Hits <sup>a</sup>	Domain Function	Score (bits); E value
EmcA (YihU)	MmsB; gnl CDD 11792	dehydrogenase; lipid metabolism	269; 3e-73
	Gnd; gnl CDD 10751	dehydrogenase; carbohydrate transport and metabolism	73.8; 2e-14
	TrkA-N domain; gnl CDD 9399	NAD binding; transport	42.1; 9e-05
	WecC; gnl CDD 10546	Dehydrogenase; cell envelope biogenesis	41.8; 1e-04
	UDPG_MGDP_dh_N; gnl CDD 23479	NAD dependent dehydrogenase	38.7; 0.001
EmcB (YihT)	LacD; gnl CDD 13006	aldolase; carbohydrate transport and metabolism	393; 1e-110
	FbaB; gnl CDD 11540	aldolase; carbohydrate transport and metabolism	48.3; 1e-06
	DhnA, Class I; gnl CDD 28138	aldolase	42.1; 7e-05
EmcC (YihS)	GlcNAc_2-epim, family; gnl CDD 12290	epimerase; carbohydrate transport and metabolism	401; 9e-113
	AGE domain; gnl CDD 1779	isomerase	335; 5e-93
EmcD (YihR)	GalM; gnl CDD 11725	mutarotase; carbohydrate transport and metabolism	188; 7e-49
	Aldose_epim; gnl CDD 2572	epimerase	154; 9e-39

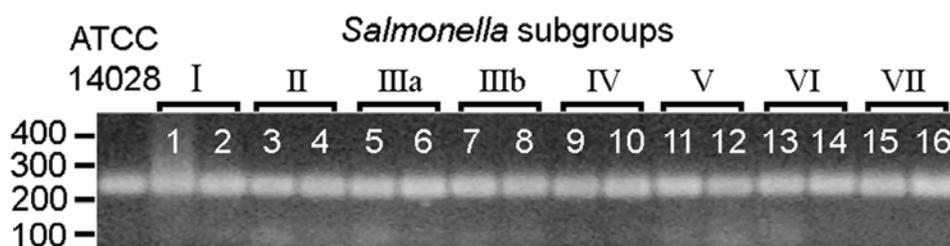
EmcE (YihQ)	$\alpha$ -glucosidases, family 31; gnl CDD 11215	hydrolase; carbohydrate transport and metabolism	566; 2e-162
EmcF (YihP)	MelB, GPH family; gnl CDD 11918	carbohydrate transport	430; 2e-121
EmcG (YihO)	MelB, GPH family; gnl CDD 11918	carbohydrate transport	447; 2e-126
EmcH (YshA)	No hits		
EmcJ (YihW)	HTH_ARSR, ARSR family; gnl CDD 17771	metal regulated transcriptional repressors	37.6; 0.001
	DeoR, deoR family; gnl CDD 24424	transcription regulators	258; 7e-70
	GlpR; gnl CDD 11065	transcriptional regulators; carbohydrate transport	239; 4e-64
	BglG; gnl CDD 13031	transcriptional antiterminator	40.1; 3e-04
	HTH_DEOR; gnl CDD 375	deoxyribose operon repressor	67.1; 2e-12
	MarR, MarR family; gnl CDD 15281	transcriptional repressor	39.5; 5e-04
EmcI <sup>b</sup> (YihV)	ribokinase_group_B, pfkB superfamily gnl CDD 28258	synthesize nucleotides and histidine	244; 1e-65
	Ribokinase; gnl CDD 28249	synthesize nucleotides and histidine	166; 2e-42
	RbsK, ribokinase family; gnl CDD 10395	carbohydrate transport and metabolism	163; 3e-41

<sup>a</sup>, Protein hits with E-values less than 0.0001 are listed along with their respective Genbank designations in parenthesis.

<sup>b</sup>, the top three hits out of twenty-two various kinase types are listed.

#### 4.3.6 Conservation of *emc* operons and biosynthesis in *Salmonellae*

The *emc* operons are conserved throughout all *Salmonella* serovars currently being sequenced (<http://globin.cse.psu.edu/enterix/enteric/enteric.html>; Florea *et al.*, 2003). To determine if the genes could be detected throughout the Salmonellae, isolates from the *Salmonella* reference collection C (SARC; Boyd *et al.*, 1996), representing all 7 subgroups of *S. enterica* along with *S. bongori*, were tested by PCR specific for *emcA* and *emcI* DNA flanking the *emc* intergenic region. The *emc* intergenic region was detected in all 16 SARC isolates (Fig. 4. 7), indicating that the divergent operon structure was conserved. In addition, all SARC isolates were tested for presence of capsule by ELISA. Several isolates had significant cross-reactivity with our capsule specific serum (data not shown), suggesting that capsule assembly and transport could be conserved. However, since each isolate likely differs in O-Ag structure, the significance of these results are unknown and require further analysis.

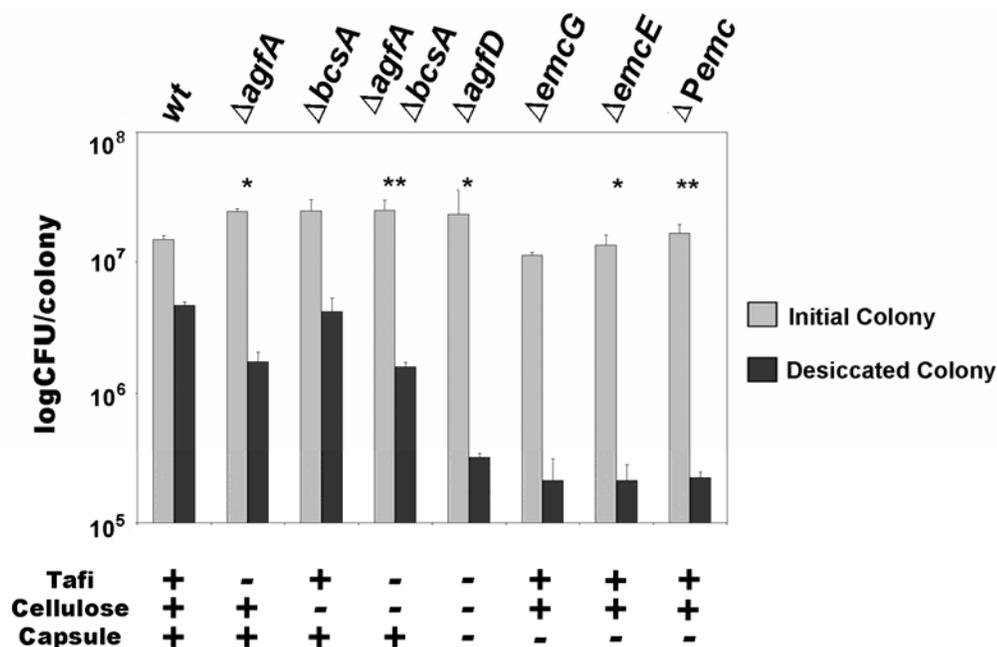


**Figure 4. 7. Conservation of *emc* operons in *Salmonellae*.**

2% agarose gel electrophoresis of PCR-amplified products from the chromosome of the SARC strains 1-16 using primers specific to regions flanking the *emcA-I* intergenic region. A sample containing no DNA was used as a negative control (data not shown).

#### 4.3.7 The O-Ag capsule promotes environmental persistence

To determine if the capsule was involved in environmental persistence, colonies of the rdar morphotype from *emc* mutant strains were subjected to desiccation by lyophilization to represent the long-term storage used in our previous study (i.e. storage on plastic for nine months; White *et al.*, 2006). The *wt* strain capable of producing Tafi, cellulose and capsule had the highest survival rate of 31.1% (Fig. 4. 8). In the absence of Tafi ( $\Delta agfA$ ), cellulose ( $\Delta bcsA$ ) or Tafi and cellulose ( $\Delta agfAbcsA$ ), survival was decreased to 7%, 16.6% and 6.3%, respectively. There was a significant loss in viability in both Tafi mutants ( $\Delta agfA$  and  $\Delta agfAbcsA$ ) but not in the cellulose mutant ( $\Delta bcsA$ ) alone when compared to *wt*. The  $\Delta agfD$  mutant was most impaired in survival (1.3%). These results closely matched the results of White *et al.*, (2006) and indicated that additional components regulated by AgfD, other than Tafi and cellulose, were responsible for survival. The capsule mutants ( $\Delta emcG$ ,  $\Delta emcE$  and  $\Delta Pemc$ ) were reduced to levels similar to the  $\Delta agfD$  mutant demonstrating that the capsule was primarily responsible for desiccation tolerance. Furthermore, we found the capsule mutants could also protect against some antimicrobials (supplementary materials). Surprisingly, the *emc* mutations did not disrupt pellicle formation, the rdar morphotype and biofilm formation similar to the other extracellular matrix components (data not shown). This indicated the capsule was not important in multicellular aggregation.



