IAI Accepts, published online ahead of print on 22 August 2011
Infect. Immun. doi:10.1128/IAI.05592-11
Copyright © 2011, American Society for Microbiology and/or the Listed Authors/Institutions. All Rights Reserved.

IAI Accepts published online ahead of print

SadA, a trimeric autotransporter from *Salmonella enterica* serovar Typhimurium, can promote biofilm formation and provides limited protection against infection.

Dhaarini Raghunathan[1]* , Timothy J Wells[1]*, Faye C Morris[1]*, Robert K Shaw[3], Saeeda Bobat[1], Sarah E Peters[4], Gavin K. Paterson[4], Karina Tveen Jensen[1], Denisse L. Leyton[1], Jessica MA Blair[1], Douglas F. Browning[1], John Pravin[1], Adriana Flores-Langarica[1], Jessica R Hitchcock[1], Claudia TP Moraes[5], Roxane MF Piazza[5], Duncan J Maskell[4], Mark A. Webber[1], Robin C May[2], Calman A MacLennan[1], Laura J Piddock[1], Adam F Cunningham[1], Ian R. Henderson[1#]

[1]School of Immunity and Infection and [2]School of Biosciences, University of Birmingham, Birmingham, UK; [3]Centre for Molecular Microbiology and Infection, Imperial College London, London, UK; [4]Department of Veterinary Medicine, University of Cambridge, Cambridge, UK; [5]Laboratory of Bacteriology, Butantan Institute, São Paulo, Brazil

Running Title: SadA of *Salmonella* provides a protective effect against infection.

Keywords: TAA, Autotransporter, Salmonella

*These authors contributed equally to the research

#Corresponding Author: School of Immunity and Infection, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.; Tel: +44 121 4144368; Email: i.r.henderson@bham.ac.uk

Present address: Department of Veterinary Medicine, University of Cambridge, Cambridge, UK

IAI Accepts published online ahead of print

29 **ABSTRACT**

30 *Salmonella enterica* is a major cause of morbidity worldwide and mortality in children and
31 immunocompromised individuals in sub-Saharan Africa. Outer membrane proteins of
32 *Salmonella* are of significance because they are at the interface between the pathogen and the
33 host, they can contribute to adherence, colonization and virulence and are frequently targets
34 of antibody-mediated immunity. In this study the properties of SadA, a purported trimeric
35 autotransporter adhesin of *Salmonella enterica* serovar Typhimurium, was examined. We
36 demonstrated that SadA is exposed on the *Salmonella* cell surface *in vitro* and *in vivo* during
37 infection of mice. Expression of SadA resulted in cell aggregation, biofilm formation and
38 increased adhesion to human intestinal Caco-2 epithelial cells. Immunisation of mice with
39 folded, full-length, purified SadA elicited an IgG response which provided limited protection
40 against bacterial challenge. When anti-SadA IgG titres were enhanced by administering
41 alum-precipitated protein a modest additional protection was afforded. Therefore, despite
42 SadA having pleiotropic functions it is not a dominant, protective antigen for antibody-
43 mediated protection against *Salmonella*.

44

IAI Accepts published online ahead of print

45 **INTRODUCTION**

46 Non-typhoidal strains of *Salmonella* (NTS) such as *Salmonella enterica* serovar
47 Typhimurium (*S.* Typhimurium) are a major cause of human gastro-intestinal infections and
48 invasive disease. While NTS cause almost 100 million cases of gastroenteritis globally every
49 year (54), invasive disease is a huge, yet under-recognised problem in regions such as sub-
50 Saharan Africa where it typically manifests as bacteraemia and meningitis in young children
51 (31) and HIV-infected individuals (29). Invasive disease is associated with high case-fatality
52 rates, and often occurs in the complete absence of gastro-intestinal symptoms. Evidence
53 points to a lack of specific antibody as a main contributing factor to severe infection and
54 bacteraemia (28, 53) making vaccine development a promising preventive option.
55 Furthermore, antibiotic resistance in these bacteria is an increasing problem (30) and
56 development of new intervention and preventative strategies is urgently required. Outer
57 membrane proteins are part of the interface between pathogen and host, are targets of
58 antibody and are therefore potential candidates for vaccine development (15, 52). An
59 improved knowledge of the surface-expressed virulence factors of *S.* Typhimurium is
60 essential to understand bacterial virulence and to identify novel vaccine targets on the
61 bacterium.

62 The *S.* Typhimurium protein SadA belongs to the family of Trimeric Autotransporter
63 Adhesins (TAAs), a subset of Type 5 secreted proteins that form a stable trimer on the
64 bacterial cell surface (2, 13, 33). TAAs, like all Type 5 secreted proteins, contain an N-
65 terminal *sec*-dependent signal sequence that allows the protein to cross the inner membrane,
66 an internal passenger domain and a C-terminal translocation domain (19, 35, 37, 38). TAAs
67 are distinguished from conventional autotransporters (ATs) by having a small 70-100 amino
68 acids (aa) translocation domain, composed of four β-strands, which forms a homo-trimer

IAI Accepts published online ahead of print

69 resulting in a full sized β-barrel pore in the outer membrane (72). This pore is essential for
70 transport of the passenger domain to the cell surface (62, 72). The β-barrel is inserted into the
71 outer-membrane via the β-barrel assembly machinery (BAM) complex and thereafter the
72 passenger domain is secreted to the cell surface (44, 48).

73 The passenger domains of TAAs share common features. Structural determinations of TAA
74 proteins such as YadA, Hia and BadA have revealed three distinct regions within the
75 passenger domain; an N-terminal head, a neck and a stalk (56, 73, 82). The stalk is an α-
76 helical region which spans the pore formed by the translocation unit in a manner analogous to
77 the classical ATs. The neck region connects the stalk to the head domain. The YadA head
78 structure contains single-stranded, left-handed helices, with the interface between them being
79 formed by periodically occurring, conserved sequence motifs (56). These motifs can be found
80 in the predicted head structures of many TAAs and variations in this region are largely
81 responsible for the functional variation observed for these proteins (56, 82).

82 Where characterised, TAAs are uniformly associated with adhesion (50) but they may also
83 have other virulence properties. The exemplar TAA is YadA from *Yersinia enterocolitica*
84 which mediates adhesion to epithelial cells and extracellular matrix (ECM) molecules and is
85 also crucial for intestinal colonisation and serum resistance (4, 23, 74). Other characterised
86 trimeric autotransporters include NadA and NhhA from *Neisseria meningitidis* which
87 facilitate binding and invasion of epithelial cells (12, 63), BadA from *Bartonella henselae*
88 that plays a role in binding to ECM proteins (40, 60, 61), Hia and Hsf from *Haemophilus*
89 *influenzae* which enable high-affinity binding to respiratory epithelial cells (14, 46) and
90 UspA1 and UspA2 from *Moraxella catarrhalis* that mediate binding to human epithelial cells
91 (47). Recently a trimeric autotransporter from uropathogenic *E. coli,* UpaG has been
92 described which is required for binding to bladder epithelial cells and ECM proteins,

IAI Accepts published online ahead of print

93 fibronectin and laminin. It also promotes autoaggregation and biofilm formation (59, 76). As
94 a role of *S.* Typhimurium SadA has not been established we investigated the cellular
95 localisation of SadA, its functions and the role antibody to SadA may play in controlling *S.*
96 Typhimurium infection.

97 **MATERIALS AND METHODS**

98 **Cells and culture conditions**

99 The bacterial strains and plasmids used in this study are listed in Table 1. All bacterial strains
100 were routinely cultured in Luria Bertani (LB) broth or M63 minimal medium (58) with
101 shaking or on agar at 37°C supplemented with kanamycin (50 µg/ml), ampicillin (100 µg/ml)
102 and/or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) where appropriate. The
103 semiadherent macrophage-like cell line J774 and the colorectal carcinoma cell line CaCo-2
104 were cultured in Dulbecco's Modified Eagle's Medium (DMEM), as previously described
105 (51, 70).

106 **Molecular biology techniques and strain construction**

107 Plasmid DNA isolation, genomic DNA isolation, PCR product purification and agarose gel
108 extractions were performed with the relevant kits from Qiagen and according to
109 manufacturer's instructions. All restriction enzymes (New England Biolabs) and T4 DNA
110 ligases (Promega) were used in accordance with the manufacturer's specifications. PCR
111 experiments were performed using Phusion® High-Fidelity DNA Polymerase (New England
112 Biolabs, F530-L) or 1x ReddyMix™ PCR master mix (Thermo scientific, Ab-0575). DNA
113 sequencing was carried out at the Functional Genomics and Proteomics Laboratory of the
114 University of Birmingham. To obtain RNA either bacterial cultures were harvested in the
115 presence of Qiagen RNAprotect Bacteria Reagent or spleen sections were homogenised using
116 QIAGEN QIAshredder and subsequently RNA was isolated using Qiagen's RNeasy Mini Kit

IAI Accepts published online ahead of print

117 and RNase-Free DNase Set in accordance with manufacturer's instructions. RNA was
118 quantitified using nanodrop, prior to cDNA synthesis using SuperScript II Reverse
119 Transcriptase and Random Hexamers (Invitrogen) and RT PCR using Taq Polymerase and
120 primers D3 and RT1. Primers used in the study are listed in Table S1.

121 The *sadA* gene was amplified by PCR from *S. enterica* SL1344 using primers D5 and D6
122 (Table S1). The PCR product containing the full-length *sadA* gene was digested with *NdeI*
123 and *EcoRI* and ligated to pET22b (Novagen) plasmid digested with the same enzymes to
124 generate pDR01. The pDR03 plasmid contained the full-length *sadA* gene, amplified with
125 primers D5 and D7 and cloned into a modified pQE60 vector (pQE60NdeI; see Table 1 for
126 details) between the restriction sites *NdeI* and *HindIII*.

127 The *sadA* mutant strains were generated using the one-step gene inactivation method
128 described for *E. coli* and *S.* Typhimurium (17, 22). Briefly, the kanamycin resistance cassette
129 was amplified using primers D1 and D2 which possessed 40 bp identical to regions within
130 *sadA*. The resulting DNA was transformed into *S. enterica* SL1344 carrying the pKD46
131 plasmid encoding the λ red recombinase. Disruption of *sadA* was confirmed by PCR using
132 primers k1, k2 and kt (17) in combination with primers D3 (upstream check primer) and D4
133 (downstream check primer) and subsequent DNA sequencing. The mutation was then P22-
134 transduced into *S. enterica* SL1344 and *S. enterica* SL3261, generating the strains *S. enterica*
135 SL1344 *sadA::aph* and SL3261 *sadA::aph,* respectively. These latter strains were used to
136 create *S. enterica* SL1344 Δ*sadA* and SL3261 Δ*sadA* by removing the kanamycin cassette as
137 previously described (17).

