IAI Accepted Manuscript Posted Online 6 February 2017
Infect. Immun. doi:10.1128/IAI.01021-16
Copyright © 2017 Kintz et al.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Accepted Manuscript Posted Online
Infection and Immunity
IAI
Infection and Immunity

1 *Salmonella* Typhi Lipopolysaccharide O-antigen Modifications Impact on Serum Resistance
2 and Antibody Recognition
3
4 Erica Kintz[a#*], Christian Heiss[b], Ian Black[b], Nicholas Donohue[a], Naj Brown[a], Mark R.
5 Davies[a**], Parastoo Azadi[b], Stephen Baker[c, d], Paul M. Kaye[a], Marjan van der Woude[a#]
6
7 [a]Centre for Immunology and Infection, Hull York Medical School and the Department of
8 Biology, University of York, York, UK
9 [b]Complex Carbohydrate Research Center, The University of Georgia, Athens, Georgia, USA
10 [c] The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford
11 University Clinical Research Unit, Ho Chi Minh City, Vietnam
12 [d]Centre for Tropical Medicine, Oxford University, Oxford, United Kingdom
13
14
15 Running Head: *S.* Typhi O-antigen Modification and Host Defence
16
17 #Address correspondence to Marjan van der Woude, Marjan.vanderwoude@york.ac.uk or
18 Erica Kintz, E.Kintz@uea.ac.uk
19
20 * Current address Norwich Medical School, University of East Anglia. Norwich, United
21 Kingdom
22 **Current address Department of Microbiology and Immunology, Peter Doherty Institute for
23 Infection and Immunity, University of Melbourne, Australia
24

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

25 **Abstract**

26 *Salmonella* Typhi is a human restricted Gram-negative bacterial pathogen, responsible for
27 causing an estimated 27 million cases of typhoid fever annually leading to 217,000 deaths,
28 and current vaccines do not offer full protection. The O-antigen side chain of the
29 lipopolysaccharide is an immunodominant antigen, can define host-pathogen interactions,
30 and is under consideration as a vaccine target for some Gram-negative species. The
31 composition of the O-antigen can be modified by activity of glycosyltransferase (*gtr*) operons
32 acquired by horizontal gene transfer. Here we investigate the role of two *gtr* operons we
33 identified in the *S.* Typhi genome. Strains were engineered to express specific *gtr* operons.
34 Full chemical analysis of the O-antigen of these strains identified *gtr*-dependent glucosylation
35 and acetylation. The glucosylated form of the O-antigen mediated enhanced survival in
36 human serum and decreased complement binding. A single nucleotide deviation from an
37 epigenetic phase variation signature sequence rendered expression of this glucosylating *gtr*
38 operon uniform in the population. In contrast, expression of the acetylating *gtrC* is controlled
39 by epigenetic phase variation. Acetylation did not affect serum survival, but phase variation
40 can be an immune evasion mechanism and thus this modification may contribute to
41 persistence in a host. In murine immunization studies, both O-antigen modifications were
42 generally immunodominant. Our results emphasize that natural O-antigen modification
43 should be taken into consideration when assessing responses to vaccines, especially O-
44 antigen based vaccines, and that the *Salmonella gtr* repertoire may confound the protective
45 efficacy of broad ranging *Salmonella* lipopolysaccharide conjugate vaccines.