**Figure 4. 8. The capsule is required for survival against desiccation stress.**

Survival of *Salmonella* Enteritidis 3b and fimbrial ( $\Delta agfA$ ), cellulose ( $\Delta bcsA$ ), extracellular matrix ( $\Delta agfD$ ) and capsule mutants ( $\Delta emcG$ ,  $\Delta emcE$ ,  $\Delta Pemc$ ) was compared after lyophilization. Colonies were grown on T agar for 6 days at 28°C, peeled off the agar surface, frozen and lyophilized for one week. Bars represent the average number of CFU per colony and standard deviations corresponding to three individual colonies. The asterisks shows statistical significance compared to *wt* survival values. (One-way ANOVA with Bonferroni's post-test \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

#### 4.4 Discussion

This study reports that *Salmonella* produces an O-Ag capsule associated with the extracellular matrix. Structural analysis yielded a repeating oligosaccharide unit similar to *Salmonella* Enteritidis LPS O-Ag with some modifications. The previously uncharacterized *yih* operons were shown to be important for capsule assembly and translocation and thus were renamed *emcA-H* and *emcIJ* for extracellular matrix capsule. The *emcIJ* operon, coding for a putative repressor of *emcA* transcription, was repressed

in the presence of AgfD. This suggested that capsule assembly and translocation was regulated along with Tafi and cellulose as part of the extracellular matrix. The *emc* genes are conserved throughout *Salmonellae*, which is important because our results indicate that the O-Ag capsule plays an important role in desiccation tolerance and thus could play a role in environmental persistence facilitating *Salmonella*'s cyclic lifestyle aiding the transmission of cells through a host, into the environment, and back into a new host (Winfield and Groisman, 2003).

The SE 3b O-Ag capsule was structurally similar to the *Salmonella* Enteritidis LPS O-Ag; however, this is not unusual. Capsular and O-Ag polysaccharides are the same in some strains of *E. coli* (Ali *et al.*, 2005; Goldman *et al.*, 1982), in *Vibrio ordalii* (Sadovskaya *et al.*, 1998) and are classified as group 4 O-Ag capsules in *E. coli* (Whitfield and Roberts, 1999). Since the major EPS identified has the biochemical characteristics of a capsule and the repeat unit is structurally similar to the SE LPS O-Ag, we propose to classify it as a group 4 O-Ag capsule. The *Salmonella* O-Ag capsule does have some structural modifications, however, most notably a glucose polymer linked to the tyvelose residue. Furthermore, the O-Ag capsule has > 2300 repeat units compared to LPS, which normally has 16-100 repeat units (Murray *et al.*, 2003; Whitfield *et al.*, 1997). The capsule and LPS differ in modifications, size, charge, and lipid attachment (Snyder *et al.*; submitted). These differences are adequate to confer immunological distinction to the capsule. Other HMW polysaccharides have conformationally dependent epitopes due to extended helices formed by large polysaccharides and their modifications (Brisson *et al.*, 1997). Thus, we presume that our serum predominately recognizes conformationally dependent epitopes on the O-Ag capsule. Recently, another report of a *Salmonella* capsule purified from the extracellular matrix was published (de Rezende *et al.*, 2005). However, this capsule does not appear to have the same chemical composition as the O-Ag capsule since rhamnose was absent from compositional analysis (de Rezende *et al.*, 2005). Furthermore, the reported capsule was important in biofilm maturation (de Rezende *et al.*, 2005), whereas the O-

Ag capsule did not significantly contribute to biofilm formation under similar conditions (data not shown). Finally, we found the *emcA* genes were inactive at 37°C in *Salmonella* Typhimurium due to reduced AgfD levels (data not shown), but the capsule reported by de Rezende (2005) was detected at both 28 and 37°C. It is possible that the second, minor EPS identified here, in association with the O-Ag capsule, could represent the capsule reported by de Rezende (2005).

We propose that the *emcABCDEFGH* and *emcIJ* operons are responsible for capsule surface assembly and translocation. From homology searches and comparisons to known gene functions we were able to make the following putative assignments of function: EmcA represents a dehydrogenase; EmcB an aldolase; EmcD an epimerase, all of which have conserved domains related to carbohydrate transport and metabolism. EmcC has homology to ManC (RfbM), a mannose-1-phosphate guanylyltransferase which is involved in the formation of an activated sugar nucleotide precursor for residues in cell surface polysaccharides (Jayaratne *et al.*, 1994). Thus EmcC may be involved in activating sugar nucleotide precursors involved in side chain modifications. EmcE has conserved domains related to carbohydrate transport, is a member of the family 31 glycosyl hydrolases, has  $\alpha$ -glucosidase activity in *E. coli* (Okuyama *et al.*, 2004) and has sequence identity to two members of the family 31 glycosylhydrolases that also have glucosyltransferase activity: CtsZ (25%), a 6-glucosyltransferase from *Bacillus globisporus* C11 (Aga *et al.*, 2002) and YicI (24%), an  $\alpha$ -xylosidase from *E. coli* (Okuyama *et al.*, 2004). Furthermore, we have shown that in the absence of EmcE, the capsule is not assembled or expressed on the cell surface. Thus EmcE could act as a glucosyltransferase that catalyzes sugar, possibly glucose transfer onto the tyvelose residue of the repeating unit and may also be required for efficient capsule translocation. EmcG is a putative membrane glucuronide transport protein; in an isogenic *emcG* mutant, the capsule was not exported. We hypothesize that EmcG is important in capsule translocation and since it is predicted to be an integral membrane lipoprotein with significant  $\alpha$ -helical content, EmcG may represent the functional

equivalent to Wza involved in group 1 and 4 capsule translocation (Whitfield and Roberts, 1999; Nesper *et al.*, 2003). EmcF is homologous to EmcG but was not sufficient to transport the capsule in the isogenic  $\Delta emcG$  mutant. For *emcH* (*yshA*), we found no predicted promoter upstream of *emcG* and thus assume that it is transcribed as a part of the *emc* operon although currently we do not have any transcriptional evidence. EmcH encodes a putative outer membrane protein (Condemine *et al.*, 2005) involved in sugar transport (Dartiglongue *et al.*, 2000) and thus could also be involved in capsule surface assembly or the translocation complex. From the divergent *emcI* operon, *emcJ* encodes a putative negative repressor and in the promoter-lux fusion assays described, when *emcI* expression was high (i.e., in the absence of AgfD), *emcA* expression was low and vice versa. It is possible that EmcJ negatively regulates the expression of *emcA*, although other regulatory effects are also feasible. For example, EmcI encodes a putative kinase and thus may have an analogous function to the kinase, Wzc, which negatively regulates colanic acid biosynthesis when autophosphorylated (Paiment *et al.*, 2002). *yihX*, encoding a phosphatase, is directly adjacent to *emcI* but predicted to have its own promoter. *yihX* may be a part of the *emc* gene cluster and represent a functional equivalent to Wzb, a phosphatase involved in group 1, group 4 capsule and colanic acid assembly translocation (Whitfield and Roberts, 1999). Biochemical enzyme analyses will be required to conclusively assign the function of each protein in the synthesis of the capsule. Since the *emc* operons contain relatively few genes coding for transferases and structural enzymes and the repeat unit is similar to LPS O-Ag we assume that the O-Ag biosynthetic genes are utilized to synthesize the capsule. We did not identify gene inactivations in any of the LPS O-Ag biosynthetic operons since P22 phage were used to transfer mutations. P22 phage are known to bind LPS O-Ag prior to infection and are unable to infect strains containing alterations in the O-Ag structure (Steinbacher *et al.*, 1997). As a result, Tn10dCm mutations causing alterations in O-Ag structure would not have been represented in the transducing lysate prepared from SE 3b. Together with biochemical, structural and bioinformatic analyses, our results indicate that the *emc*

operons are important for O-Ag capsule surface assembly and transport and putatively encode the functional equivalent of Wza, Wzc and possibly Wzb proteins involved in group 1 and 4 capsule translocation.

Capsule expression was co-regulated with the extracellular matrix by AgfD from the Tafi operon. *emcI* expression was repressed in the presence of AgfD and this coincided with *emcA* activation. Furthermore, bioinformatic predictions (Softberry, Mount Kisco, NY) revealed several known operator binding sites in the *emc* intergenic region (supplementary material) which suggest that capsule synthesis may be activated in the presence of amino acids, low oxygen and low glucose, similar to conditions for *agfD* activation (Gerstel and Romling, 2003). A putative binding site for AgfD (Brombacher *et al.*, 2003) that is highly conserved amongst *Salmonellae* (A.P. White and M. G. Surette, in preparation) was not found in the *emc* intergenic region suggesting the repressive effect is probably indirect.

The conservation of the *emc* operons throughout *Salmonellae* suggests that the capsule may play an important role in the lifecycle of *Salmonella*. The extracellular matrix genes and their expression for multicellular pattern formation is a highly conserved physiological phenomenon of *Salmonellae* (A.P. White and M. G. Surette, in preparation). Considering the gene conservation and AgfD dependent regulation under the specified conditions, the capsule is likely involved in this multicellular process. Other members of the *Enterobacteriaceae* family produce Tafi and cellulose (Zogaj *et al.*, 2003) and several sequenced *E. coli* strains also possess the *emc* operons (<http://globin.cse.psu.edu/enterix/enteric/enteric.html>; Florea *et al.*, 2003). Since each LPS O-Ag repeating unit is expected to be unique, it is possible that each species or serovar uses their own O-Ag biosynthetic enzymes for capsule synthesis and the *emc* operon for assembly and translocation such as we found in SE 3b. Although *Salmonella* and *E. coli* have been rigorously studied as planktonic cells grown in broth, the O-Ag capsule was probably only recently discovered as a result of the recent advances of knowledge concerning the importance of multicellular behaviour in bacteria.

Our previous studies have shown that the extracellular matrix promotes environmental persistence of *Salmonella* in the face of desiccation stress (White *et al.*, 2006). We used lyophilization as a simple and efficient test of bacterial components important for long-term survival. Our results closely mirrored those of White *et al.*, performed on colonies that had been stored for 9 months on plastic. We demonstrated that the capsule is primarily responsible for desiccation tolerance in SE 3b. Although Tafi did contribute to desiccation tolerance we cannot rule out that this is due to a pleiotropic effect considering a Tafi mutant has reduced capsule production. We hypothesize that the main role of Tafi is to organize the extracellular matrix, perhaps maximizing coverage of cells by the capsule or locking it onto the cell surface via a non-covalent interaction, whereas the main role of the capsule may be to protect the cells against desiccation stress when faced with adverse conditions.