138 **Protein localization and Western blotting**

IAI Accepts published online ahead of print

139 Cellular fractions were prepared as described previously (59). Cellular fractions and purified
140 proteins were electrophoresed on 10% SDS-PAGE gels and stained with Coomassie blue or
141 transferred to PVDF membrane for western blotting as previously described (77). Western
142 blotting was performed with 1:1,000 dilution of the anti-SadA antibody and 1:10,000 anti-
143 rabbit IgG alkaline phosphotase before detection with NBP/BCIP (Sigma) as the substrate.
144 Proteins were localised by immunofluorescence as described previously (77). Briefly, 13 mm
145 glass coverslips coated with 1 mg/ml poly-L-lysine were overlaid with LB-cultured bacteria.
146 Subsequently, coverslips were incubated with PBS containing 4% formalin followed by a
147 PBS-BSA blocking buffer. SadA was detected with anti-SadA antibody (1:100 in PBS-BSA)
148 and subsequently GAR alexa fluor488 anti-rabbit secondary antibody (1:100 in PBS-BSA)
149 before visualisation on a Leica DMRE fluorescence microscope-DC200 digital camera
150 system. Captured images are representative of randomly selected fields of view.

151 **Purification of full length SadA protein**

152 *E. coli* BL21 pDR01 was grown with IPTG induction until an OD_{600}=1. Cells were pelleted
153 by centrifugation, washed and resuspended in 50 mM HEPES, 150 mM Sodium chloride, 1
154 mM PMSF pH 7.4. Cells were lysed by sonication and the cell debris and inclusion bodies
155 pelleted by centrifugation at 20,000 × *g*. The pellet was the resuspended in 50 mM HEPES,
156 150 mM NaCl, 8 M urea, 1 mM PMSF pH 7.4 and incubated at room temperature for 1 h.
157 Subsequently, insoluble material removed by centrifugation at 50,000 × *g* and the supernatant
158 recovered. This SadA-containing solution was run on SDS-PAGE gels, bands corresponding
159 to SadA were excised and gel slices homogenised in 0.5 ml of 50 mM HEPES, 0.1% SDS,
160 pH 7.4. SadA was electro-eluted in a Novagen elution system as per manufacturer's
161 instructions. The eluted protein was diluted to 10 ml 50 mM HEPES, 0.1% SDS, pH 7.4 and
162 then dialysed 3 times against 5 l of 50 mM HEPES, 150 mM NaCl, 0.5% Elugent detergent

IAI Accepts published online ahead of print

163 (Calbiochem). Refolding by dialysis was performed over 3 days. The concentration of the
164 protein was estimated using a tannin assay. BSA made up in the final refolding buffer was
165 used as standards. Tryptophan fluorescence readings were taken at 280 nm excitation,
166 emission scanned over 300 to 450 nm, 0.5 mm slit width, ~ 1 nm bandwidth, 1 s response
167 time, 1 nm increments using a QuantaMaster instrument (Photon Technologies International).
168 These purified SadA protein preparations were tested with an endotoxin assay (Sigma E-
169 Toxate Kit) to measure levels of lipopolysaccharide (LPS). Antibodies against SadA were
170 raised in New Zealand white rabbits using the purified refolded protein described above.

171 **Autoaggregation and biofilm Assays**

172 Autoaggregation was observed as described previously (78). The rate of autoaggregation was
173 determined by the mean decrease in optical density over time. Biofilm formation on
174 polystyrene surfaces was studied using 96-well microtitre plates as described previously (66).
175 All the experiments were performed in triplicate on three separate days. Flow chamber
176 experiments were performed as previously described (78) with the exception that the media
177 also contained BacLight green bacterial stain (Invitrogen) to visualise the biofilms. Biofilm
178 development was monitored at 18 h and 24 h after inoculation. All experiments were
179 performed in triplicate.

180 **Adherence, Invasion/Intracellular survival Assays**

181 Tissue culture assays were performed as previously described. Briefly, overnight bacterial
182 cultures were then washed twice in 1 × PBS (Invitrogen) and resuspended in serum-free
183 culture medium (~10^8 CFU/ml). 24-well plates were seeded with 10^5 J774 cells or 10^6 Caco-2
184 cells each well. For adherence assays, the washed cells were infected with 1 ml of bacterial
185 cultures and incubated at 37°C in a 5% CO_2 atmosphere for 2 h. Subsequently, the cells were
186 washed several times with 1 × Hanks Balanced Salt Solution (HBSS) to remove the non-

IAI Accepts published online ahead of print

187 adherent bacteria prior to disruption with 1 × DPBS, 1% (vol/vol) Triton-X 100 solution.
188 Serial dilution of this solution on to LB agar plates supplemented with suitable antibiotics
189 was used to enumerate CFUs. To estimate the number of intracellular/invasive bacteria, the
190 infected cells were washed several times with HBSS and then treated with 100 µg/ml
191 gentamicin for 2 h at 37°C and 5% CO_2 to kill the extracellular bacteria. The number of
192 CFUs were calculated as above. The results were the mean of three individual experiments
193 conducted in triplicate on three separate days. Leaf infection and confocal fluorescence
194 microscopy were performed as previously described (7). Briefly, supermarket leaves were
195 affixed to a 30 mm Petri dish base and immersed in 4 ml of the appropriate bacterial culture
196 prior to incubation at 20°C for one hour, with no agitation. Non adherent bacteria were
197 removed by washing and numbers of leaf-adherent bacteria were quantified by plating on
198 agar plates. A two-tailed, paired Student's *t*-test was used to assess significance, using $P <$
199 0.05 as a cutoff. ECM binding assays were performed as previously described (77) with all
200 ECM molecules (Collagen I,III,IV, fibronectin, elastin and laminin) purchased from Sigma.

201 **Serum Bactericidal Assay**

202 The serum bactericidal assays were performed as described previously (53). Killing of *S.*
203 *enterica* SL1344, SL1344Δ*sadA* and *E. coli* strains expressing SadA was investigated with
204 normal serum obtained from healthy African and European individuals. Cultures of bacteria
205 in log-growth phase were prepared in 1 × PBS and 10 µl of each sample was added to 90 µl
206 of freshly-thawed, undiluted serum to give a final bacterial concentration of 10^6 CFU/ml. The
207 mixtures were incubated at 37°C with gentle rocking (20 rpm) and 10 µl samples were
208 withdrawn at 45, 90 and 180 min. Serial dilutions of the samples were plated onto LB agar
209 plates to determine the viable bacterial counts.

210 **In vivo assessment of virulence and immune response**

IAI Accepts published online ahead of print

211 *Caenorhabditis elegans* strain Bristol N2 were cultured using standard methods and survival
212 assays were performed as described previously (1, 3). The worms were grown on Nematode
213 Growth Medium (NGM) agar plates and fed with *E. coli* strain OP50. Larval stage 4 (L-4) *C.*
214 *elegans* were picked and transferred onto assay plates. The plates were incubated at 25°C and
215 scored daily for survival. Death was defined as a failure to respond to mechanical stimulus. A
216 minimum of 100 worms were used on three independent occasions. A Kaplan Meier estimate
217 was used to determine the probability of *C. elegans* survival to the next day. Survival curves
218 were generated by plotting probability of survival against time and were then compared using
219 the log rank test to establish whether the difference between two curves was statistically
220 significant.

221 Mice were infected intraperitoneally (i.p.) with ~5 x 10^5 cfu and sacrificed at the described
222 times post-infection. For oral infection, mice were give 5 x 10^6 bacteria by oral gavage and
223 sacrificed 4 or 6 days later. After sacrifice blood, spleens and liver were harvested and
224 bacterial counts in organs were measured by homogenising the tissues and plating onto LB
225 agar as described (15). In some experiments mice were immunized via the i.p. route with 10
226 g purified SadA protein with or without alum-precipitation as described previously (16).
227 After immunization for the prescribed times mice were bled and were subsequently
228 challenged with ~5 x 10^5 cfu *S. enterica* SL1344 i.p. and sacrificed two days later. Organs
229 and blood were isolated at sacrifice and numbers of bacteria were enumerated as above.
230 Serum was isolated from blood by centrifugation and assessed by ELISA for SadA-specific
231 IgM and total IgG titers. Maxisorp 96 well plates (Nunc) were coated with 5 µg/ml of
232 purified SadA in carbonate coating buffer. After washing plates were blocked for 1 h at 37°C
233 with 1% BSA in PBS. Subsequently serum samples were added in blocking buffer and
234 serially diluted and incubation continued for 1 h at 37°C. Secondary goat anti-mouse IgM or

IAI Accepts published online ahead of print

235 IgG conjugated to alkaline-phosphatase (Southern Biotech) were diluted in blocking buffer
236 and added for 1 h at 37°C. Signal was detected using the SIGMAFAST p-Nitrophenyl
237 phosphate system and plates were read at 405 nm. In some cases bacteria were administered
238 after opsonisation. Before being used for opsonisation, all serum was heated to 56°C for 20
239 min to inactivate complement. The heat inactivated sera and organisms were mixed (1:200)
240 with gentle agitation for 30 min at room temperature, the bacterial concentration was adjusted
241 to the concentration used for infection (~5 x 10^5 cfu) and the bacterial suspension was
242 administered via the i.p. route; bacteria were cultured to ensure no loss of viability.

243 **RESULTS**

244 **Comparative sequence analysis of SadA positional orthologues.**

245 Analysis of the *S. enterica* SL1344 genome identified *sadA*, a 4,383 bp gene that encodes a
246 TAA protein. The 1461 aa protein is a typical TAA, possessing all of the expected features of
247 this family. Analysis of the amino acid sequence revealed a putative signal sequence
248 possessing a conserved N-terminal extended signal peptide region (ESPR), which is common
249 to many autotransporter proteins (5, 18, 20, 49), and is predicted to be cleaved after amino
250 acid 50. The analysis also revealed a 1333 aa passenger domain and a C-terminal
251 translocation unit indicated by the Pfam:YadA (PF03895) domain. The translocator domain is
252 only 78 aa, which is consistent with other TAAs that form a homo-trimer to make a full sized
253 β-barrel. The passenger regions of TAAs have three conserved structural features; the head,
254 neck and stalk (45, 50). The head structure is composed of multiple Pfam:Hep_Hag
255 (PF05658) motifs while the neck consists of a single Pfam:HIM (PF05662) motif. The stalk
256 is composed of multiple small repeats that are not conserved between TAAs and therefore
257 does not have a Pfam identifier (50). SadA contains these motifs (Fig. 1) suggesting that it

IAI Accepts published online ahead of print

258 has an N-terminal head followed by a long region of alternating neck and stalk segments
259 similar to other long TAAs such as BadA from *Bartonella henselae* (73).

260 To find orthologues of SadA, the full protein sequence was used to probe the available
261 sequenced bacterial genomes using BLASTP. Sequences identified by this method were then
262 screened to determine if they had the same domain organisation as SadA, as well as sharing
263 over 50% similarity. This analysis identified orthologues of SadA in *E. coli* (including
264 *Shigella sonnei*), *Citrobacter youngae* and *S. enterica* genomes. In each case, the *sadA* gene
265 was found to be in the same genomic location adjacent to the *lld* locus suggesting *sadA* was
266 acquired prior to the divergence of these species (Fig. 1A). A multiple alignment of the full
267 length proteins revealed that the proteins from *Salmonella* were very well conserved, sharing
268 over 98% similarity with each other where-as the proteins from *E. coli* were more variable
269 (Fig. 1B). A phylogenetic tree was created from the multiple alignments identifying that the
270 sequences grouped according to species and pathotypes (11). SadA orthologues from
271 intestinal *E. coli* isolates were more conserved than proteins from extra-intestinal,
272 uropathogenic and environmental *E. coli* strains (Fig. 1B).