46

47

48

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

49 **INTRODUCTION**

50 *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi) is responsible for an
51 estimated 27 million new cases of typhoid fever and 217,000 deaths annually (1). Infection
52 with *S*. Typhi occurs via the fecal-oral route; after ingestion, bacteria cross the intestinal
53 epithelium, enter the bloodstream and spread systemically (2). In some cases, *S*. Typhi is
54 capable of colonizing the gall bladder, leading to chronic asymptomatic shedding and
55 contributing to the infection cycle. Two vaccines are currently licensed for use against *S*.
56 Typhi: the parenterally administered Vi capsular polysaccharide subunit vaccine and the
57 orally administered live-attenuated Ty21a vaccine. Neither vaccine offers complete
58 protection (3) and there is on-going research into new vaccine formulations against *S*. Typhi
59 and other *Salmonella* infections (3, 4). Conjugate vaccines, combining carrier proteins with
60 the Vi polysaccharide antigen are under development. However, Vi expression can be up- or
61 down-regulated and Vi-negative isolates have been isolated from typhoid patients (5, 6).
62 Lipopolysaccharide (LPS) is a Gram-negative bacterial virulence factor, a component of the
63 outer membrane and, in the absence of Vi, is the predominant *S.* Typhi surface carbohydrate.
64 Notably, the efficacy of the *S.* Typhi Ty21 vaccine is associated in part to expression of LPS
65 (7).
66
67 LPS is composed of a lipid A tail, which anchors the LPS into the membrane, a core
68 oligosaccharide and O-antigen side chain. The surface-exposed O-antigen side chain protects
69 the bacterial cell from the actions of the innate immune system (8). The O-antigen is
70 immunogenic, and may be a functional target for novel vaccines (9, 10). The *S. enterica*
71 subspecies is comprised of over 2,600 serovars, which is based on differences in the antigenic
72 properties of the O and H (flagellar) antigens, and forms the basis of the Kauffman-White

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

73 serotyping system (11). Antibodies are raised during natural infection against the LPS and the
74 detection of *S.* Typhi O-antigen antibodies forms the basis of the diagnostic Widal test for
75 typhoid (12). Furthermore, significant cross-reactivity between serovars sharing certain O-
76 antigen epitopes exists, which may be exploited in developing pan-*Salmonella* vaccines (13).
77 Therefore, gaining insight into occurrence and significance of variation in *S.* Typhi O-antigen
78 composition may enhance understanding of *S.* Typhi pathogenesis, and support the
79 development of diagnostic and intervention tools and therapies.
80
81 Modifications in LPS, including those in the O-antigen, play a role in many chronic bacterial
82 infections (14). O-antigen structures can be modified through several processes, and recently
83 we identified and characterised numerous *Salmonella gtr* (glycosyltransferase) operons (15).
84 Using the amino acid sequence identity of the GtrC O-antigen modifying proteins we were
85 able to group the *gtr* operons into ten different "families" and proposed that each family
86 performs a different O-antigen modification (15). We additionally noted that a single
87 *Salmonella* isolate may harbour multiple *gtr* operons and several families of these *gtr* operons
88 can undergo phase variation (15, 16), thus generating further potential complexity of the O-
89 antigen presented by a population. If, as a result, clonal bacterial populations have a non-
90 uniform O-antigen composition, this could serve as a means of immune evasion (17-19).
91
92 The significance of *gtr*-mediated O-antigen modification for *Salmonella* biology is not fully
93 understood. In *S.* Typhimurium specific modifications have been implicated in gut
94 colonization (20) and in phage resistance (21). To better understand the extent and impact of
95 O-antigen variation, we aimed to characterize the activity and expression of the *gtr* repertoire
96 in *S.* Typhi. Given that *S.* Typhi causes a systemic infection and that evading the innate

Accepted Manuscript Posted Online

97 immune response can contribute to persistence, we measured the effect of *gtr* modification on
98 serum sensitivity. Further, we assessed the antibody response towards each O-antigen
99 modification in serum from a murine immunization model.
100

101 MATERIALS AND METHODS

102 Bacterial strains and culture conditions

103 Strains were grown in LB unless mentioned otherwise. For *S.* Typhi BRD948, medium was
104 supplemented with an Aro mix (final concentrations: 40 µM L-phenylalanine, 40 µM L-
105 tryptophan, 1 µM para-aminobenzoic acid, and 1 µM 2,3-dihydroxybenzoic acid, tyrosine 40
106 µM). Antibiotic were used at the following concentrations: tetracycline (15 µg/ml),
107 ampicillin (100 µg/ml), chloramphenicol (34 µg/ml for vectors or 8 µg/ml for chromosomal
108 inserts) or kanamycin (30 µg/ml). *S* Typhi BRD948 and its derivatives were confirmed to be
109 Vi+ by serum agglutination (Vi antiserum, Serum Statens Institute). For analysis of LacZ
110 expression, strains were grown on minimal M9 media (Sigma, Gilingham, UK) with 0.2%
111 glucose (*S.* Typhimurium) or LB (*S.* Typhi) with 40 µg/ml X-gal (5-Bromo-4-chloro-3-
112 indolyl-ß-D-galactoside, Melford). Strains are listed in Supplemental Table S1.
113