Curiously, this study revealed a link between flagella and capsule expression. Flagella can detect and respond to wetness (Bokranz *et al.*, 2005) and thus this connection may involve the cell's ability to detect its hydration state. Considering that the O-Ag capsule is important in desiccation tolerance, this might indicate that flagella are important in relaying the wetness signal to the *emc* genes. Further experimentation is required to determine the details of this relationship.

The *Salmonella* O-Ag capsule plays an important role in mediating protective effects against desiccation and some antimicrobials (supplementary material). This may be relevant for the proposed life cycle of *Salmonella* spp. aiding the transmission of encapsulated cells through a host, into the environment, and back into a new host, by enhancing survival in non-host environments (Winfield and Groisman, 2003). It is possible the O-Ag capsule is a contributing factor to the survival of *Salmonella* spp. on desiccated foods (Hiramatsu *et al.*, 2005). Furthermore, other capsules (Vuong *et al.*, 2004) and the extracellular matrix (Guard-Petter *et al.*, 1996) are important in virulence. Thus the O-Ag capsule reported here may also have an important role during the infection process but this remains to be tested. Nevertheless, we have demonstrated that

the O-Ag capsule is an important component of the *Salmonella* extracellular matrix involved in environmental persistence.

Table 4.III. Primers used in this study.

Product	Reaction	Primer Sequence (5'-3')
Tn10dCm <sup>a</sup>	PCR1	Forward: cat01 (CAGGGTCGTTAAATAGCCGC) Reverse: arb1 (GGCCACGCGTCGACTAGTACNNNNN NNNNNGATAT)
	PCR2	Forward: cat02 (CCGTTGCTTCTCAAATGCC) Reverse: arb2 (GGCCACGCGTCGACTAGTAC)
	Sequencing	cat02
$\Delta$ emcG <sup>a</sup>	PCR 1	Forward: emcG-A (AGCCCAAGCTTAAAAACCCATC CATGA) Reverse: emcG-B (TCTAATCATGATCCGCGTTAATTA ATTAACGCCACTCACTTTACTTTACCCGTTAAAC A)
	PCR 2	Forward: emcG-C (AGTAAAGTGAGTGGCGTTAATTA ATTAACGCGGATCATGATTAGACATAATCGCCTCT CA) Reverse: emcG-D (TCGGCGGGCTGTTAATATGG)
	Screening	Forward: (ACTCGGGATGCCTGCATATT) Reverse: emcG-A

<i>ΔemcE</i> <sup>a</sup>	PCR 1	Forward: emcE-A (ACCGTAAGCTTTTACGCCTGCGT TGCGC) Reverse: emcE-B (AATTCTCTACCACAACGTTAATTA ATTAACCTGCAAAACGACGACCTGCACCATAACC GT)
	PCR 2	Forward: emcE-C (GTCGTCGTTTTGCAGGTTAATTA TTAACGTTGTGGTAGAGAATTCATTGTAATCTCCG T) Reverse: emcE-D (TCGGTTAGATGCCGCCAGTG)
	Screening	Forward: (ATTGACATGTTTCGCGGCAA) Reverse: emcE-A
<i>Pemc</i> <sup>a</sup>	PCR 1	Forward: Pemc-A (AGGGGAAGCTTGCTCTACTTGCT CCGCC) Reverse: Pemc-B (CGAAATGTTTAAAAAAAAAAAGC TGTC AATGTATTGACCCCCCCCCCAAATACGAAA AAAACCCCCCCCCCCTGCTCATT AATGAC)
	PCR 2	Forward: Pemc-C (GCATTAATGAGCAGGGGGGGGG GGGTTTTTTTTTCGTATTG GGGGGGGGGGGTCAATA CATTGACAGCTTTTTTTTTTAAACATTTTCG) Reverse: Pemc-D (AGCCTGACGCGCCAGCGTAA)
	Screening	Forward: (GCATTTTTCTGTCTGTTTGTGATCG) Reverse: (GTGACGGCTGTCAATTTTTTTGA)
<i>PemcA-I</i> <sup>b</sup>		Forward: emc1 (GCCCTCGAGCCGATAAATGCTATA ACTG) Reverse: emc2 (GCCGGATCCCAAGCAATACGAACC AT)

$\Delta agfD^a$	PCR 1	Forward: agfD-A (GACGAATTCGTGTGTTATGCCGCC ATGGG) Reverse: agfD-B (GGA CTGCAGTAAACATGATG)
	PCR 2	Forward: agfD-C (GCCCTGCAGCAAACGATAATCTC AGGCGG) Reverse: agfD-D (GCCAAGCTTTGTCCGTGACGTTGA GCTGG)
	Screening	Forward: agfD K/O 2 (CACTTGCTTTAAGATTTGTA ATGGC) Reverse: agfD K/O 1 (ATTCGCTTTCCATTTGTCG)

<sup>a</sup>, PCR primers were purchased from Alpha DNA ([www.alphadna.com](http://www.alphadna.com)).

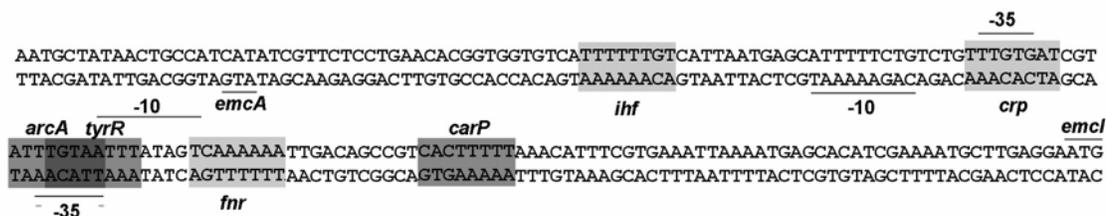
<sup>b</sup>, PCR primers were purchased from University Core DNA and Protein Services ([www.dnaservices.myweb.med.ucalgary.ca](http://www.dnaservices.myweb.med.ucalgary.ca); University of Calgary)

## 4.5 Supplementary Material

### 4.5.1 Predicted operator sites in the *emc* intergenic region

The 158 bp *emcA-I* intergenic region was analyzed for operator sites using BPROM software (Softberry, Mount Kisco, NY). The results revealed six putative sites: *tyr*, *arc* and *car* upstream of *emcI* and *fnr*, *crp* and *ihf* upstream of *emcA* (Fig. S1). In the presence of aromatic amino acids, the tyrosine repressor protein (TyrR) binds to the *tyr* box resulting in transcriptional repression (Pittard *et al.*, 2005). The putative repressive effect of TryR is supported by the located of the *tyr* box in the putative -10 region of the *emcI* putative promoter. This suggests that *emcA* transcription is dependent on the presence of amino acids via transcriptional repression of *emcI*. An *arc* box upstream *emcI* and a *fnr* box upstream *emcA* suggest that oxygen levels affect capsule synthesis. The two-component *acrBA* system, activated in microaerophilic conditions and repressed in an RpoS dependent manner (Sevcik *et al.*, 2001), is

responsible for anaerobic repression (Iuchi *et al.*, 1990). The putative repression of the *emcI* promoter by the Arc system is supported by the location of the *arc* box in the -10 region. FNR is a major regulator controlling the physiological switch between aerobic and anaerobic growth conditions and the expression of many genes are known to be involved in anaerobic respiration, while those repressed are involved in aerobic respiration (Kang *et al.*, 2005). This suggests that *emcI* transcription is repressed and thus capsule synthesis inaugurates concurrent with oxygen tension during the stationary phase of growth. The catabolite repression boxes, *car*, located upstream of *emcI* and *crp* located upstream of *emcA* suggests capsule synthesis is affected by glucose concentration. In low glucose the *car* site would be unbound (Hoang *et al.*, 2004) and thus transcription of *emcI* would be inhibited permitting active transcription from *emcA*. Additionally, in low glucose, cAMP levels are high supporting CRP-*cap* binding (de Crombrughe *et al.*, 1984), further activating *emcA* transcription. Finally, an *ihf* box upstream *emcA* indicates the integration host factor (IHF), which in *E. coli* is involved in control of transcription of over 100 genes of widely varying function including virulence (McLeod and Johnson, 2001), affects a wide variety of genes including *agfD* (Gerstel *et al.*, 2003), also has an effect on *emcA* transcription. The putative transcription factor binding sites in the *emc* intergenic region predict that transcription is favoured in the presence of amino acids during oxygen tension, limiting glucose and in the stationary phase of growth. These predictions are substantiated by maximal *emcA* expression and minimal *emcI* expression conditions of 1% tryptone, under microaerophilic conditions and in the stationary phase of growth experimentally determined for *lux* assays (data not shown).



**Figure 4. 9. Predicted operator sites in the *emc* intergenic region.**

The 158 bp sequence of the predicted *emc* intergenic region is depicted. The putative translational start sites are underlined on the coding strand and labeled with the first gene of the operon. The putative operator sites upstream the translational start sites are highlighted light grey for *emcA* and dark grey for *emcI* where *arcA* and *tyrR* overlapping sequence is indicated by a darker shade of dark grey. The putative -10 and -35 regions of the promoter are underlined on the coding strands for each operon.