273 One of the positional orthologues identified was the *E. coli* CFT073 UpaG (76). However,
274 UpaG and SadA differ substantially in protein sequence. UpaG is 317 aa longer than SadA,
275 due to extra amino acids in the N-terminal head, stalk and neck domains of the protein. The
276 head domain in particular differed in sequence from the analogous region in UpaG. Although
277 the full length protein sequences shared 56.8% similarity, the head domain shared only 30.4%
278 similarity. In addition to this, the UpaG head sequence is over twice the length of the SadA
279 head region (Fig. 1C).

280 **SadA is surface expressed in *S. enterica* SL1344.**

IAI Accepts published online ahead of print

281 SadA was overexpressed in *E. coli* BL21 pDR01, purified and refolded as described in the
282 Materials and Methods (Fig. 2A). The fluorescence spectrum of the refolded protein was
283 typical of proteins containing multiple tryptophans with a broad band at half-height. The λ_{max}
284 was found at 336 nm which indicates that the tryptophans are in a folded conformation (Fig.
285 2B). The refolded material was analysed by mass-spec ID analysis and was identified as
286 SadA and analysis by silver staining confirmed that the sample was >99% pure and had no
287 detectable LPS. An endotoxin detection kit (Sigma, E-toxate) also verified that the sample
288 had no detectable LPS (<0.05 endotoxin units (EU) in 40ug protein). This purified protein
289 was used for infection studies (see later) and to raise polyclonal antibodies to SadA.
290
291 As SadA belongs to the TAA family it was expected to adopt a 426 kDa homotrimeric
292 conformation associated with the outer membrane with the passenger domain displayed on
293 the cell surface. The protein expressed in *E. coli,* and the purified refolded form, are ca. 400
294 kDa indicating SadA adopts a native form when expressed in *E. coli*. To confirm SadA was
295 expressed and surface localised in *S. enterica* SL1344 a number of assays were undertaken.
296 First, RT-PCR reactions were used to detect *sadA* expression in *S. enterica* SL1344 grown at
297 37°C in LB; notably no transcript was detected for the isogenic *sadA* null mutant (Fig. 2C).
298 Next the presence of SadA in the outer membrane was confirmed using the anti-SadA
299 specific antibody; thus a band ca. 400 kDa was detected in outer membrane preparations of
300 wild-type *S.* enterica SL1344 but not in an isogenic *sadA* mutant grown in LB at 37°C. Outer
301 membrane localisation of SadA was restored by introducing plasmid pDR03 into *S. enterica*
302 SL1344 Δ*sadA* (Fig. 2C); in addition to the trimeric form a lower band, consistent with
303 monomeric SadA, was also detected when SadA was over-expressed. Finally, the surface
304 localisation of SadA was confirmed by immunofluorescence microscopy (Fig. 2E). *S.*
305 *enterica* SL1344 displayed abundant surface labelling whereas the *S. enterica* SL1344 Δ*sadA*

IAI Accepts published online ahead of print

306 mutant strain was devoid of any labelling. The labelling was restored in the complemented
307 mutant *S. enterica* SL1344 Δ*sadA* pDR03. Interestingly, the labelling is punctate in both
308 wild-type and complemented mutant and in *E. coli* pDR01 strains (data not shown)
309 suggesting localisation is a phenomenon of spatial expression and not a product of variations
310 in O-antigen length. Punctate labelling has been observed previously for the prototypical
311 TAA, YadA (32) though other TAAs seem to have a variety of surface expression patterns,
312 including polar (76) and whole cell labelling (32). As some TAAs are known to bind
313 immunoglobulins (24) two further controls were performed. First the wild-type strain was
314 incubated with the fluorescently labelled secondary antibody only, and second the wild-type
315 strain was incubated with a different primary antibody (anti-Pet) prior to the addition of
316 fluorescently labelled secondary antibody. In both controls no labelling was detected (data
317 not shown).

318 **Expression of SadA promotes biofilm formation and autoaggregation.**

319 Many of the TAAs characterised previously promote biofilm formation and autoaggregation
320 (24, 76). To determine whether SadA also shared these phenotypic properties wild-type *S.*
321 *enterica* SL1344, the Δ*sadA* mutant strain and the complemented mutant strain (*S. enterica*
322 SL1344 Δ*sadA* pDR03) were tested for their ability to promote biofilm formation and
323 aggregation. No statistically significant difference was seen in aggregation between the *S.*
324 *enterica* SL1344 wild-type, the Δ*sadA* mutant or the complemented strain (Fig. 3A).
325 Similarly, in the polystyrene biofilm assay no difference in biofilm formation was seen
326 between *S. enterica* SL1344, the Δ*sadA* isogenic mutant or the complemented *S. enterica*
327 SL1344 Δ*sadA* pDR03 over-expressing SadA (Fig 3B).

IAI Accepts published online ahead of print

328 Previous investigations have demonstrated the function of Type 5 secreted molecules can be
329 obscured in *in vitro* assays by occlusion of the functional portion of the protein by other
330 surface structures such as capsular polysaccharide or smooth lipopolysaccharide (LPS) (34,
331 64, 68, 69, 75). To determine if this was the case for SadA, we took two different
332 approaches. First, SadA was expressed in *E. coli* M15 lacking smooth LPS and second we
333 introduced a *galE* null mutation into *S. enterica* SL1344 and its *sadA* mutant derivatives,
334 resulting in strains defective in O-antigen production. In contrast to wild-type *S. enterica*
335 SL1344, *E. coli* M15 pDR03 demonstrated significant levels of autoaggregation
336 (OD_{600}=0.3/h) whereas the strain carrying the empty vector (pQE60NdeI) did not settle (Fig
337 3A; >OD_{600}=0.1/h). Similarly, the *S. enterica* SL1344 Δ*sadA* Δ*galE* double mutant strain did
338 not aggregate (>OD_{600}=0.1/h) but the mutant expressing SadA did aggregate (Fig. 3C;
339 OD_{600}=0.45/h). These data clearly demonstrate the capacity of SadA to induce
340 autoaggregation.

341 To determine whether the LPS could be blocking the capacity of SadA to mediate biofilm
342 formation, *E. coli* M15 was tested in the polystyrene biofilm assay. When compared to the
343 empty vector control, biofilm formation was significantly increased when SadA was
344 expressed from *E. coli* M15 pDR03 ($P<0.001$, Fig. 3B). Furthermore, under continuous flow
345 conditions SadA mediated a strong biofilm forming phenotype in *S. enterica* lacking long O-
346 antigen side chains; this phenotype was absent from an isogenic mutant lacking SadA
347 expression (Fig 3D). This verifies that SadA can induce biofilm formation phenotypes in *S.*
348 *enterica*.

349 **SadA increases adherence and invasion of *E. coli* K12 to intestinal epithelial cells**

350 All characterised TAAs have been found to have a role in adhesion. To determine if the SadA
351 protein is also involved in adhesion, the strains described above were tested for their ability to

IAI Accepts published online ahead of print

352 adhere or invade two epithelial cell lines: the human intestinal CaCo-2 cells and murine J774
353 macrophages. No difference was detected between the ability of *S. enterica* SL1344 and *S.*
354 *enterica* SL1344 *sadA::aph* to adhere to or invade Caco-2 cells. However, when SadA was
355 expressed in *E. coli* M15 the ability of these bacteria to adhere to ($P<0.05$) and invade
356 ($P<0.01$) Caco-2 cells was significantly increased (Fig. 4). In fact, *E. coli* M15 pDR03 was
357 five times more adherent, and over 100 times more invasive than the empty vector control *E.*
358 *coli* M15 pQE60. No difference in adhesion to or invasion of J774 macrophages was
359 observed for any of the strains tested (data not shown). This suggests that SadA can promote
360 bacterial adherence to intestinal cells but that in *Salmonella* other factors can compensate for
361 the loss of *sadA*.

362 Many TAAs promote binding to extracellular matrix (ECM) molecules (50, 61, 63, 74),
363 including the SadA homologue, UpaG, which binds to fibronectin and laminin (76). To
364 determine if expression of SadA also conferred this phenotype, *S. enterica* SL1344 wild-type
365 and Δ*sadA* mutant, as well as *E. coli* overexpressing SadA were tested for their ability to
366 adhere to ECM molecules as previous performed (71). Our studies were unable to detect any
367 SadA mediated adhesion to ECM molecules collagen I, collagen III,collagen IV, elastin,
368 fibronectin, and laminin (data not shown).

369 Finally, ingestion of salad leaves contaminated with *S. enterica* strains has been associated
370 with outbreaks of disease. Specific adherence of *S. enterica* has been demonstrated (7, 8, 67).
371 Thus, we investigated the ability of SadA to mediate binding of *S. enterica* SL1344, the
372 Δ*sadA* isogenic mutant, and *E. coli* strains expressing SadA to rocket leaves. While strains
373 were capable of binding to the leaves there was no significant difference between those
374 strains expressing SadA and those which did not produce SadA (Fig. S1).

IAI Accepts published online ahead of print

375 **SadA does not promote serum resistance**

376 YadA, the prototypic TAA, and other members of the Type 5 secretion family, have been
377 shown to confer the ability to resist complement-mediated killing in serum (24, 36). Such
378 serum resistance is an important phenotype of invasive *salmonellae* as it enables the bacteria
379 to survive in the blood (53). *S. enterica* SL1344, the isogenic Δ*sadA* mutant strain and the
380 complemented version (*S. enterica* Δ*sadA* pDR03) were tested for survival in human serum
381 derived from healthy individuals. No difference in serum resistance between the *Salmonella*
382 strains was observed, all being equally resistant. SadA was also unable to confer serum
383 resistance on *E. coli* with both *E. coli* M15 pQE60 and M15 pDR03 strains remaining serum
384 sensitive (Fig. S2).

385 **Evaluation of the role of SadA in the virulence of *Salmonella* Typhimurium**

386 As SadA had been shown to be involved in adhesion, aggregation and biofilm formation we
387 wanted to test its role in virulence. A common model organism used to study *Salmonella*
388 infection is *C. elegans*, a hermaphroditic worm, which has been used to identify *Salmonella*
389 virulence factors in the past (1). *C. elegans* N2 wildtype worms were infected with either *S.*
390 *enterica* SL1344 or *S. enterica* SL1344 *sadA::aph* and the relative survival rates were
391 compared. No significant difference in survival rate was observed (Fig. S3).

392 Since, the worm model showed no attenuation of virulence, we assessed the virulence of a
393 *sadA* mutant in two murine models of infection. First, the *S. enterica sadA::aph* mutation was
394 transferred to the *S. enterica* SL3261 strain, using P22 transduction. This allowed any
395 potential role for virulence to be assessed over a longer period when CD4 T cells are required
396 for the resolution of infection. Wild-type C57BL/6 mice were infected i.p. with *S. enterica*
397 SL3261 or the isogenic *sadA::aph* mutant. The mutant strain exhibited no attenuation
398 compared to the parental *S. enterica* SL3261 in the liver or spleen, at all time points (Fig. S3).