114 Molecular biology, strain construction and mutagenesis

115 Standard molecular biology techniques were used (22). Details on vectors and primers used
116 in these studies are provided in Supplemental Table S1. Strains containing a *lacZ* reporter
117 fusion on the chromosome at the *attB* site were generated using the CRIM system (23) using
118 vector pMV243 (24). Allelic replacement was used to introduce mutations to the
119 chromosome (25). Antibiotic resistance cassettes were obtained from Tn10 for TcR and
120 pKD4 for KmR (25). Unmarked strains were generated by removal of the pKD13 derived

Infection and Immunity
IAI
Infection and Immunity
Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online
Infection and Immunity
IAI
Infection and Immunity
Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

121 KmR cassette by Flp recombinase expressed from pCP20 (26). Vectors, and primer details
122 are provided in Table S1.
123
124 **Analysis of gene expression.**
125 β-galactosidase assays on strains with *lacZ* reporter fusions were performed as described by
126 Miller (27). Cultures derived from two independent colonies were grown in M9 minimal
127 media with glucose. Samples were collected at least in triplicate between OD_{600} 0.3-1.5 for
128 measurement of β-galactosidase activity, given in Miller Units. The switch frequency of
129 phase variation was calculated for two independent colonies each for a Lac+ or Lac-
130 phenotype as described (28). The switch frequency is expressed as number of cells that have
131 changed expression state (M) over total number of cells (N), divided by the number of
132 generations (g).
133
134 **LPS extraction and visualization**
135 Crude LPS extracts were prepared as described (15), separated on a Tricine SDS-PAGE and
136 O-antigen visualized using a silver stain (15) or Western blot. These crude extracts can
137 incidentally contain contaminating macromolecules that are apparent on the gels or Westerns.
138 For Western blots, the samples were transferred to PVDF and blocked with 5% milk PBS-T.
139 O12, OMA polyvalent antiserum mixture to salmonella O-antigen from Group A, B, D, E, L
140 (Serum Statens Institute) or $O12_2$ sera were used to probe blots as indicated using secondary
141 goat anti rabbit IgG-HRP [Sigma (A0545)]. When mouse serum was used, goat anti-mouse
142 IgG-HRP (A1068) served as secondary. Millipore's Luminato Western HRP substrate was
143 used for detection.
144

Accepted Manuscript Posted Online

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

145 **Serum sensitivity assay**

146 Human serum was purchased from Sigma (H4522). This serum failed to agglutinate the *S.*
147 Typhi strains. *S.* Typhi strains were grown over night and then diluted to an OD_{600} = 0.5 in
148 PBS. 5ul of bacteria were added to 45ul of serum diluted in PBS and incubated one hour at
149 37˚C. Dilutions were performed to determine colony-forming units. Survival was determined
150 by comparing counts to those obtained from a control that was incubated in 45ul PBS.
151 Experiments were performed in duplicate and repeated at least three times.

152

153 **C3 binding to bacterial surface**

154 Experiments were performed using stationary phase bacteria. Methods were adapted from
155 MacClennan et al. (29), following quantities similar to the serum sensitivity experiments.
156 Briefly, bacteria were incubated with full strength human serum followed by incubation with
157 Dako's polyclonal rabbit anti-human C3c complement FITC antibody (F-0201). A Beckman
158 Coulter CyAn ADP analyser was used to collect data. Bangs FITC-5 MESF kit (Cat# 555)
159 allowed for standardization of fluorescence across experiments.