#### 4.5.2 The O-Ag capsule protects against diffusion dependent antibiotics

To determine if presence of the O-Ag capsule could mediate resistance to different antibiotics (Table SI), various Etest® strips were used to compare minimum inhibitory concentrations (MICs) between wt,  $\Delta bcsA$  and  $\Delta bcsA$  Tn10dCm::*emcG* strains. The O-Ag capsule-deficient *emcG* strain was more susceptible to ceftriaxone, gentamicin and colistin than the O-Ag capsule-positive strains (Table SII). In contrast, no MIC differences between strains were detected for tobramycin, sulfamethoxazole-trimethoprim or ciprofloxacin. Biofilm related EPS, contributes to antibiotic resistance (Mah and O'Toole, 2001). Similarly, the *Salmonella* Enteritidis 3b O-Ag capsule does limit the killing effects of the antimicrobial colistin, and to some extent ceftriaxone and gentamicin. In contrast the O-Ag capsule did not confer resistance to the action of sulfamethoxazole-trimethoprim, ciprofloxacin or tobramycin. Colistin is a large cationic detergent comprised of a cyclic peptide with a long hydrophobic tail. Like other polymyxins, it disrupts the bacterial cell membrane by interacting with phospholipids causing cell leakage and death. Both ceftriaxon and gentamicin are dependent on porin mediated penetration through the outer membrane. Although

biofilm related antibiotic resistance is multifactorial (Mah and O'Toole, 2001), it is possible the O-Ag capsule limits the transport of the larger cationic colistin to the OM decreasing its penetration across the membrane. Alternatively, other antimicrobial resistance factors could be activated as a consequence of O-Ag capsule expression. Further experimentation is required to detail the exact molecular details of resistance.

Table 4.IV. Antibiotic characteristics.

Antibiotic	Type	Molecular weight*
Colistin (polymyxin E)	cationic detergent	1759.90
Ceftriaxone	3 <sup>rd</sup> generation cephalosporin	598.54
Gentamicin	aminoglycosides	463.58
Trim/sul (bactrim)	PABA analogue	290.32
Ciprofloxacin	quinolone	331.34
Tobraycin	aminoglycosides	467.51

\*, taken from SIGMA-Aldrich ([www.sigmaaldrich.com/Area\\_of\\_Interest/Biochemicals/Antibiotic\\_Explorer](http://www.sigmaaldrich.com/Area_of_Interest/Biochemicals/Antibiotic_Explorer)).

Table 4.V. Etest® for MIC determination of *Salmonella* Enteritidis 3b strains.

Antibiotic	MIC* (ug/mL)	<i>wt</i>	$\Delta$ <i>bcsA</i>	$\Delta$ <i>bcsAemcG::Tn10dCm</i>
Colistin	N/D	64	64	12
Ceftriaxone	9	.094	.047	.023
Gentamicin	4	1	1	.25
Trim/sul (bactrim)	2	.064	.064	.064
Ciprofloxacin	1	.012	.012	.023
Tobramycin	4	1	1	1

\*, taken from the Clinical and Laboratory Standards Institute MIC Interpretive standards for *Enterobacteriaceae* Table 2A; N/D; not determined for *Salmonellae*.

## **5.0 A unique acidic polysaccharide of the *Salmonella* extracellular matrix is associated with multicellular behavior.**

### **5.1 Introduction**

The surfaces of many natural isolates of bacteria are covered by EPS. EPS are key components of biofilm matrices and promote adherence of bacteria to surfaces and to each other. Several examples indicate that in their absence, cells lack the ability to form a mature biofilm (Danese *et al.*, 2000; O'Toole *et al.*, 2000; Stoodley *et al.*, 2002; Sutherland, 2001). Additionally, EPS have important roles in immune evasion, tolerance toward antibacterial agents (Roberts, 1996) and desiccation (Ophir and Gutnick, 1994), the latter because the molecules are highly hydrated holding up to 95% water (Roberson and Firestone, 1992). EPS also facilitate swarming, a multicellular surface motility behavior in *Proteus mirabilis* by providing the cells with lubricant reducing friction over the cell surface (Gygi *et al.*, 1995).

*Salmonella* has a cyclic lifestyle consisting of passage through a host into the environment and back into a new host (Winfield and Groisman, 2003). This has been attributed to *Salmonella's* ability to survive harsh conditions outside of its host thereby providing opportunities to spread from one host to another (Winfield and Groisman, 2003). For example, *Salmonella* has been detected within cow farms, pig farms, and slaughterhouses; both before and after sacrifice (Winfield and Groisman, 2003) despite routine disinfections (Davies and Wray, 1995). This is expected to provide opportunities for the microbe to spread from one host to another (Winfield and Groisman, 2003). Since environmental persistence or survival is a critical stage to the cycle, *Salmonella* must employ factors to enhance its survival under adverse conditions such as those found outside an animal where temperature and pH fluctuate and there are stresses such as limited nutrient availability, desiccation, antimicrobials and predation.

There is a strong correlation between biofilm formation, pellicle formation, rdar (red, dry and rough) morphology, bacterial aggregation and the extracellular matrix

(Allen-Vercoe *et al.*, 1997; Anriany *et al.*, 2001; Collinson *et al.*, 1993; Romling *et al.*, 1998; Romling and Rohde, 1999; Romling *et al.*, 2003; Romling, 2005; Solano *et al.*, 2002; White *et al.*, 2005). Bacteria with a rdar morphology are connected in a dense, rigid cellular network that spreads across the agar surface and can be peeled off intact (Anriany *et al.*, 2001; Collinson *et al.*, 1993; Romling *et al.*, 1998; White *et al.*, 2005). These characteristics are a result of a highly coordinated and organized multicellular behavior (Romling *et al.*, 1998; Romling *et al.*, 2000; White *et al.*, 2005).

Until recently, the *Salmonella* extracellular matrix has not been clearly defined, but is minimally composed of Tafi (Collinson *et al.*, 1993; Romling *et al.*, 1998), cellulose (Zogaj *et al.*, 2001), O-Ag capsule and another acidic EPS (Gibson, submitted; White *et al.*, 2003). Temporal gene expression studies indicate that formation of the extracellular matrix is initiated by Tafi expression (White *et al.*, 2005) where Tafi organizes cells by mediating cell-to-cell short range interactions and cellulose mediates long range cell-to-cell interactions (Romling *et al.*, 2000). The associated rdar morphotype is highly conserved amongst *S. enterica* (Romling *et al.*, 2003; White *et al.*, 2005) and has been shown to confer resistance to USDA approved levels of chlorine which kill their planktonic counterparts (Scher *et al.*, 2005; White *et al.*, 2005). Furthermore, Tafi stabilizes the O-Ag capsule on the cell surface, which plays a role in desiccation tolerance (Gibson, submitted).

The extracellular matrix components, Tafi, cellulose and O-Ag capsule are regulated by AgfD (CsgD). Thus AgfD is considered the regulatory checkpoint in multicellular behavior characterized by the rdar morphotype (Gerstel *et al.*, 2003; Gerstel and Romling, 2003). AgfD is influenced by the global regulatory proteins MlrA (Brown *et al.*, 2001), RpoS (Olsen *et al.*, 1993; Romling *et al.*, 1998), OmpR (Sauer *et al.*, 2004; Vidal *et al.*, 1998), CpxR (Prigent-Combaret *et al.*, 2001), RcsB (Jubelin *et al.*, 2005), a thermosensor Crl (Arnqvist *et al.*, 1992; Bougdour *et al.*, 2004) and the architectural proteins H-NS (Arnqvist *et al.*, 1994; Olsen *et al.*, 1993) and IHF (Gerstel and Romling, 2003).

During previous studies which identified an O-Ag capsule co-regulated with the *Salmonella* extracellular matrix, we discovered another novel acidic EPS (Gibson, submitted). The purpose of this study was to identify the EPS and reveal genes important for regulation or expression using a Tn10dCm mutagenesis approach. Using this technique we did not identify the polysaccharide assembly operon, but we found other mutations inactivating genes involved in quorum sensing AI-2 degradation, flagella repression and Tafi and TolA expression as important to EPS surface expression. All the mutants had their multicellular behavior disrupted as determined by pellicle formation, morphology on TCR and calcofluor binding. Furthermore, all the mutants had significantly reduced Tafi expression or none at all. These results implicate quorum sensing AI-2 degradation and flagella repression in the complex regulation of the extracellular matrix.

## 5.2 Material and methods

### 5.2.1 Bacterial strains, growth conditions and culture media

*Salmonella enterica* subspecies *enterica* serovar Enteritidis 27655-3b (Feutrier *et al.*, 1986) and mutant strains were routinely grown at 37°C for 24 h in 1% Tryptone broth or on T agar supplemented with CR or (Collinson *et al.*, 1991) supplemented with 200 µg/mL calcofluor (SIGMA-ALDRICH) for 24 and 48 h at 37° C and imaged at 312 nm using a MultiImage™ Light Cabinet (Alpha Innotech Corporation). For EPS purification experiments, *Salmonella* Enteritidis  $\Delta bcsA$  Tn10::*galE* (White *et al.*, 2003) was grown on M9MM + 0.5% glucose at 28°C for 5 days. For transposon mutagenesis, *Salmonella* Enteritidis 3b transduction mixtures and  $\Delta bcsA$  Tn10dCm mutants were incubated at 37° C overnight in Lb medium or broth supplemented with 50 µg/ml kanamycin, 12 µg/mL chloramphenicol, and 10 mM EGTA.

### 5.2.2 EPS purification

Cells were scraped off agar surfaces, resuspended in 1% phenol and incubated at RT for 20 min. The cellular debris was pelleted by centrifugation (16000 X g, 4°C, 5 h). The aqueous phase of the supernatant was removed and four volumes of ice-cold acetone were added on ice while constantly stirring with a glass rod for 10 min. The precipitated material was allowed to pellet at -20°C overnight and the acetone was decanted. The EPS was washed with acetone and air-dried at RT overnight. The material was solubilized in dH<sub>2</sub>O with gentle agitation and heat. This solution was dialyzed (MW 6-8) overnight with several dH<sub>2</sub>O changes and lyophilized. Samples were further purified by incubating in an equal volume of 3% CTAB. The material was centrifuged (2500 X g) and the precipitate was dissolved in 10% NaCl and reprecipitated by the addition of 2 volumes of acetone. This precipitate was resuspended in 10% NaCl, dialyzed three times versus 1% NaCl and then three times against dH<sub>2</sub>O and lyophilized.