IAI Accepts published online ahead of print

399 As SadA mediates binding and invasion of intestinal epithelial cells, but had no apparent role
400 in disease when administered by the i.p. route we hypothesised SadA may play a role in
401 establishment of *S.* Typhimurium infection in the intestine. To test this, mice were orally
402 challenged with *S. enterica* SL1344 or the isogenic *sadA::aph* mutant. No significant
403 difference was observed between the numbers of wild-type and mutant bacteria present in
404 either spleen or liver at four of six days post infection (Fig. S3). These data suggest that SadA
405 is not required for the development or maintenance of either oral or systemic *Salmonella*
406 infection in mice.

407 **Immunization with alum-precipitated SadA induces marked IgG titers and provides**
408 **limited protection against *S.* Typhimurium infection .**

409 As SadA is surface-expressed on the bacterium, we tested whether immunisation with
410 purified SadA protein could reduce bacterial colonization after infection. Mice were
411 immunised according to the schedule shown in Fig. 5A, being immunized twice (Day 0 and
412 Day 30) with PBS, purified SadA or alum-precipitated SadA (SadA-Alum) before i.p.
413 challenge with *S. enterica* SL1344 14 days after the second immunization. Mice were
414 sacrificed two days post-challenge and bacterial numbers in spleens and livers recorded. In
415 addition serum samples were taken before and after challenge with the bacteria. Mice
416 immunised with purified SadA-Alum had 6.5 fold lower numbers of bacteria in the spleen
417 ($P<0.05$) and 10-fold fewer bacteria in the liver ($P<0.01$) when compared to the PBS control.
418 Mice immunised with purified unadjuvanted SadA had 10-fold fewer bacterial numbers in the
419 liver ($P<0.01$) but no significant differences in splenic bacterial numbers (Fig. 7B) compared
420 to control mice.

421 Assessment of antibody titers in mice prior to infection showed that SadA-immunized mice
422 induced marked IgG titers against SadA and that the group that had the highest IgG titers had

IAI Accepts published online ahead of print

423 the lowest bacterial burdens. Whilst, alum-precipitating SadA augmented the IgG response it
424 was surprising to see a potent anti-SadA IgG response in mice that had received the protein
425 without adjuvant (Fig. 5). Finally IgM-titers to SadA were examined in serum taken pre and
426 post-challenge from the PBS control mice. As expected no IgM response was seen in mice
427 pre-challenge however two of the three control mice had a SadA-specific IgM response 2
428 days post-challenge with *S. enterica* SL1344 indicating SadA was being expressed *in vivo*
429 during infection (Fig. 5).

430 To prove the protective effect of anti-SadA antibody, and to rule out the influence of non-
431 specific effects or the effects of innate immunity, bacteria were opsonised before infecting
432 mice. After 3 days, mice which were challenged with opsonised bacteria demonstrated a
433 marginal but significant reduction in bacterial numbers when compared to mice challenged
434 non-opsonised bacteria (Fig. 5E). The reduction in bacterial numbers was similar to that
435 observed for mice immunised with SadA. Taken together, the data indicate that SadA is
436 expressed *in vivo* during infection, can elicit a rapid immunological response to itself, but
437 pre-existing IgG antibody to SadA only provokes a modest protective effect against challenge
438 in this host.

439 **DISCUSSION**

440 Proteins that use the Type V secretion system represent an increasingly important family of
441 secreted virulence factors in Gram negative bacteria, not only for their contribution to
442 virulence but also as targets for vaccine development (9, 80). Two Type Va, or
443 autotransporter (AT) proteins have previously been characterised in *S.* Typhimurium; MisL
444 and ShdA. These AT proteins have a role in *S.* Typhimurium intestinal colonisation and
445 persistence via binding to various ECM molecules (21, 41-43). In this study we characterise
446 SadA, the first trimeric autotransporter adhesin (TAA) to be characterised in detail in *S.*

IAI Accepts published online ahead of print

447 *enterica*. We demonstrate that SadA possesses functional properties associated with biofilm
448 formation, aggregation and adhesion to intestinal epithelial cells. Loss of SadA from the
449 bacterium does not affect the course of infection and whilst antibody to SadA can reduce
450 bacterial colonization this effect is modest.

451 Although SadA is a clear positional orthologue of the previously characterised TAA UpaG
452 from *E. coli* CFT073, this study has revealed that they have differing functions. SadA is
453 similar to UpaG in that it promotes biofilm formation and bacterial cell-cell aggregation, but
454 nonetheless it differs from UpaG in adhesion phenotypes. In contrast to UpaG, we were
455 unable to detect SadA mediated binding to any ECM molecules (76). Despite the lack of
456 ECM binding, SadA does promote significant adhesion and invasion of Caco-2 epithelial
457 cells, whereas UpaG was reported to increase binding to T24 epithelial cell lines, albeit very
458 slightly (76). These differences are most likely to be due to sequence differences in the
459 passenger domain. The passenger domain of UpaG and SadA varied significantly (46.2%
460 identity) in contrast to the translocator (79.7% identitiy) and signal sequences (72.5%
461 identity). Further analysis of the passenger domain revealed that the motifs in the predicted
462 head region were more variable than motifs in the stalk and neck structures. The head is
463 responsible for the adhesion phenotype in other TAAs (50), and thus it is not surprising that
464 SadA and UpaG have differing adhesion phenotypes.

465 In contrast to the high sequence variability between SadA and positional orthologues in *E.*
466 *coli* strains, the orthologues of SadA in *S. enterica* strains are highly conserved (over 97%
467 amino acid similarity). This is even more pronounced in the SadA orthologues from the four
468 sequenced *S.* Typhimurium strains which share 100% amino acid identity. Similarly the
469 majority of SadA orthologues in *E. coli* strains from intestinal isolates are also highly
470 conserved. The fact that the protein is also in the same genomic location in all strains

IAI Accepts published online ahead of print

471 (including in *C. youngae*) suggests that the gene has been maintained since before *Salmonella*
472 and *E. coli* diverged. These facts suggest SadA plays an important function for the bacterium.
473 The conserved nature of the protein also infers that either SadA is not under immunological
474 pressure despite surface localisation or that the amino acid sequence must remain highly
475 conserved to maintain the functionality of the protein.

476 SadA was expressed in *S. enterica* SL1344 at low levels *in vitro*. Interestingly, SadA is likely
477 to be surface-exposed *in vivo* since IgM antibody to it was detectable within 2 days of
478 infection. Our ongoing experiments indicate that SadA expression may be increased at 42°C,
479 the avian body temperature (data not shown). As *Salmonella* is also highly associated with
480 birds (26), it may be that SadA plays a more significant role in avian colonisation.

481 SadA mediated bacterial cell aggregation and biofilm formation. These phenotypes have been
482 implicated in persistence and pathogenicity for many bacterial pathogens (6, 57, 65) and are
483 also associated with many AT and TAA proteins (79) although no structural domains for
484 these conserved functions have yet been defined. Many other AT and TAA proteins that do
485 mediate aggregation and biofilm formation have other, more specific virulence functions such
486 as adhesion to epithelial cells (76, 78) or extracellular matrix molecules (77), serum
487 resistance (24), and toxin and esterase activity. Likewise, SadA has a further, more specific
488 function, promoting adhesion to and invasion of human intestinal epithelial cells (Caco-2)
489 when expressed in an *E. coli* background. Initial attachment and invasion of human intestinal
490 epithelial cells is vital for the initiation of infection (55). As SadA can mediate adhesion and
491 invasion of Caco-2 cells when expressed in *E. coli* K-12 it may play a role in the initial
492 attachment and colonisation of *Salmonella* in the host. Previous studies with TAA proteins
493 that bind epithelial cells have suggested that this is due to the ability of the proteins to bind
494 ECM components (76). As we could not detect any SadA-mediated binding to ECM

IAI Accepts published online ahead of print

495 molecules it is likely that SadA has other binding targets on the epithelial cell surface or
496 binds to ECM molecules not tested in this study.

497 Although SadA is expressed in *S.* Typhimurium *in vitro* no difference in cell aggregation,
498 biofilm formation or adhesion to Caco-2 epithelial cells was detected between the wild-type
499 and *sadA* mutant strain. Large bacterial surface structures such as caspsule, LPS or fimbriae
500 have been shown to block the function of AT proteins such as Antigen 43 and EhaA (34, 64,
501 78). We demonstrated that by removing the O-antigen from *S.* Typhimurium we could restore
502 the aggregation and biofilm formation phenotypes of SadA. This suggests that large surface
503 structures can block the function of SadA and expression of surface factors needs to be
504 carefully orchestrated in the bacterium for TAAs such as SadA to play a role in colonisation
505 and infection. Thus, SadA may also be important under particular conditions where LPS
506 production is reduced, such as during macrophage infection (25).

507 No difference in virulence between the *S.* Typhimurium wildtype and *sadA* mutant strains
508 was seen in any of the assays performed. This may be due to functional redundancy with
509 other expressed outer membrane proteins which compensate for the deletion of *sadA*. This is
510 also seen for other AT proteins characterised from *S.* Typhimurium. Although shown to be
511 critical for maintenance of faecal shedding, there is no effect on virulence when MisL and
512 ShdA are deleted (21, 43). Indeed, the roles of MisL, ShdA and SadA may be compensatory;
513 loss of one AT may not reduce virulence but loss of all three may severely attenuate *S.*
514 *enterica*. Studies to test such functional redundancy are currently underway in our laboratory.