160

161 **Generation of murine immune serum**

162 Female *Slc11a1*^{D169} (Nramp1^{s}) C57BL/6 CD45.1 mice were obtained from Charles River
163 UK, housed under specific pathogen-free conditions and used at 6–10 weeks of age. All
164 experiments were approved by the University of York Animal Welfare and Ethical Review
165 Body and performed under UK home Office license. For immunizations, bacteria from
166 overnight cultures was diluted to an OD_{600} = 1.0 in PBS. 200ul was given IP, resulting in an
167 inoculum ranging from 8.12 x 10^7 to 2.56 x 10^8 CFU. 28 days after immunization mice were
168 exsanguinated by cardiac puncture under terminal anaesthesia. Serum was obtained by

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

169 allowing the collected blood to coagulate overnight at 4°C, then centrifuging at 2000rpm for
170 2 minutes. The serum layer could then be removed and stored at -20°C until use. Nine mice
171 were immunised with the STy-basal strain and seven immunised with the STy- FM strain.
172

173 **RESULTS**

174 The genomes of the *S*. Typhi strains Ty2, CT18 and P-stx-12 (isolated from a chronic carrier
175 in India) (30) each contain two different *gtr* operons (15). These share sequence identity
176 between the *S*. Typhi strains. One operon is a family 3 *gtr* type with high identity (99%
177 amino acid) to the *S*. Typhimurium family 3 operon (STM0557-0559) that mediates α1->4
178 glucosylation of the O-antigen galactose sugar (20). The second *gtr* operon could be grouped
179 with the family 2 GtrCs and shared 77% amino acid identity with a similar operon in the
180 invasive *S*. Typhimurium isolate D23580. This GtrC operon has been hypothesised to
181 acetylate the rhamnose residue of the O-antigen (21); the *S*. Typhi O-antigen has a rhamnose
182 residue, but no acetylation has been described.
183

184 To assess the role of the *S*. Typhi *gtr* operons, we generated a set of four otherwise isogenic *S*.
185 Typhi strains with a defined *gtr* expression pattern: STy-Basal (both *gtr* operons deleted);
186 STy-Acetyl (expressing only family 2); STy-Gluc (expressing only family 3); and STy-FM
187 (both *gtr* operons expressed). LPS from these isogenic strains was extracted and compared by
188 Western blotting (Fig 1; Fig S1). The O-antigen of all strains reacted with commercial
189 *Salmonella* serum, confirming all strains expressed O-antigen (Fig. 1A) and that production
190 of the long antigen structure was not affected. Factor $O12_2$ serum targets the α1-4
191 glucosylation of the galactose (31), and the O-antigens of the parent *S*. Typhi strain, STy-
192 Gluc and STy-FM reacted with this serum; strains lacking expression of family 3 *gtr* did not

Accepted Manuscript Posted Online
Infection and Immunity
IAI

193 react with this serum (Fig. 1A). Silver staining showed that strains expressing family 3 *gtr*
194 had a distinct O-antigen laddering pattern compared to isolates that lacked family 3 *gtr* (Fig.
195 S1). These data indicate the family 3 *gtr* operon of *S.* Typhi performs the same O-antigen
196 modification as the family 3 *gtr* operon of *S.* Typhimurium, namely α1-4 glucosylation of
197 the galactose. However, no visible shift in the O-antigen pattern for STy-Acetyl compared to
198 STy-Basal was observed, providing further evidence the family 2 GtrC does not act as a
199 glucosyltransferase.
200
201 **Chemical analysis of the O-antigen from *S.* Typhi strains**
202 To define the composition and linkages of the *gtr*-dependent modifications various chemical
203 analyses were performed on LPS isolated from the *S*. Typhi parent strain and isogenic
204 variants. Full details are available with Supplemental Figures S2 and S3, and Tables S2-S4. A
205 methylation analysis of the LPS of these strains showed only minor differences in the
206 proportion of the linkages present in the polysaccharide (Table 1). The *S*. Typhi parent strain
207 and both STy-Gluc, STy-FM showed glucosylation on *O*-4 of galactose, at 62.3%, 82.4% and
208 82.5%, respectively, deduced from the galactose linkages. Nuclear magnetic resonance
209 (NMR) analysis revealed that the extent of glucosylation was comparable to that derived
210 from the methylation analysis (Table S2, Fig. S2) [parent (55.9%), STy-Gluc (72.8%) and
211 STy-FM (71.2%)].
212
213 In contrast to the methylation analysis, in which *O*-acetyl groups are removed during the acid
214 hydrolysis, NMR on untreated LPS (no lipid A removal) permits identification of acetylation.
215 We recorded an *O*-acetylation signal from both parent *S.* Typhi and STy-FM untreated LPS
216 (both encode family 2*gtr)* (Fig. S2), but this signal was absent in the spectra from STy-Gluc.