### 5.2.3 Compositional analysis

Glycosyl composition analysis was performed by gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The monosaccharides were identified by their retention times in comparison to standards, and the carbohydrate character was authenticated by the mass spectra.

### 5.2.4 Generation of polyclonal serum against EPS

Crudely purified EPS was subjected to SDS-PAGE and resolved by electrophoresis. The stacking gel containing the EPS material was excised and broken up using a tissue homogenizer. The EPS material was conjugated to KLH (PIERCE) and BSA (SIGMA) following procedures of Conlan *et al* (Conlan *et al.*, 1999). The conjugation was confirmed by SDS-PAGE, staining for proteins and immunoblot

analysis using serum generated against whole Tafi (Collinson *et al.*, 1991). The EPS-KLH conjugate prepared in Emmulsigen™ adjuvant (MPV Laboratories) was used to immunize a New Zealand White rabbit. Subcutaneous and intramuscular injections of 200 µg were performed three times at two-week intervals with a final boost of 100 µg. Three weeks following the final booster injection, serum was collected and the titre determined by ELISA (Engvall, 1976), using the EPS-BSA conjugate.

### 5.2.5 Transposon Mutagenesis

*Salmonella* Enteritidis 3b harboring pNK (Bender and Kleckner, 1992) was infected with a P22 HT 105/1 int-201 phage (Schmieger, 1972) lysate of *Salmonella* Typhimurium ATCC 14028 containing Tn10dCm on F' (Elliott and Roth, 1988). The resulting transductants were pooled (20,000-30,000 colonies) and a P22 *Salmonella* Enteritidis 3b  $\Delta bcsA$  Tn10dCm transducing fragment library was created using the method of Maloy (Townsend *et al.*, 2001). The P22 *Salmonella* Enteritidis Tn10dCm transducing fragment library was used to create Tn10dCm insertions via homologous recombination of Tn10dCm-containing transducing fragments with the *Salmonella* Enteritidis 3b chromosome in the absence of the plasmid-encoding transposase. The resulting stable Tn10dCm mutants were picked into 55 clear, flat-bottomed, sterile, 96-well, cell culture plates (Costar®, Corning, N.Y.) and stored in 5% glycerol at -70°C.

### 5.2.6 EPS mutant screening and gene identification

The resulting stable Tn10dCm mutants were picked into 55 clear, flat-bottomed, sterile, 96-well, cell culture plates (Costar®, Corning, N.Y.) and stored in 5% glycerol at -70°C. Tn10dCm mutants were inoculated from glycerol stocks using a sterile 48-pronged metal stamp into 100 µl 1% tryptone, pH 7.2 broth in clear flat-bottom sterile 96-well cell culture plates. Growth was measured at A<sub>595</sub> using an ELx 808 Biotek Ultra microplate reader (Biotek) after 24 h growth at 37°C. Whole cells were heat fixed at 85°C in a hybridization oven for 6 h or until all the media had evaporated.

ELISA experiments were performed according to the method of Engvall (Engvall, 1976) in high-binding, flat bottom, polystyrene 96-well plates (Costar) using rabbit polyclonal serum generated against purified EPS followed by goat anti-rabbit immunoglobulin G-alkaline phosphate (Cedarlane Laboratories Ltd.). The absorbance in each well was measured at  $A_{405}$  and the final  $A_{405}/A_{595}$  values were calculated and compared to relative % of EPS on the *wt* surface where *wt* was 100% and background was *Salmonella* Enteritidis 3b in-frame *ΔagfD* mutant (Gibson, submitted). Mutants with less than 10% capsule on the cell surface were re-screened, streaked onto EBU plates to render the mutants phage-free and re-screened for maintenance of the phenotype. Genomic DNA for PCR, was prepared following the method of Walsh *et al* (Walsh *et al.*, 1991). Sites of Tn10dCm insertion in the chromosome were amplified by arbitrary primed PCR described by Welsh (Welsh and McClelland, 1990).

### 5.2.7 Tafi expression

For ELISA, cells were scraped off T agar plates and resuspended in 10 mM Tris-HCl (pH 7.2) and adjusted to  $A_{600}$  0.1 in each well and heat fixed at 90°C for 4 h. AgfA was detected using AgfA-specific monoclonal antibody ascites followed by goat-anti-mouse G-alkaline phosphatase conjugates (Cedarlane Laboratories Ltd.). For western analysis, Tafi fimbrial protein cell pellet samples were prepared from 1  $A_{600}$  as previously described (White *et al*, 2003). Tafi were detected using polyclonal serum generated to whole Tafi (Collinson *et al*, 1991) followed by goat-anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR, Ltd.). Immunoreactive material was visualized using an Osysey™ Biosciences scanner (LI-COR, Ltd.).

### 5.2.8 SDS-PAGE, protein staining and immunoblotting

Cells were resuspended in 1 mL of 10 mM Tris-HCl (pH 8.0) and adjusted to  $A_{600}$  0.1 in SDS-PAGE sample buffer and boiled for 10 min prior to electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) with

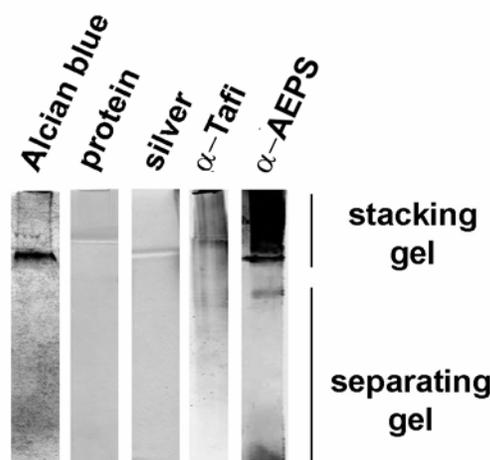
a 5% stacking gel and 12% resolving gel. Purified EPS were electrophoresed by SDS-PAGE and stained with Gelcode™ (Bio-Rad), Alcian blue (SIGMA-ALDRICH), LPS silver stained (Bio-rad) or electrophoretically transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell™ (Bio-Rad Laboratories) in buffer as recommended by the manufacturer. Immunoblots were detected using rabbit polyclonal immune serum generated to whole Tafi or purified EPS followed by goat-anti-rabbit immunoglobulin conjugated to IRDye800 (LI-COR, Ltd.). SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) with a 5% stacking gel and 12% resolving gel. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose using a Mini Trans-Blot Electrophoretic Transfer Cell™ (Bio-Rad Laboratories) in buffer recommended by the manufacturer.

### 5.3 Results

#### 5.3.1 A *Salmonella* acidic EPS associated with the extracellular matrix

We recently found that *Salmonella* Enteritidis 3b produced an O-Ag capsule co-regulated with the extracellular matrix components Tafi and cellulose (Gibson, submitted). From these and other studies it was apparent that an acidic EPS was also present (Gibson, submitted; White *et al.*, 2003). Therefore, to characterize the EPS associated with the extracellular matrix, polysaccharides were purified from a cellulose deficient (*ΔbcsA*) and O-Ag capsule (*Tn10::galE*) deficient strain of *Salmonella* Enteritidis 3b. To analyze the EPS biochemically, it was resolved by SDS-PAGE and stained directly or transferred to nitrocellulose for immunoblotting. A diffuse band corresponding to the stacking gel did not react with protein- or LPS-specific stains and was stained by the acidic polysaccharide stain, Alcian blue (Fig. 5.1), similar to other EPS (Gibson, submitted; Junkins and Doyle, 1992; Waldor *et al.*, 1994). The diffuse material was recognized by serum generated against whole Tafi and purified EPS (Fig.

5.1). Together, these results indicated the EPS was the acidic EPS found associated with Tafi (White *et al.*, 2003) and the O-Ag capsule (Gibson *et al.*, submitted).



**Figure 5. 1. The *Salmonella* EPS associated with the extracellular matrix.** SDS-PAGE and immunoblot analysis of 10  $\mu$ g of purified acidic EPS (AEPS) from *Salmonella* Enteritidis 3b  $\Delta$ bcsA Tn10::galE.

### 5.3.2 Composition of the novel EPS

Compositional analysis indicated that the EPS is composed of Gal, Xyl, GluA and Glu in a stoichiometric ratio of 5:1:0.6:0.2 (Table 5.I). Also present were four monosaccharides that did not match any of the standards where three of these monosaccharides were methylated. As well the presence of 3-hydroxymyristic acid was observed. Since the EPS lacked detectable levels of other LPS constituents such as KDO, glucosamine, and core sugars the detection of the fatty acid is consistent with it being part of a lipid anchor. The total carbohydrate was only 33% indicating other non-carbohydrate substituents were present and may be a part of the EPS molecule, but further analysis will be required to identify these components. Although the EPS was

purified from *Salmonella* Enteritidis  $\Delta bcsA$  Tn10::*galE* which can not generate Gal in the absence Gal deficient agar media, one of the major sugars identified was Gal. Since Gal is not present in tryptone, we presume the mutant was able to scavenge Gal from the agar. Thus EPS from *Salmonella* Enteritidis  $\Delta bcsA$  Tn10::*galE* is probably impeded by the *galE* mutation but is not prevented.

Table 5.I. Glycosyl composition analysis of *Salmonella* Enteritidis 3b EPS.