515 A surprising finding from this study was that SadA alone was sufficient to drive a switched
516 IgG response to itself in the absence of exogenous adjuvant. This coupled to the finding that
517 during infection in naive mice anti-SadA IgM was detectable within 48 hours suggests that

IAI Accepts published online ahead of print

518 this trimeric autotransporter is likely to be recognized by selective B cell subsets, such as B1b
519 cells that are known to recognize Salmonella proteins such as OmpD (27). Nevertheless, IgG
520 antibody to SadA afforded limited protection of only a ten-fold reduction even when given
521 twice as an alum-precipitated protein. Recent studies have shown that antibodies targeting the
522 LPS of *S.* Typhimurium inhibit complement-mediated killing in immuno-compromised
523 individuals (52) despite being an immunodominant target of the immune response. Therefore,
524 collectively, the data from this and the described studies indicate that identifying the most
525 effective targets of antibody-mediated protection is likely to be a complex process and may
526 involve targeting multiple antigens in parallel.
527

528 **ACKNOWLEDGEMENTS**

529 This work was supported by project grants from the BBRSC and MRC to IRH. TJW is a
530 recipient of the Fellowship Bridging Award through the Wellcome Trust Value in People
531 scheme.
532

IAI Accepts published online ahead of print

533 **REFERENCES**

534 1. **Aballay, A., and F. M. Ausubel.** 2000. *Salmonella typhimurium* infection induces program
535 cell death in *Caenorhabditis elegans*. Molecular Biology of the Cell **11:**254a-254a.
536 2. **Alvarez, B. H., M. D. Hartmann, R. Albrecht, A. N. Lupas, K. Zeth, and D. Linke.** 2008. A new
537 expression system for protein crystallization using trimeric coiled-coil adaptors. Protein
538 Engineering Design & Selection **21:**11-18.
539 3. **Bailey, A. M., A. Ivens, R. Kingsley, J. L. Cottell, J. Wain, and L. J. Piddock.** 2010. RamA, a
540 member of the AraC/XylS family, influences both virulence and efflux in *Salmonella enterica*
541 serovar Typhimurium. J Bacteriol **192:**1607-1616.
542 4. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic-analysis of virulence plasmid from a
543 Serogroup-9 *Yersinia-enterocolitica* strain - Role of outer-membrane protein-P1 in resistance
544 to human-serum and autoagglutination. Infection and Immunity **48:**782-786.
545 5. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of
546 signal peptides: SignalP 3.0. Journal of Molecular Biology **340:**783-795.
547 6. **Berge, A., B. M. Kihlberg, A. G. Sjoholm, and L. Bjorck.** 1997. Streptococcal protein H forms
548 soluble complement-activating complexes with IgG, but inhibits complement activation by
549 IgG-coated targets. Journal of Biological Chemistry **272:**20774-20781.
550 7. **Berger, C. N., R. K. Shaw, D. J. Brown, H. Mather, S. Clare, G. Dougan, M. J. Pallen, and G.**
551 **Frankel.** 2009. Interaction of *Salmonella enterica* with basil and other salad leaves. ISME J
552 **3:**261-265.
553 8. **Berger, C. N., R. K. Shaw, F. Ruiz-Perez, J. P. Nataro, I. R. Henderson, M. J. Pallen, and G.**
554 **Frankel.** 2009. Interaction of enteroaggregative *Escherichia coli* with salad leaves.
555 Environmental Microbiology Reports **1:**234- 239.
556 9. **Bowe, F., E. C. Lavelle, E. A. McNeela, C. Hale, S. Clare, B. Arico, M. M. Giuliani, A. Rae, A.**
557 **Huett, R. Rappuoli, G. Dougan, and K. H. G. Milis.** 2004. Mucosal vaccination against
558 serogroup B meningococci: Induction of bactericidal antibodies and cellular immunity
559 following intranasal immunization with NadA of *Neisseria meningitidis* and mutants of
560 *Escherichia coli* heat-labile enterotoxin. Infection and Immunity **72:**4052-4060.
561 10. **Boyer, H. W., and D. Roulland-dussoix.** 1969. A complementation analysis of the restriction
562 and modification of DNA in *Escherichia coli.* Journal of Molecular Biology **41:**459-472.
563 11. **Chaudhuri, R. R., M. Sebaihia, J. L. Hobman, M. A. Webber, D. L. Leyton, M. D. Goldberg, A.**
564 **F. Cunningham, A. Scott-Tucker, P. R. Ferguson, C. M. Thomas, G. Frankel, C. M. Tang, E. G.**
565 **Dudley, I. S. Roberts, D. A. Rasko, M. J. Pallen, J. Parkhill, J. P. Nataro, N. R. Thomson, and**
566 **I. R. Henderson.** 2010. Complete genome sequence and comparative metabolic profiling of
567 the prototypical enteroaggregative *Escherichia coli* strain 042. PLoS One **5:**e8801.
568 12. **Comanducci, M., S. Bambini, B. Brunelli, J. Adu-Bobie, B. Arico, B. Capecchi, M. M. Giuliani,**
569 **V. Masignani, L. Santini, S. Savino, D. M. Granoff, D. A. Caugant, M. Pizza, R. Rappuoli, and**
570 **M. Mora.** 2002. NadA, a novel vaccine candidate of *Neisseria meningitidis*. Journal of
571 Experimental Medicine **195:**1445-1454.
572 13. **Cotter, S. E., N. K. Surana, and J. W. St Geme.** 2005. Trimeric autotransporters: a distinct
573 subfamily of autotransporter proteins. Trends in Microbiology **13:**199-205.
574 14. **Cotter, S. E., H. J. Yeo, T. Juehne, and J. W. S. Geme.** 2005. Architecture and adhesive
575 activity of the *Haemophilus influenzae* Hsf adhesin. Journal of Bacteriology **187:**4656-4664.
576 15. **Cunningham, A. F., F. Gaspal, K. Serre, E. Mohr, I. R. Henderson, A. Scott-Tucker, S. M.**
577 **Kenny, M. Khan, K.-M. Toellner, P. J. L. Lane, and I. C. M. MacLennan.** 2007. *Salmonella*
578 induces a switched antibody response without germinal centers that impedes the
579 extracellular spread of infection. J Immunol **178:**6200-6207.

IAI Accepts published online ahead of print

580 16. **Cunningham, A. F., K. Serre, E. Mohr, M. Khan, and K. M. Toellner.** 2004. Loss of CD154
581 impairs the Th2 extrafollicular plasma cell response but not early T cell proliferation and
582 interleukin-4 induction. Immunology **113:**187-193.
583 17. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in
584 *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences of
585 the United States of America **97:**6640-6645.
586 18. **Desvaux, M., L. M. Cooper, N. A. Filenko, A. Scott-Tucker, S. M. Turner, J. A. Cole, and I. R.**
587 **Henderson.** 2006. The unusual extended signal peptide region of the type V secretion
588 system is phylogenetically restricted. FEMS Microbiol Lett **264:**22-30.
589 19. **Desvaux, M., N. J. Parham, and I. R. Henderson.** 2004. Type V protein secretion: Simplicity
590 gone awry? Current Issues in Molecular Biology **6:**111-124.
591 20. **Desvaux, M., A. Scott-Tucker, S. M. Turner, L. M. Cooper, D. Huber, J. P. Nataro, and I. R.**
592 **Henderson.** 2007. A conserved extended signal peptide region directs posttranslational
593 protein translocation via a novel mechanism. Microbiology **153:**59-70.
594 21. **Dorsey, C. W., M. C. Laarakker, A. D. Humphries, E. H. Weening, and A. J. Baumler.** 2005.
595 *Salmonella enterica* serotype Typhimurium MisL is an intestinal colonization factor that
596 binds fibronectin. Molecular Microbiology **57:**196-211.
597 22. **Eaves, D. J., L. Randall, D. T. Gray, A. Buckley, M. J. Woodward, A. P. White, and L. J. V.**
598 **Piddock.** 2004. Prevalence of mutations within the quinolone resistance-determining region
599 of gyrA, gyrB, parC, and parE and association with antibiotic resistance in quinolone-resistant
600 *Salmonella enterica*. Antimicrobial Agents and Chemotherapy **48:**4012-4015.
601 23. **El Tahir, Y., P. Kuusela, and M. Skurnik.** 2000. Functional mapping of the *Yersinia*
602 *enterocolitica* adhesin YadA. Identification of eight NSVAIG-S motifs in the amino-terminal
603 half of the protein involved in collagen binding. Molecular Microbiology **37:**192-206.
604 24. **El Tahir, Y., and M. Skurnik.** 2001. YadA, the multifaceted *Yersinia* adhesin. International
605 Journal of Medical Microbiology **291:**209-218.
606 25. **Eriksson, S., S. Lucchini, A. Thompson, M. Rhen, and J. C. Hinton.** 2003. Unravelling the
607 biology of macrophage infection by gene expression profiling of intracellular *Salmonella*
608 *enterica*. Mol Microbiol **47:**103-118.
609 26. **Foley, S. L., A. M. Lynne, and R. Nayak.** 2008. *Salmonella* challenges: Prevalence in swine
610 and poultry and potential pathogenicity of such isolates. J. Anim Sci. **86:**E149-162.
611 27. **Gil-Cruz, C., S. Bobat, J. L. Marshall, R. A. Kingsley, E. A. Ross, I. R. Henderson, D. L. Leyton,**
612 **R. E. Coughlan, M. Khan, K. T. Jensen, C. D. Buckley, G. Dougan, I. C. M. MacLennan, C.**
613 **Lopez-Macias, and A. F. Cunningham.** 2009. The porin OmpD from nontyphoidal *Salmonella*
614 is a key target for a protective B1b cell antibody response. Proceedings of the National
615 Academy of Sciences of the United States of America **106:**9803-9808.
616 28. **Gondwe, E. N., M. E. Molyneux, M. Goodall, S. M. Graham, P. Mastroeni, M. T. Drayson,**
617 **and C. A. MacLennan.** 2010. Importance of antibody and complement for oxidative burst
618 and killing of invasive nontyphoidal Salmonella by blood cells in Africans. Proceedings of the
619 National Academy of Sciences of the United States of America **107:**3070-3075.
620 29. **Gordon, M. A.** 2008. *Salmonella* infections in immunocompromised adults. Journal of
621 Infection **56:**413-422.
622 30. **Gordon, M. A., S. M. Graham, A. L. Walsh, L. Wilson, A. Phiri, E. Molyneux, E. E. Zijlstra, R.**
623 **S. Heyderman, C. A. Hart, and M. E. Molyneux.** 2008. Epidemics of invasive *Salmonella*
624 *enterica* serovar enteritidis and *S-enterica* serovar typhimurium infection associated with
625 multidrug resistance among adults and children in Malawi. Clinical Infectious Diseases
626 **46:**963-969.
627 31. **Graham, S. M., E. M. Molyneux, A. L. Walsh, J. S. Cheesbrough, M. E. Molyneux, and C. A.**
628 **Hart.** 2000. Nontyphoidal *Salmonella* infections of children in tropical Africa. Pediatr Infect
629 Dis J **19:**1189-1196.