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia
Infection and Immunity

Accepted Manuscript Posted Online

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

217 A further evaluation identified a 4-linked 3-*O*-acetyl rhamnose and a 4-linked 2-*O*-acetyl-
218 rhamnose (Fig. S3, Table S4). The two states likely reflect a single modification event, with
219 subsequent migration of the acetyl group (32). The parent *S.* Typhi strain exhibited the same
220 pattern of peaks in all NMR spectra as the STy-FM strain, with only slight disparities in
221 intensity of the acetylated positions (50% and 67%, respectively), with *O*-2 and *O*-3 of
222 rhamnose in approximately equal abundance. Taken together we confirm that the *S.* Typhi
223 family 3 *gtr* operon mediates α1>4 glucosylation of the galactose and show that the family 2
224 *gtr* operon acts as a rhamnose acetyltransferase (Fig. 1C.)
225
226 **The expression patterns of the two *S.* Typhi *gtr* operons differ**
227 The expression of multiple *gtr* operons in *S. enterica* is controlled by phase variation (16).
228 This regulation is associated with a signature sequence in the regulatory region of *gtrA*
229 comprised of two binding sites for the transcriptional regulatory protein OxyR and four
230 GATC sites. The GATC sites are the target sequence for Dam, a "maintenance" adenine
231 DNA methyltransferase. DNA methylation-dependent interaction of OxyR at the *gtr* binding
232 sites leads to epigenetic phase variation of *gtr* expression (15, 16). In *S.* Typhi, this signature
233 sequence is present in the regulatory region of the family 2 *gtr* regulatory region and,
234 therefore, we predicted this operon to undergo phase variation. In contrast, the family 3
235 operon has the OxyR binding sequences but only three GATC sequences, with GAAA at the
236 fourth, promoter proximal, GATC sequence (Fig. 2).
237
238 The expression of both *gtr* operons was assessed using single copy transcriptional *lacZ*
239 fusions. In *S.* Typhimurium and *S.* Typhi backgrounds, family 2 expression was controlled by
240 phase variation. The switch frequency of the ON phase to the OFF phase was similar to that

IAI Infection and Immunity

Accepted Manuscript Posted Online

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

241 for the reverse switch (Table 2). Therefore, in the absence of selective pressure, a clonal
242 population should consist of similar numbers of cells with and without the family 2-mediated
243 modification. In contrast, the strain with the *lacZ* reporter for the family 3 *gtr* operon gave
244 rise to only Lac+ colonies, indicating the *S.* Typhi family 3 *gtr* operon is expressed but not
245 controlled by phase variation. Mutating GAAA back to GATC restored phase variation to
246 this family 3*gtr* operon (data not shown). These data indicate that a clonal *S.* Typhi
247 population is likely to have a uniform O-antigen glycosylation pattern, but heterogeneous at
248 the single cell level with respect to O-antigen acetylation.
249

250 **Effect of *gtr* modifications on serum sensitivity**

251 *S.* Typhi spreads systemically during typhoid fever; therefore the infecting organisms must
252 have reliable mechanisms for survival in the presence of components of the innate immune
253 system. The O-antigen can contribute to serum survival. We next assessed whether *gtr*
254 expression and its effects on O-antigen composition affects serum resistance in *S.* Typhi.
255