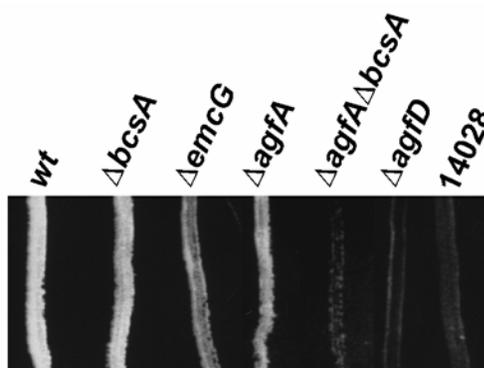
Glycosyl residue	Mass ( $\mu\text{g}$ )	Mole % <sup>1</sup>
Xylose	8.3	15.0
Glucuronic acid	6.1	8.5
Galactose	49.2	73.9
Glucose	1.8	2.7
Unkown sugar	+	+
Methylated unknown sugars	+	+
3OH-tetradecanoic acid	+	+
Sum	65.4	100.00

<sup>1</sup>; values are expressed as mole percent of total carbohydrate where the total mass of the carbohydrate was calculated to be 33%

### 5.3.3 Mulicellular behavior is important for EPS surface expression

Calcofluor is a fluorescent agent that non-specifically binds to  $\beta$ -glucans and is often used to examine EPS expression on bacteria including cellulose expression on *Salmonella* (Romling *et al.*, 1998; Solano *et al.*, 2002; White *et al.*, 2005). Calcofluor binding was examined on *Salmonella* Enteritidis 3b strains (Fig. 5.2). The *wt*,  $\Delta bcsA$  (cellulose) and  $\Delta emcG$  (O-Ag capsule) isogenic mutants were fluorescent when grown on media containing calcofluor but an  $\Delta agfD$  isogenic mutant was not (Fig. 5.2). This

suggested *Salmonella* Enteritidis 3b produced an additional EPS other than cellulose and O-Ag capsule that was positively influenced by the extracellular matrix transcriptional regulator, AgfD. However, an in-frame double mutant ( $\Delta agfA\Delta bcsA$ ; Tafi and cellulose) with multicellular behavior abolished did not fluoresce but the individual Tafi ( $\Delta agfA$ ) or cellulose ( $\Delta bcsA$ ) mutants were fluorescent suggesting that multicellular behavior itself and not necessarily AgfD alone was influencing EPS expression.



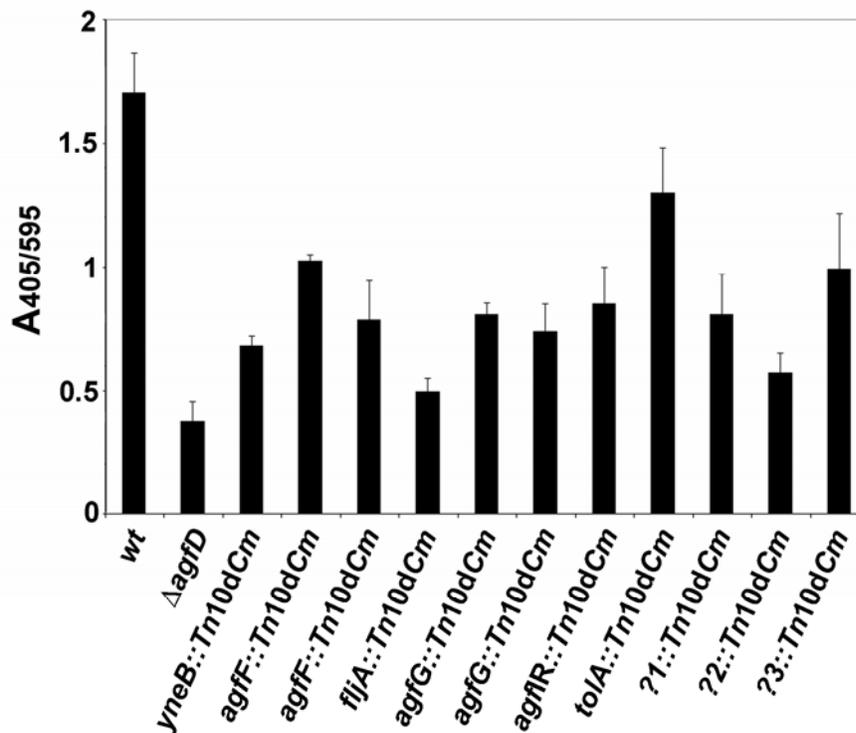
**Figure 5.2. Calcofluor binding of *Salmonella* Enteritidis 3b and extracellular matrix mutants.**

*Salmonella* Enteritidis 3b and in-frame mutations in extracellular matrix components ( $\Delta bcsA$ , cellulose;  $\Delta emcG$ , O-Ag capsule;  $\Delta agfA$ , Tafi;  $\Delta agfA\Delta bcsA$ , Tafi and cellulose;  $\Delta agfD$ ; transcriptional regulator) were grown on T agar supplemented with calcofluor agent at 37°C for 24 hr. *Salmonella* Typhimurium 14028 grown at 37°C for 24 hr was used as a negative control.

#### 5.3.4 Quorum sensing AI-2 degradation, flagella repression and Tafi and Tola expression are important for EPS surface expression.

*Tn10dCm* mutagenesis was used to inactivate and identify prospective genes involved in EPS assembly and expression. The serum generated to the purified acidic EPS was used to screen *Tn10dCm* mutants by ELISA for loss of EPS expression. Since the *Salmonella* Enteritidis 3b *agfD* mutant no longer bound calcofluor and whole

cell ELISA analysis using serum generated to EPS resulted in the reduction of absorbance (Fig. 5. 3) we used an  $\Delta agfD$  mutant strain as a negative control. We identified eleven mutants that had significantly decreased surface associated EPS (Fig. 5.3, Table 5.II). One of the mutants identified had a Tn10dCm insertion in the *yneB* (*lsrF*) gene within an operon containing genes involved in transport and degradation of the quorum sensing autoinducer molecule, AI-2. YneB is a putative aldolase shown to be involved in the degradation of AI-2 (Taga *et al*, 2003). Another mutant had an inactivated *fljA* gene, which encodes a flagellar repression protein. Five mutants had inactivated genes from the *agf* operon and one mutant had an inactivated *tolA* gene, which had previously been found to be important in *agfBA* expression (Vianney *et al*, 2005). Although none of the mutations was within a putative polysaccharide operon involved in biosynthesis, we identified mutants that indicate that quorum sensing AI-2 degradation, flagella repression and Tafi expression are important for EPS expression.



**Figure 5. 3. Immunological analysis of EPS expression on *Salmonella* Enteritidis 3b *ΔbcsA* Tn10dCm mutants.**

*Wt* and Tn10dCm EPS mutants were analyzed using serum generated against EPS by whole-cell ELISA. Bars correspond to averaged values with standard deviations from triplicate samples. *Salmonella* Enteritidis 3b *ΔagfD* was used as a negative control for EPS production.

Table 5.II. *Salmonella* Enteritidis 3b Tn10dCm mutants deficient in EPS production.

Gene flanking Tn10dCm	% EPS <sup>a</sup>	Function <sup>b</sup>
<i>yneB</i> ( <i>lsrF</i> ) (NP462959)	2.3	putative aldolase involved in AI-2 degradation (Taga <i>et al</i> , 2003)
<i>agfF</i> (NP460111)	3.1, 4.9	Tafi assembly component
<i>fljA</i> (NP461697)	0.9	<i>fliC</i> repressor
<i>agfG</i> (NP460110)	2.7, 3.3	AgfA stabilization
Intergenic region of <i>agf</i>	3.6	Tafi synthesis
<i>tola</i> (NP459732)	6.9	IM protein of membrane spanning complex
UK 1	3.2	?
UK 2	1.5	?
UK 3	4.6	?

<sup>a</sup>, % EPS was calculated by subtracting the background A<sub>405</sub> of the  $\Delta agfD$  strain from all strains and then dividing by A<sub>405</sub> of the *wt*.

<sup>b</sup>, Some functions are putative.

### 5.3.5 Multicellular behavior of EPS mutants

There is a strong correlation among multicellular behavior, pellicle formation and rdar morphology. To determine the effect that the absence of EPS had on multicellular behavior we examined the Tn10dCm mutants for pellicle formation, rdar morphology on T agar supplemented with CR and fluorescence on T agar supplemented with calcofluor (Table 5. III). As expected, the Tafi mutants did not produce a pellicle, were pink and mucoid when grown on TCR indicative of saw morphology (Romling *et al*; 1998). Furthermore, none of the mutants bound calcofluor indicating the absence of EPS. Unexpectedly, however the *yneB* and *fljA* mutants had a similar phenotype to the Tafi mutants: there was no pellicle formation, colonies were pink and mucoid on TCR agar and did not bind calcofluor. The *tola* mutant produced a delicate pellicle, was red

and semi-smooth on TCR and bound calcofluor. These results indicate that multicellular behavior is important for EPS expression.

Table 5.III. Multicellular behavior of *Salmonella* Enteritidis 3b  $\Delta bcsA$  Tn10dCm mutants deficient in EPS production.