IAI Accepts published online ahead of print

630 32. **Grosskinsky, U., M. Schutz, M. Fritz, Y. Schmid, M. C. Lamparter, P. Szczesny, A. N. Lupas, I.**
631 **B. Autenrieth, and D. Linke.** 2007. A conserved glycine residue of trimeric autotransporter
632 domains plays a key role in *Yersinia* adhesin A autotransport. J Bacteriol **189:**9011-9019.
633 33. **Hartmann, M. D., O. Ridderbusch, K. Zeth, R. Albrecht, O. Testa, D. N. Woolfson, G. Sauer,**
634 **S. Dunin-Horkawicz, A. N. Lupas, and B. H. Alvarez.** 2009. A coiled-coil motif that sequesters
635 ions to the hydrophobic core. Proceedings of the National Academy of Sciences of the
636 United States of America **106:**16950-16955.
637 34. **Hasman, H., T. Chakraborty, and P. Klemm.** 1999. Antigen-43-mediated autoaggregation of
638 *Escherichia coli* is blocked by fimbriation. Journal of Bacteriology **181:**4834-4841.
639 35. **Henderson, I. R., R. Cappello, and J. P. Nataro.** 2000. Autotransporter proteins, evolution
640 and redefining protein secretion. Trends Microbiol **8:**529-532.
641 36. **Henderson, I. R., J. Czeczulin, C. Eslava, F. Noriega, and J. P. Nataro.** 1999. Characterization
642 of Pic, a Secreted Protease of *Shigella flexneri* and Enteroaggregative *Escherichia coli*. Infect.
643 Immun. **67:**5587-5596.
644 37. **Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen.** 2004.
645 Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev **68:**692-
646 744.
647 38. **Henderson, I. R., F. Navarro-Garcia, and J. P. Nataro.** 1998. The great escape: structure and
648 function of the autotransporter proteins. Trends Microbiol **6:**370-378.
649 39. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella-typhimurium* are
650 non-virulent and effective as live vaccines. Nature **291:**238-239.
651 40. **Kaiser, P. O., T. Riess, C. L. Wagner, D. Linke, A. N. Lupas, H. Schwarz, G. Raddatz, A.**
652 **Schafer, and V. A. J. Kempf.** 2008. The head of *Bartonella* adhesin A is crucial for host cell
653 interaction of *Bartonella henselae*. Cellular Microbiology **10:**2223-2234.
654 41. **Kingsley, R. A., D. A. Ghanem, N. Puebla-Osorio, A. M. Keestra, L. Berghman, and A. J.**
655 **Baumler.** 2004. Fibronectin binding to the *Salmonella enterica* serotype typhimurium ShdA
656 autotransporter protein is inhibited by a monoclonal antibody recognizing the A3 repeat.
657 Journal of Bacteriology **186:**4931-4939.
658 42. **Kingsley, R. A., R. L. Santos, A. M. Keestra, L. G. Adams, and A. J. Baumler.** 2002. *Salmonella*
659 *enterica* serotype Typhimurium ShdA is an outer membrane fibronectin-binding protein that
660 is expressed in the intestine. Molecular Microbiology **43:**895-905.
661 43. **Kingsley, R. A., K. van Amsterdam, N. Kramer, and A. J. Baumler.** 2000. The shdA gene is
662 restricted to serotypes of Salmonella enterica subspecies I and contributes to efficient and
663 prolonged fecal shedding. Infection and Immunity **68:**2720-2727.
664 44. **Knowles, T. J., A. Scott-Tucker, M. Overduin, and I. R. Henderson.** 2009. Membrane protein
665 architects: the role of the BAM complex in outer membrane protein assembly. Nat Rev
666 Microbiol **7:**206-214.
667 45. **Koretke, K. K., P. Szczesny, M. Gruber, and A. N. Lupas.** 2006. Model structure of the
668 prototypical non-fimbrial adhesin YadA of *Yersinia enterocolitica*. Journal of Structural
669 Biology **155:**154-161.
670 46. **Laarmann, S., D. Cutter, T. Juehne, S. J. Barenkamp, and J. W. St Geme.** 2002. The
671 *Haemophilus influenzae* Hia autotransporter harbours two adhesive pockets that reside in
672 the passenger domain and recognize the same host cell receptor. Molecular Microbiology
673 **46:**731-743.
674 47. **Lafontaine, E. R., L. D. Cope, C. Aebi, J. L. Latimer, G. H. McCracken, and E. J. Hansen.** 2000.
675 The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella*
676 *catarrhalis* to human epithelial cells in vitro. Journal of Bacteriology **182:**1364-1373.
677 48. **Lehr, U., M. Schütz, P. Oberhettinger, F. Ruiz-Perez, J. Donald, T. Palmer, D. Linke, I.**
678 **Henderson, and I. Autenrieth.** 2010. C-terminal amino acid residues of the trimeric

IAI Accepts published online ahead of print

679 autotransporter adhesin YadA of *Yersinia enterocolitica* are decisive for its recognition and
680 assembly by BamA. Molecular Microbiology**:**no-no.
681 49. **Leyton, D. L., M. d. G. De Luna, Y. R. Sevastsyanovich, K. Tveen Jensen, D. F. Browning, A.**
682 **Scott-Tucker, and I. R. Henderson.** 2010. The unusual extended signal peptide region is not
683 required for secretion and function of an *Escherichia coli* autotransporter. Fems
684 Microbiology Letters **pre-pub:**no no.
685 50. **Linke, D., T. Riess, I. B. Autenrieth, A. Lupas, and V. A. J. Kempf.** 2006. Trimeric
686 autotransporter adhesins: variable structure, common function. Trends in Microbiology
687 **14:**264-270.
688 51. **Ma, H. S., J. E. Croudace, D. A. Lammas, and R. C. May.** 2006. Expulsion of live pathogenic
689 yeast by macrophages. Current Biology **16:**2156-2160.
690 52. **MacLennan, C. A., J. J. Gilchrist, M. A. Gordon, A. F. Cunningham, M. Cobbold, M. Goodall,**
691 **R. A. Kingsley, J. J. G. van Oosterhout, C. L. Msefula, W. L. Mandala, D. L. Leyton, J. L.**
692 **Marshall, E. N. Gondwe, S. Bobat, C. Lopez-Macias, R. Doffinger, I. R. Henderson, E. E.**
693 **Zijlstra, G. Dougan, M. T. Drayson, I. C. M. MacLennan, and M. E. Molyneux.** 2010.
694 Dysregulated humoral immunity to nontyphoidal *Salmonella* in HIV-infected African adults.
695 Science **328:**508-512.
696 53. **MacLennan, C. A., E. N. Gondwe, C. L. Msefula, R. A. Kingsley, N. R. Thomson, S. A. White,**
697 **M. Goodall, D. J. Pickard, S. M. Grahami, G. Dougan, C. A. Hart, M. E. Molyneux, and M. T.**
698 **Drayson.** 2008. The neglected role of antibody in protection against bacteremia caused by
699 nontyphoidal strains of *Salmonella* in African children. Journal of Clinical Investigation
700 **118:**1553-1562.
701 54. **Majowicz, S. E., J. Musto, E. Scallan, F. J. Angulo, M. Kirk, S. J. O'Brien, T. F. Jones, A. Fazil,**
702 **R. M. Hoekstra, and I. C. E. D. Burd.** 2010. The global burden of nontyphoidal *Salmonella*
703 *gastroenteritis*. Clinical Infectious Diseases **50:**882-889.
704 55. **Monack, D. M., A. Mueller, and S. Falkow.** 2004. Persistent bacterial infections: The
705 interface of the pathogen and the host immune system. Nature Reviews Microbiology **2:**747-
706 765.
707 56. **Nummelin, H., M. C. Merckel, Y. El Tahir, P. Ollikka, M. Skurnik, and A. Goldman.** 2003.
708 Structural studies of *Yersinia* adhesin YadA. Genus *Yersinia*: Entering the Functional Genomic
709 Era **529:**85-88.
710 57. **Ochiai, K., T. Kuritaochiai, Y. Kamino, and T. Ikeda.** 1993. Effect of coaggregation on the
711 pathogenicity of oral bacteria. Journal of Medical Microbiology **39:**183-190.
712 58. **Ojha, A., M. Anand, A. Bhatt, L. Kremer, W. R. Jacobs, and G. F. Hatfull.** 2005. GroEL1: A
713 dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in
714 mycobacteria. Cell **123:**861-873.
715 59. **Parham, N. J., U. Srinivasan, M. Desvaux, B. Foxman, C. F. Marrs, and I. R. Henderson.**
716 2004. PicU, a second serine protease autotransporter of uropathogenic *Escherichia coli*.
717 Fems Microbiology Letters **230:**73-83.
718 60. **Riess, T., S. G. E. Andersson, A. Lupas, M. Schaller, A. Schafer, P. Kyme, J. Martin, J. H.**
719 **Walzlein, U. Ehehalt, H. Lindroos, M. Schirle, A. Nordheim, I. B. Autenrieth, and V. A. J.**
720 **Kempf.** 2004. *Bartonella* adhesin A mediates a proangiogenic host cell response. Journal of
721 Experimental Medicine **200:**1267-1278.
722 61. **Riess, T., S. G. E. Andersson, A. Lupas, M. Schaller, J. H. Walzlein, U. Ehehalt, H. Lindroos,**
723 **M. Schirle, A. Nordheim, I. B. Autenrieth, and V. A. J. Kempf.** 2004. *Bartonella henselae*
724 adhesin A (BadA), a novel non-fimbrial adhesin mediating a proangiogenic host cell
725 response. International Journal of Medical Microbiology **294:**94-94.
726 62. **Roggenkamp, A., N. Ackermann, C. A. Jacobi, K. Truelzsch, H. Hoffmann, and H.**
727 **Heesemann.** 2003. Molecular analysis of transport and oligomerization of the *Yersinia*
728 *enterocolitica* adhesin YadA. Journal of Bacteriology **185:**3735-3744.

IAI Accepts published online ahead of print

729 63. **Scarselli, M., D. Serruto, P. Montanari, B. Capecchi, J. Adu-Bobie, D. Veggi, R. Rappuoli, M.**
730 **Pizza, and B. Arico.** 2006. *Neisseria meningitidis* NhhA is a multifunctional trimeric
731 autotransporter adhesin. Molecular Microbiology **61:**631-644.
732 64. **Schembri, M. A., D. Dalsgaard, and P. Klemm.** 2004. Capsule shields the function of short
733 bacterial adhesins. Journal of Bacteriology **186:**1249-1257.
734 65. **Schembri, M. A., K. Kjaergaard, and P. Klemm.** 2003. Global gene expression in *Escherichia*
735 *coli* biofilms. Molecular Microbiology **48:**253-267.
736 66. **Schembri, M. A., and P. Klemm.** 2001. Biofilm formation in a hydrodynamic environment by
737 novel FimH variants and ramifications for virulence. Infection and Immunity **69:**1322-1328.
738 67. **Shaw, R. K., C. N. Berger, B. Feys, S. Knutton, M. J. Pallen, and G. Frankel.** 2008.
739 Enterohemorrhagic *Escherichia coli* exploits EspA filaments for attachment to salad leaves.
740 Applied and Environmental Microbiology **74:**2908-2914.
741 68. **Sherlock, O., M. A. Schembri, A. Reisner, and P. Klemm.** 2004. Novel roles for the AIDA
742 adhesin from diarrheagenic *Escherichia coli*: cell aggregation and biofilm formation. J
743 Bacteriol **186:**8058-8065.
744 69. **Sherlock, O., R. M. Vejborg, and P. Klemm.** 2005. The TibA adhesin/invasin from
745 enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and
746 biofilm formation. Infect Immun **73:**1954-1963.
747 70. **Smollett, K., R. K. Shaw, J. Garmendia, S. Knutton, and G. Frankel.** 2006. Function and
748 distribution of EspG2, a type III secretion system effector of enteropathogenic *Escherichia*
749 *coli*. Microbes Infect **8:**2220-2227.
750 71. **Sokurenko, E. V., V. A. McMackin, and D. L. Hasty.** 1995. Bacterial adhesion measured by
751 growth of adherent organisms. Methods Enzymol **253:**519-528.
752 72. **Surana, N. K., D. Cutter, S. J. Barenkamp, and J. W. St Geme.** 2004. The *Haemophilus*
753 *influenzae* Hia autotransporter contains an unusually short trimeric translocator domain.
754 Journal of Biological Chemistry **279:**14679-14685.
755 73. **Szczesny, P., D. Linke, A. Ursinus, K. Bar, H. Schwarz, T. M. Riess, V. A. J. Kempf, A. N.**
756 **Lupas, J. Martin, and K. Zeth.** 2008. Structure of the head of the *Bartonella* adhesin BadA.
757 Plos Pathogens **4:**-.
758 74. **Tertti, R., M. Skurnik, T. Vartio, and P. Kuusela.** 1992. Adhesion protein Yada of *Yersinia*
759 species mediates binding of bacteria to fibronectin. Infection and Immunity **60:**3021-3024.
760 75. **Ulett, G. C., R. I. Webb, and M. A. Schembri.** 2006. Antigen-43-mediated autoaggregation
761 impairs motility in *Escherichia coli*. Microbiology **152:**2101-2110.
762 76. **Valle, J., A. N. Mabbett, G. C. Ulett, A. Toledo-Arana, K. Wecker, M. Totsika, M. A.**
763 **Schembri, J. M. Ghigo, and C. Beloin.** 2008. UpaG, a new member of the trimeric
764 autotransporter family of adhesins in uropathogenic *Escherichia coli*. Journal of Bacteriology
765 **190:**4147-4161.
766 77. **Wells, T. J., T. N. McNeilly, M. Totsika, A. Mahajan, D. L. Gally, and M. A. Schembri.** 2009.
767 The *Escherichia coli* O157:H7 EhaB autotransporter protein binds to laminin and collagen I
768 and induces a serum IgA response in O157:H7 challenged cattle. Environmental
769 Microbiology **11:**1803-1814.
770 78. **Wells, T. J., O. Sherlock, L. Rivas, A. Mahajan, S. A. Beatson, M. Torpdahl, R. I. Webb, L. P.**
771 **Allsopp, K. S. Gobius, D. L. Gally, and M. A. Schembri.** 2008. EhaA is a novel autotransporter
772 protein of enterohemorrhagic *Escherichia coli* O157 : H7 that contributes to adhesion and
773 biofilm formation. Environmental Microbiology **10:**589-604.
774 79. **Wells, T. J., M. Totsika, and M. A. Schembri.** 2010. Autotransporters of *Escherichia coli*: a
775 sequence based characterisation. Microbiology.
776 80. **Wells, T. J., J. J. Tree, G. C. Ulett, and M. A. Schembri.** 2007. Autotransporter proteins: novel
777 targets at the bacterial cell surface. Fems Microbiology Letters **274:**163-172.