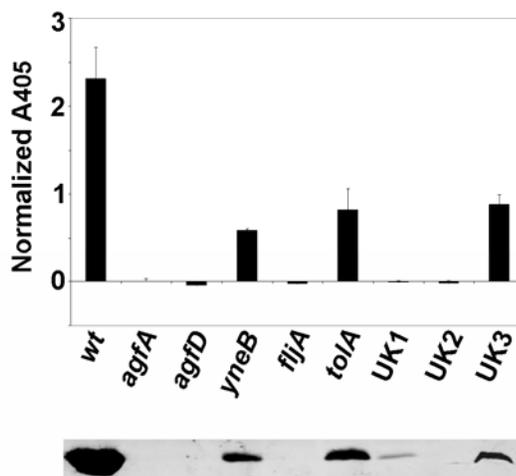
<i>Salmonella</i> Enteritidis 3b strain	Pellicle Formation	Morphology on TCR <sup>b</sup>	Fluorescence on T + Calcofluor
<i>wt</i>	+++	rdar	+
$\Delta bcsA^a$	+	bdar	+
$\Delta agfA^a$	-	pink and smooth	+
$\Delta agfA\Delta bcsA^a$	-	saw	-
$\Delta agfD^a$	-	saw	-
<i>yneB</i>	-	saw	-
<i>agfF</i>	-	saw	-
<i>fljA</i>	-	saw	-
<i>agfG</i>	-	saw	-
Intergenic region of <i>agf</i>	-	saw	-
<i>tolA</i>	+	red and semi-smooth	+
UK 1	+/-	dark pink and smooth	+/-
UK 2	-	pink and smooth	-
UK 3	+	dark orange and semi- smooth	+

<sup>a</sup>, inframe isogenic mutants

<sup>b</sup>, Colony phenotypes of analogous *Salmonella* Typhimurium strains grown on Luria agar without salt supplemented with 40  $\mu$ g of CR per mL and 20  $\mu$ g of Coomassie brilliant blue per mL) rdar, red, dry and rough; bdar, brown, dry and rough; saw; smooth and white. Phenotypes are as reported previously (White et al 2003; Romling 1998 and 2000).

### 5.3.6 Quorum sensing AI-2 degradation, flagella repression and TolA expression influences Tafi expression

The *yneB* and *fljA* mutant had a saw morphotype suggesting Tafi expression was negatively affected whereas the *tolA* mutant exhibited a disrupted rdar morphotype also indicative of decreased Tafi expression. Immunological analysis of the *yneB*, *fljA* and *tolA* mutants using serum specific to AgfA confirmed there was reduced Tafi expression (Fig. 5.4). A mutation in *fljA* results in the repression of Tafi expression whereas *yneB* and *tolA* mutations had reduced Tafi expression. The results indicate that flagella repression completely disrupts Tafi expression. In contrast, quorum sensing AI-2 degradation and TolA expression do not have a complete effect on Tafi expression but rather negatively influence Tafi expression. These results suggest that quorum sensing AI-2 degradation, flagella repression and TolA expression have a role in the complex regulatory circuit involved in Tafi expression.



**Figure 5. 4. Immunological analysis of Tafi expression on *Salmonella* Enteritidis 3b EPS mutants.**

*Wt*, in-frame  $\Delta agfA$  and  $\Delta agfD$  mutants and Tn10dCm EPS mutants were analyzed for Tafi expression using AgfA specific mAb by whole-cell ELISA (top) and western analysis of cell pellet samples treated with FA (bottom). The background  $A_{405}$  of the *agfA* strain was subtracted from the  $A_{405}$  of all strains to normalize the data. Standard deviations correspond to averaged values from triplicate samples.

#### 5.4 Discussion

The *Salmonella* Enteritidis 3b extracellular matrix is a polymeric complex with multiple polysaccharides. It has been known that the extracellular matrix is composed of Tafi, cellulose, O-Ag capsule and another acidic EPS. In this study, we have identified the composition of the acidic EPS. While screening for EPS mutants, it was anticipated that an operon required for the EPS biosynthesis would be identified, but instead several mutations disrupting multicellular behavior were identified. During this screen, it was discovered that AI-2 degradation, flagella repression and TolA expression are important in Tafi expression and thus multicellular behavior. These results indicate that multicellular behavior is important for the expression of the novel acidic EPS associated with the extracellular matrix.

The EPS found associated with the extracellular matrix is composed of Gal, Xyl, GluA and Glu. The ratio suggests that 5 Gal residues are present for every Xyl. Since Xyl residues and other pentoses are relatively uncommon in bacterial EPS (Sutherland, 1994), the EPS reported here is intriguingly unique. Although GluA was relatively low, it was enough to confer acidic character on the EPS as previously observed using an uronic acid assay (data not shown; White *et al.*, 2003) and affect staining by Alcian blue. Acidic monosaccharides are relatively common in bacterial EPS as a side-chain providing a range of different cationic interactions including those with some antimicrobials (Sutherland, 1994). Glu was also present in a minor amount, which may suggest it is a side chain. Furthermore four other unknown monosaccharides, three of which were methylated, were detected suggesting the structure is complex and preliminary linkage analysis suggests it could be branched. As well, the data indicates that the structure may be decorated with other non-carbohydrate substituents. Common components are O-acetyl, pyruvyl, succinyl groups; less commonly propionyl, hydroxybutanyl and glyceryl groups as well as sulphate, P and aa some of which also could contribute to the acidic nature (Sutherland, 1994). The EPS may be capsular

since a 3-hydroxymyristic acid was detected with purified fractions. Further studies are underway to determine the structure of this novel EPS.

Multicellular behavior characterized by Tafi and cellulose expression is required for EPS expression. We identified nine mutants in a cellulose deficient background strain that had EPS expression lower than that of *wt*. All of these mutations had multicellular behavior disrupted via a reduction in Tafi expression. Five of these mutants had direct *agf* gene inactivations whereas the other mutations in *yneB*, *fljA* and *tola* had reduced Tafi expression via pleiotropic mutations. Both the *yneB* and *fljA* mutations resulted in the loss of multicellular behavior indicated by a saw morphology and the absence of pellicle formation whereas the *tola* mutation resulted in a red and mucoid morphology able to form a pellicle that was easily disrupted. These results indicate that multicellular behavior is a prerequisite for EPS expression.

The *fljA*, *yneB* and *tola* mutations affected Tafi expression. The *fljA* mutant resulted in the complete absence of immunologically detectable levels of Tafi whereas the *yneB* and *tola* mutations resulted in decreased Tafi expression. The *fljA* gene encodes a product that represses FliC, the major flagellin subunit. Therefore the *fljA* mutant hyperexpresses flagella and this resulted in the loss of EPS expression. This is similar to *V. cholerae* EPS expression which is controlled by a flagellum-dependent pathway of expression (Lauriano *et al.*, 2004). In this scenario, the absence of flagella triggers EPS expression via a sodium-driven motor dependent signal cascade. *Salmonella* uses a proton-driven motor homologous to this system of flagellar rotation (Asai *et al.*, 2003) and thus could activate EPS expression in a similar manner. However, since the *fljA* mutation completely abolished Tafi expression and multicellular behavior it is likely that multicellular behavior itself similar to the other mutants resulted in the loss of EPS expression. It is unknown, however, how flagella repression results in Tafi deficiency. The *V. cholera* EPS also has a quorum sensing-dependent pathway independent of the flagella pathway of expression where expression is activated by a two component signal transduction pathway upon signal interaction (Hammer *et al.*, 2003).

We also found that quorum sensing was important in EPS expression however, in an atypical manner. We identified a mutation in *yneB* (*lsrF*) that resulted in a decrease in EPS expression and thus it appears that AI-2 degradation, rather than AI-2 activation, is important in generating EPS expression. In *Salmonella* the quorum sensing signal, AI-2 is synthesized by LuxS and extracellular levels peak in mid-to late exponential phase and decline rapidly in stationary phase, which is a consequence of its import by the products of the *lsr* operon. The *lsr* operon contains seven genes, *lsrACDBFGE* and transcription is activated by phospho-AI-2 repressing a divergent two gene operon responsible for AI-2 phosphorylation. YneB (LsrF) encodes a putative aldolase and has a role in the degradation of the AI-2 (Taga *et al*, 2003). It is unknown why bacteria produce, release and then internalize AI-2. One hypothesis could be that AI-2 signal is transformed into a different cytoplasmic signal (Xavier and Bassler, 2005). Our results show that in the absence of YneB, EPS is significantly decreased on the cell surface, multicellular behavior is disrupted and Tafi expression is reduced. One speculation could be that the degraded AI-2 signals are involved in the regulation scheme. Whether this is via AgfD or not is yet to be determined. Tafi regulated quorum sensing has been investigated previously, however these experiments using culture from stationary phase where the AI-2 would have already been internalized and thus no AI-2 would have been present in the spent media explaining their negative results. Further investigation is required to determine how Tafi is influenced by AI-2 degradation. Lastly, TolA mutants have already been shown to express less *agfBA* (Vianney *et al*, 2005). We confirmed that TolA, an IM protein that forms a membrane-spanning complex and is important for envelope integrity, is also important for Tafi expression. This expression is not via AgfD however (Vianney *et al*, 2005). These results implicate two new factors, YneB and FljA in the complex regulatory circuit of Tafi expression.

The biosynthetic gene operon for EPS was not identified in this screen likely as a result of using a transposon library created in a cellulose minus background strain.

Previously, we found that cellulose was important in stabilizing the EPS close to the cell surface (White *et al.*, 2003). Calcofluor binding indicates that a Tafi mutant itself can fluoresce but not a Tafi and cellulose mutant resulting in the disruption of multicellular behavior. Thus we presume that this loss in calcofluor binding is due to the loss of the EPS identified here since the loss of cellulose and O-Ag capsule alone does not result in the loss of calcofluor binding. It is unknown if the loss of EPS expression is due to regulatory cross-talk as a result of multicellular behavior or if both Tafi and cellulose are simply required to stabilize EPS on the cell surface of the colony.