IAI Accepts published online ahead of print

81. **Wray, C., and W. J. Sojka.** 1978. Experimental *Salmonella-typhimurium* infection in calves. Research in Veterinary Science **25:**139-143.

82. **Yeo, H. J., S. E. Cotter, S. Laarmann, T. Juehne, J. W. St Geme, and G. Waksman.** 2004. Structural basis for host recognition by the *Haemophilus influenzae* Hia autotransporter. Embo Journal **23:**1245-1256.

IAI Accepts published online ahead of print

785

786 **Table 1.** Strains and Plasmids used in this study

Strains	Characteristics	Reference
S. enterica		
SL1344	Wild type *Salmonella enterica* Typhimurium	(81)
SL1344 *sadA::aph*	Part of *sadA* gene replaced with kanamycin resistance cassette	This study
SL1344Δ*sadA*	SL1344 *sadA::aph* with *aph* cassette resolved	This study
SL3261	Attenuated *Salmonella enterica* Typhimurium. SL1344Δ*aroA*	(39)
SL3261 *sadA::aph*	Part of *sadA* gene replaced with kanamycin resistance cassette	This study
SL3261 Δ*sadA*	SL3261 *sadA::aph* with *aph* cassette resolved	This study
SL3261 *galE::aph*	The *galE* gene replaced with kanamycin resistance cassette	This study
SL3261 Δ*sadA, galE::aph*	The *galE* gene replaced with kanamycin resistance cassette	This study
E. coli		
M15 pREP4	For regulated high-level expression with pQE vectors	Qiagen
HB101	Non-aggregating *E. coli* laboratory strain	(10)
BL21	*E. coli* DE3 protein expression strain	Invitrogen
Plasmids		
pET22b	IPTG-inducible expression vector	Invitrogen
pDR01	*sadA* gene in the *Nde*I – *EcoRI* sites of pET22b	This study
pQE60	IPTG-inducible expression vector	Qiagen
pQE60 *Nde*I	pQE60 with added *Nde*I restriction site in the multiple cloning site and previously existing *Nde*I site removed	This study
pDR03	pQE60*NdeI* carrying *sadA* gene in *Nde*I – *Hind*III site	This study

787
788

IAI Accepts published online ahead of print

789

790 **Figure 1.** *In silico* analysis of SadA protein. (A) Schematic diagram comparing SadA and
791 UpaG proteins. Structural domains are shown as coloured boxes and Pfam motifs as follows;
792 Signal peptide, pink; Head, yellow; Neck, orange; Stalk, black; Translocation domain, green.
793 (B) Unrooted phylogram of full length SadA orthologues from 32 genomes. Genomes are
794 colour coded according to pathogenesis groups. Bootstrap values from 1000 replicates are
795 shown. (C) Genomic region containing *sadA* in *S.* Typhimurium LT2 and alignment to the
796 orthologues region in *E. coli* CFT073 and *E. coli* K-12.

797

798 **Figure 2.** Purification, expression and localisation of SadA. (A) Stained SDS-PAGE gel of
799 total cell protein extracts from (i) *E. coli* BL21 pET22b and (ii) *E. coli* BL21 pDR01 (SadA+)
800 and (iii) the protein sample after electro-elution and purification. L: PageRuler Plus Protein
801 Ladder (B) Tryptophan fluorescence emission of re-folded SadA. An excitation wavelength
802 of 280 nm was used and emission was scanned from 300-450 nm with 1 nm increments. (C)
803 RT-PCR gel using *sadA* specific primers of (i) wild-type *S. enterica* SL1344 and (ii) isogenic
804 *sadA* mutant grown in LB at 37°C. L: Hyperladder 1 (Bioline) (D) Western immunoblot
805 analysis using SadA-specific antibody from outer membrane preparations of (i) wild-type *S.*
806 *enterica* SL1344, (ii) mutant *S. enterica* SL1344 Δ*sadA* and (iii) complemented *S. enterica*
807 Δ*sadA* pDR03. (E) Localisation of SadA on the cell surface. *S. enterica* SL1344, *S. enterica*
808 SL1344 Δ*sadA* and *S. enterica* Δ*sadA* pDR03 were probed with anti-SadA antibody followed
809 by GAR alexa fluor488. Phase contrast and fluorescence micrographs demonstrate labelling
810 of the wild-type and complemented but not the mutant strain. Two controls were performed
811 where SL1344 was treated with secondary antibody only or a different primary antibody
812 followed by the secondary to rule out binding to immunoglobulins. Neither controls had any
813 labelling (not shown). Bar= 0.5 µm

IAI Accepts published online ahead of print

814

815 **Figure 3.** Autoaggregation and biofilm formation mediated by SadA (A) Settling profiles of
816 *S. enterica* SL1344, *S. enterica* SL1344 Δ*sadA, S. enterica* SL1344 Δ*sadA* pDR03, *E. coli*
817 M15 pQE60 and *E. coli* M15 pDR03. Images show settling from static liquid suspensions in
818 the SadA expressing *E. coli* M15 strain. (B) Biofilm formation of the same five strains in
819 polystyrene plates. All strains were cultured in conditions suitable for inducing *sadA*
820 expression from pDR03. The data is the average of 8 replicate samples, performed on three
821 separate occasions. (C) Settling profiles of an *S. enterica galE sadA* double mutant and the
822 double mutant expressing SadA (pDR03). (D) Flow cell biofilms demonstrating spatial
823 distribution of double mutant *S. enterica* SL3261 Δ*sadA galE::aph* with either pQE60 or
824 pDR03 after 18 hours growth. Shown are representative horizontal sections within each
825 biofilm and vertical sections representing the yz-plane and xz-plane at the positions indicated
826 by the white dotted lines.

827

828 **Figure 4.** Adherence and invasion of *S. enterica* SL1344, *S. enterica* SL1344 *sadA::aph, E.*
829 *coli* M15pQE60 and *E. coli* M15pDR03 (SadA+) to intestinal CaCo-2. The *E. coli* strain
830 expressing SadA had increased adhesion ($p<0.05$) and invasion ($p<0.01$) over the *E. coli*
831 strain with the empty plasmid.