Although researchers generally use calcofluor to indicate cellulose production in *Salmonella*, caution should prevail particularly with clinical strains such as the strain used here, *Salmonella* Enteritidis 27655-3b strain previously isolated from human faeces (Feutrier *et al.*, 1986). Often individual strains capable of forming EPS are able to produce several different EPS (Branda *et al.*, 2005). For example, in *P. aeruginosa* alginate was generally believed to be the major EPS in the extracellular matrix of the biofilm formed by strains isolated from the lungs of Cystic fibrosis patients but recently two other distinct EPS have also been discovered to be major components of the biofilm each with their own genetic loci (Friedman and Kolter, 2004b). Commonly, EPS appears to be a component that is lost upon domestication of laboratory strains (Friedman and Kolter, 2004a). Therefore, it is possible that laboratory strains such as *Salmonella* Typhimurium 14028 do not express EPS other than cellulose and cellulose mutations result in the complete loss of calcofluor binding. However, others have shown that *Salmonella* Typhimurium 14028 does produce an EPS associated with the extracellular matrix yet the composition is unlike the composition of the EPS found here (de Rezende *et al.*, 2005). Strain-to-strain variability may account for the diversity of EPS in the *Salmonella* extracellular matrix.

The *Salmonella* Enteritidis 3b extracellular matrix is comprised of at least three EPSs: cellulose, O-Ag capsule and an acidic EPS. In this study, we have identified the composition of the acidic EPS. Furthermore, we found that multicellular behavior is

required for EPS expression. During this screen we unexpectedly discovered that AI-2 degradation and flagella repression are important in Tafi expression revealing two more factors involved in the complex regulation of Tafi expression.

## 6.0 GENERAL DISCUSSION AND OVERALL CONCLUSIONS

Tafi and EPS form a recalcitrant extracellular matrix that probably enhances the persistence of *Salmonella* cells in the environment promoting passage to future hosts. Tafi are innately aggregative and uniquely insoluble providing cellular communities with a sticky, resistant, proteinaceous web. The extracellular nature of Tafi assembly is divergent from all other known fimbrial assembly pathways and resembles silk spun from a spider into a sticky, recalcitrant web. This assembly mechanism may in fact allow the fibers to branch and create an amorphous web used to organize the cells within a community and to stabilize the EPS close to the cell surface within this cellular community. This serves the microbial community well, by providing a protective, hydrated, charged diffusion-limited shell against desiccation stress, some antimicrobials and disinfectants. Thus the formation of the extracellular matrix individual components as a co-regulated process probably allows for a co-ordinated multicellular behavior utilized as a survival strategy.

The *Salmonella* extracellular matrix is important for biofilm formation. *Salmonella* biofilms could be a continuous source of contamination of foods coming in contact with them when formed on contact surfaces and this could have implications for the food processing industry. *Salmonella* strains entering the food processing environment either through the meat or through carriers handling meat may survive in the premises by forming biofilms on various surfaces. Since *Salmonella* biofilms compared to planktonic cells are more resistant to disinfectants such as chlorine, the biofilm cells would survive the usual cleaning procedures based on studies using

planktonic cells. Thus, cleaning and disinfection protocols in food processing units should consider *Salmonella* biofilms and determine effective ways of decontamination.

The *Salmonella* extracellular matrix may represent a fundamental survival strategy utilized by many other microorganisms. The majority of organisms have the ability to form protein and polysaccharide polymers on their cellular surface generating an extracellular matrix that encases individual cells into a biofilm. In other organisms the biofilm is initiated by fimbriae and the EPS is important for maturation. In *Salmonella*, Tafi are required for the initiation of biofilm formation and without Tafi cells are no longer aggregative and are more susceptible to desiccation stress and chlorine, probably indirectly from the loss of EPS. Although the O-Ag capsule was not found to be important for biofilm formation under the conditions studied here, it is possible that the acidic EPS is similar to other EPSs and are important for the maturation of the biofilm. Thus, the principle function of the extracellular matrix appears to enable individual cells to stick together on surfaces and withstand stresses in the environment. Undoubtedly, this would contribute to long-term survival in the environment and ensure passage to future hosts perpetuating the cycle lifecycle of *Salmonella*. This multicellular phenomenon has been hypothesized by some to represent the functional equivalent to sporulation for non-sporulating Gram-negative bacteria, like *Salmonella*.

## 7.0 FUTURE INVESTIGATIONS

Tafi structure and assembly mechanisms of polymerization are still unresolved. With the current technologies utilized to elucidate the molecular structures of proteins, it is nearly impossible to study Tafi subunits due to their insolubility and innate propensity to aggregate *in vitro* when purified. Thus, creative ways are needed to investigate Tafi structure(s) and the mechanism in which Tafi changes conformations.

The key to understanding Tafi assembly is certainly through AgfA's conformational changes during the process of polymerization. *In vivo*, AgfA's

conformational changes are directed by the assembly factors. It is important to know if the minor assembly subunits form a complex by interacting with each other but this is made difficult due to the aggregative nature of the AgfA. Possibly these studies would be facilitated by direct mutagenesis of AgfA and binding of purified subunits. Furthermore, the minor assembly factors need to be further investigated. This could include structural studies of each or reconstitution studies of all the subunits together. However, this is made difficult due to the aggregative properties of AgfA. Furthermore, AgfB and AgfF are difficult to overexpress *in vitro* and thus obtaining abundant amounts of proteins required for structural studies is difficult. Additionally, more research directed at elucidating AgfC's role in cellulose binding may provide insight into co-ordinated assembly of Tafi and cellulose.

The O-Ag capsule associated with the extracellular matrix appears to be functionally important for desiccation tolerance and survival against some antimicrobials. The *emc* operons also appear to be induced in the presence of bile (unpublished results). It is known that *Salmonella* forms biofilms in gallbladders and an EPS is associated with these biofilms. It would be interesting to determine if *emc* mutants can still form biofilms in gallbladders. Thus this would implicate the O-Ag capsule important in host persistence as well.

The biochemistry of the *emc* gene products and their role in capsule assembly and transport needs to be confirmed. Furthermore, we presume that O-Ag biosynthetic genes are used for the biosynthesis of the O-Ag capsule but this should be confirmed by measuring gene activity in the stationary phase of growth. Additionally, *Salmonella* Enteritidis 3b LPS Man configuration needs to be confirmed by NMR and mass spectrometry to understand the nature of the O-Ag capsule antibody specificity and the roles of the *emc* gene products.

It would be interesting to investigate the relationship between flagella, Tafi and capsule expression. In a Tafi mutant, flagella are hyperexpressed (unpublished results) and cell surface capsule expression is decreased. How flagella are upregulated in the

absence of Tafi is curious. We hypothesize that decreased capsule expression is due to the loss of Tafi stabilizing it on the cell surface. To verify this, *emc* expression could be measured in an  $\Delta agfA$  mutant. Similarly, in a flagella mutant, it appears that there is less capsule cell surface expression. Is this because flagella are required to somehow lock the capsule on the cell surface or it is due to regulatory signals generated by surface sensing? Curiously, the opposite was true for AEPS expression where a hyperexpressing flagella mutant expressed less AEPS. The regulatory details of these complex relationships need to be further investigated.

AEPS appears to be different from other EPS as Xyl is not a common component of bacterial EPS. Thus genes for its synthesis and regulation could be determined in a similar manner to that performed in this thesis with modification. We used a cellulose minus background but rather the *wt* strain should be used for screening mutants since the EPS appears to require the extracellular components for tight cell association. This would facilitate gene knockouts and further experimentation directed at functional analyses such as biofilm maturation or survival against antimicrobials.

The role of quorum sensing AI-2 degradation in EPS expression would be interesting to investigate. Since extracellular matrix formation is a multicellular behaviour, it is not entirely surprising that quorum sensing is involved although in an unusual manner since it is degradation of the signal that appears to activate expression and not the signal itself. It will be important to determine how *yneB* disruption is linked to EPS and Tafi expression. Thus studies of *yneB* will be required to determine its precise role and what signal is generated by its activity. Following this, perhaps it can be determined how this signal is relayed to EPS and Tafi expression potentially via AgfD. Additionally, other signaling mechanisms are involved in the complex regulatory circuit of the extracellular matrix formation and thus it would be interesting to determine if either the O-Ag capsule or EPS are regulated by c-di-GMP similar to cellulose. This could be done by examining in frame mutations of genes encoding

GGDEF and EAL domain containing proteins and measuring *emc* promoter fusions to measure activity.

It has been hypothesized by some that the extracellular matrix encased cells are hyperinfective. In fact, there have been at least two victims in our lab who can attest to the severity of disease manifestation caused by this strain. However, further research is required to determine if the extracellular matrix contributes to the infectious state of *Salmonella*. Serum collected from two infected volunteers contained antibodies that recognized HMW material in the stacking gel indicative of the O-Ag capsule or EPS but not Tafi. The question still remains open if the extracellular matrix is solely for environmental survival or if it also a virulence factor. Since *Salmonella* Enteritidis can not be used in a mouse infection model, extracellular matrix mutants need to be studied in *Salmonella* Typhimurium and used for mouse infection studies.

Finally, the data from this research should be exploited to find new ways to disinfect *Salmonella* and disarm it from perpetuating in the environment. Presumably, the extracellular matrix at some point must be degraded once it needs to leave the biofilm or once ingested by the host. Thus, it is possible that *Salmonella* also contains enzymes required to proceed with the matrix dissolution. Determining these factors may lead to their exploitation and use in the prevention and or disinfection process. Alternatively, the future for antimicrobial design would be to prevent biofilm and extracellular matrix formation by disrupting some quorum sensing pathway. This could be with natural autoinducer analogues that mimic the autoinducing pheromones and thus prevent the behaviors associated with their induction such as biofilm formation and EPS production.

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