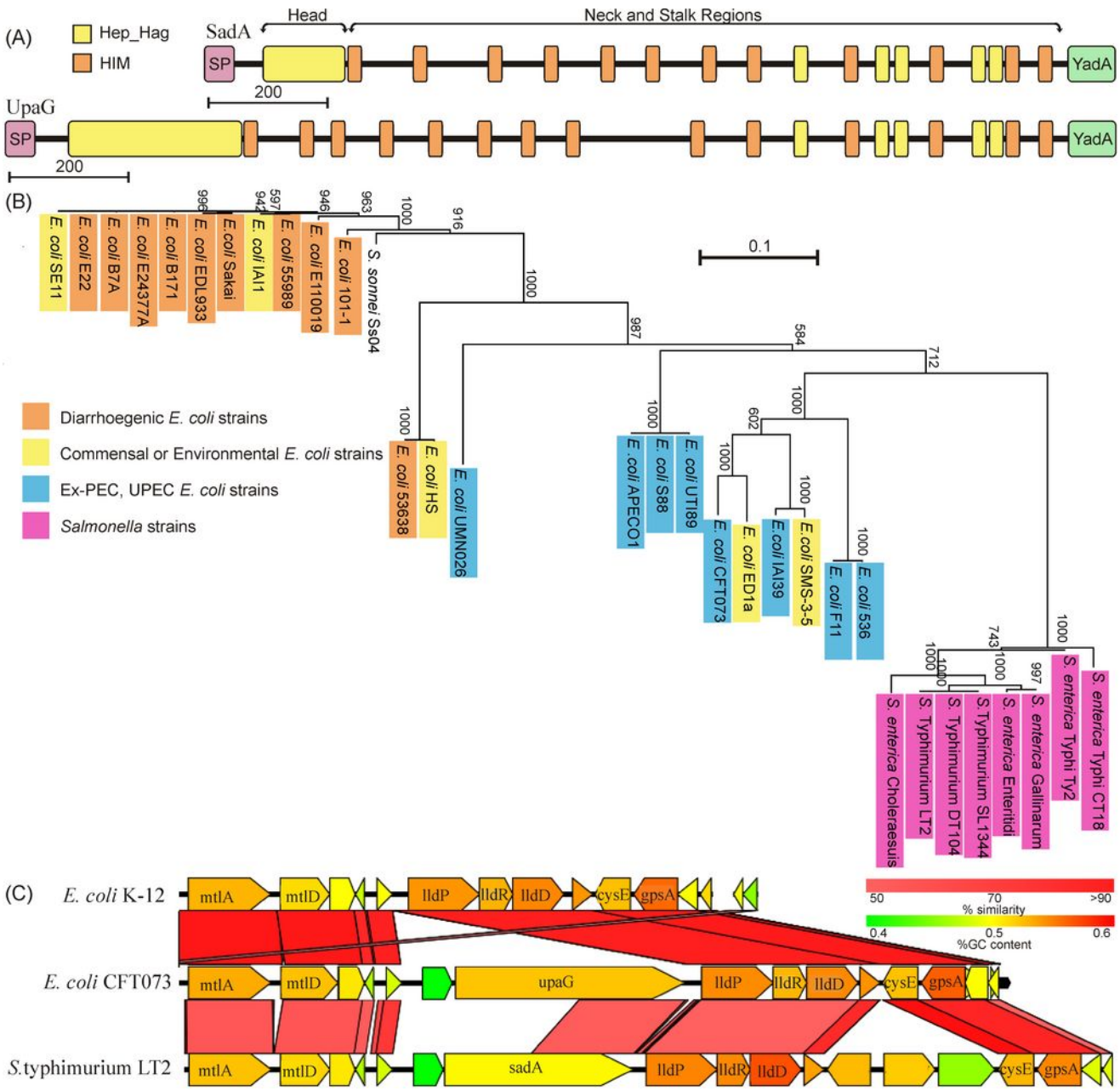
832

833 **Figure 5.** SadA induces an antibody response in mice (A) Experimental design of challenge
834 experiment (B) Colony forming units (CFUs) recovered from the liver and spleen of mice
835 two days after challenge. Mice dosed with SadA-Alum had lower levels of *S. enterica* in both
836 spleen and liver compared to the control where-as mice dosed with SadA only had lower
837 bacterial numbers in the liver. (C) Sera taken from mice prior to challenge with *S. enterica*
838 SL1344 were tested for relative amounts of SadA-specific IgG. SadA specific IgG was found

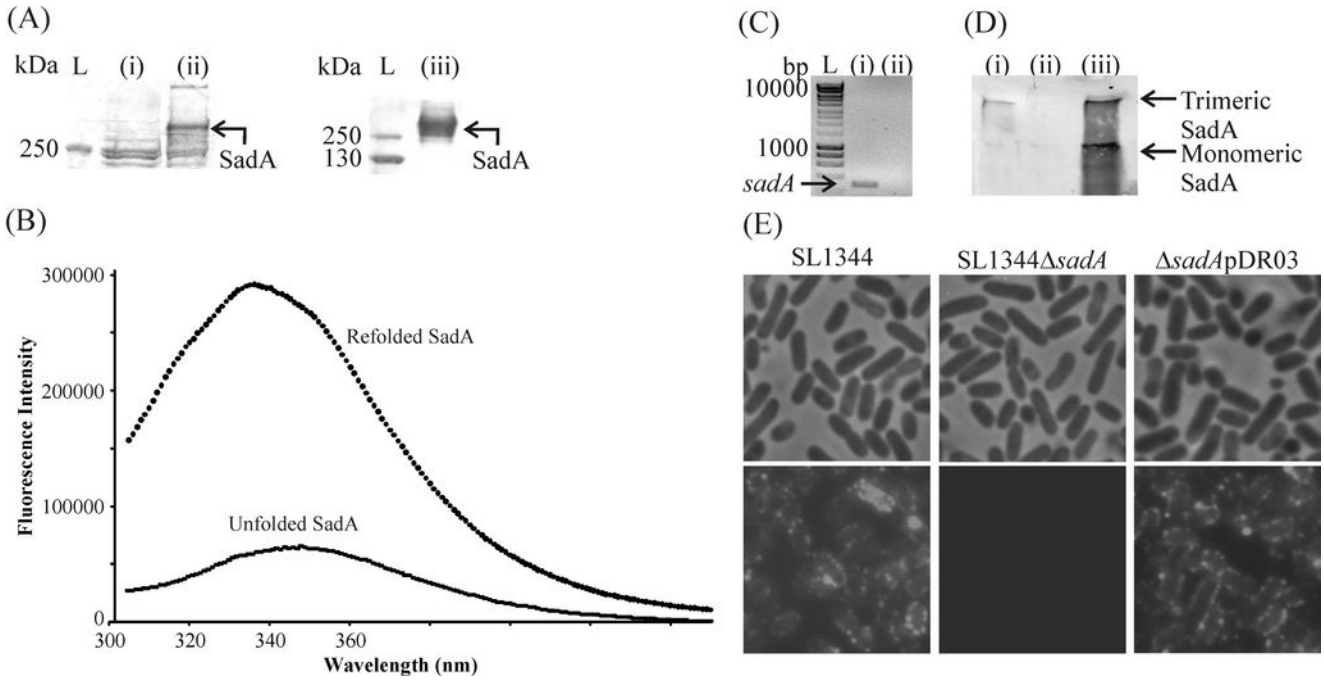
IAI Accepts published online ahead of print

839 in mice dosed with SadA and SadA-Alum. (D) SadA specific IgM was found in post-
840 challenge serum from PBS control mice but not in pre-challenge serum indicating SadA
841 expression *in vivo* during *S. enterica* SL1344 infection. (E) CFUs recovered from the spleens
842 of mice infected with opsonised *S. enterica* SL1344. Bacteria opsonised with the serum from
843 SadA immunised mice had a reduction in bacterial numbers compared to the PBS serum
844 control.
845

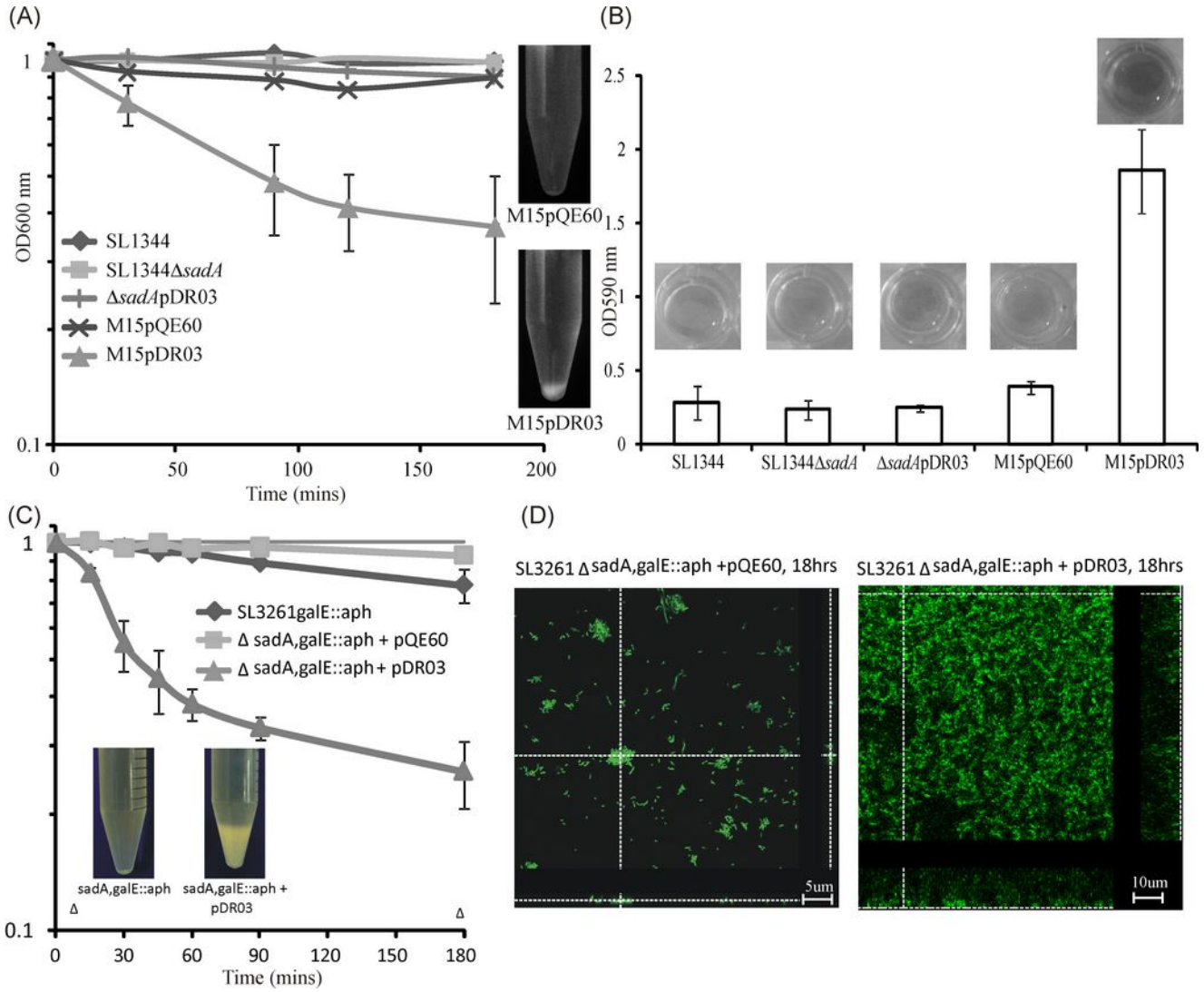
IAI Accepts published online ahead of print



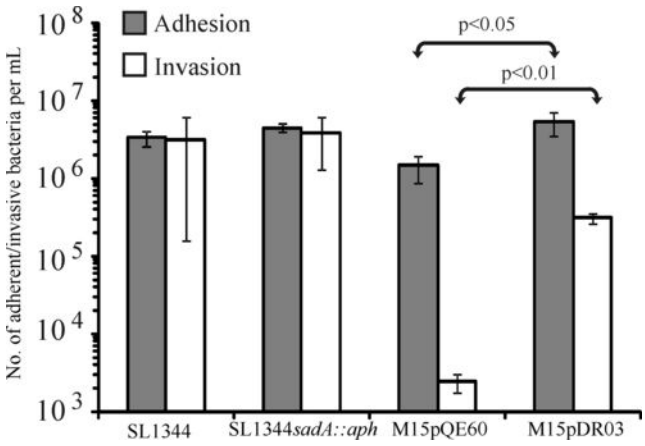
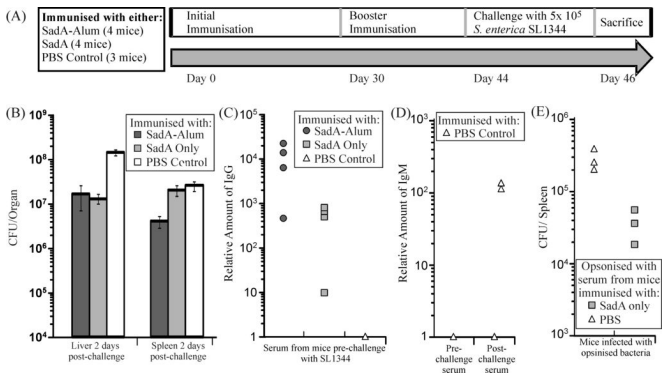
IAI Accepts published online ahead of print



IAI Accepts published online ahead of print



IAI Accepts published online ahead of print



IAI Accepts published online ahead of print