256 Serum sensitivity of the various isogenic *S.* Typhi was measured using a commercially
257 available human serum (Fig. 3A). The two strains containing the family 3-mediated
258 glucosylation had greater resistance to serum killing than the STy-Basal or STy-Acetyl
259 strains that both lack glucosylation. Acetylation of the O-antigen did not afford any
260 significant survival benefit compared to STy-basal in any of the conditions tested. Taken
261 together, these results imply that complement-mediated killing of *S*. Typhi is altered by the
262 *gtr*-dependent glucosylation of the O-antigen.
263

IAI Infection and Immunity

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

264 To corroborate differences in serum sensitivity between glucosylated and non-glucosylated
265 strains, we next assessed C3 complement protein binding to the strains. C3 initiates alternate
266 pathway activation, leading to the formation of membrane attack complex (MAC). After
267 incubation in naïve human serum, cells were exposed to FITC conjugated anti-C3 antibody
268 and surface-bound C3 measured with flow cytometry. Non-glucosylated strains (STy-Basal
269 and STy-Acetyl) exhibited significantly more surface-bound fluorescence than glucosylated
270 strains (STy-Gluc and STy-FM) (Fig. 3B). Acetyl modification did not significantly alter C3-
271 binding (comparison between STy-Basal to STy-Acetyl and STy-Gluc to STy-FM).
272
273 **Recognition of *S.* Typhi O-antigen from a murine immunization model**
274 We assessed whether O-antigen modification affected the specificity of antibodies generated
275 in a murine immunization model. Mice were immunized with either STy-Basal (no O-
276 antigen modification) or STy-FM (acetylated and glycosylated O-antigen) and serum was
277 collected. LPS from the four strains with defined *gtr* expression was used in a Western blot
278 assay and probed with serum from individual mice (Fig. 4). This approach allowed
279 identification of the O-antigen moieties recognized by antibody, and discrimination from
280 antibody directed to the shared lipid A and core.
281
282 Irrespective of the immunizing strain, there was variation between the responses of individual
283 mice (Fig. 4A & B). Immunization with non-modified O-antigen uniformly resulted in
284 recognition of lipid A + core (Fig. 4A). Some animals (3/9, i.e. panel 10) failed to show any
285 additional recognition of the O-antigen structures. When an O-antigen response was evident,
286 the composition of O-antigen from the immunizing strain was most frequently recognized

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

287 (5/9), and in some cases the only O-antigen that was recognized (3/9; i.e. panel 3) Notably,

288 only one serum recognized all four forms of *S.* Typhi O-antigen (e.g. panel 2).

289

290 In contrast, in mice immunized with the STy-FM strain expressing a fully modified O-

291 antigen, the unmodified form of O-antigen was only recognized in 1/7 mice (e.g. panel 7),

292 suggesting a general immunodominance of the *gtr*-dependent modifications (Fig. 4B).

293 In only 3/7, e.g. panel 4, all three modified O-antigen forms were recognized. The remaining

294 mice produced antibodies specific for either the glucosylated or the acetylated form despite

295 having been immunized with a strain expressing both modifications. These data predict that

296 *gtr*-mediated modifications mostly are immunodominant over the unmodified form (Sty-

297 Basal), but that neither acylation nor glucosylation is preferentially recognized after

298 immunization with a strain expressing O-antigen with both modifications.

299

300 **DISCUSSION**

301 The occurrence of strain and serovar-dependent O-antigen modification in the *Salmonellae*

302 has long been recognized (33), but only recently has the potential for *gtr*-mediated

303 modification been described in this genus (15). Here, we investigated two *gtr* operons in the

304 human host-restricted pathogen *S.* Typhi. Our data show that the family 3 *gtr* operon

305 catalyses a α 1->4 glucosylation of the galactose, as has been previously described for a

306 family 3 *gtr* operon for the serovar *S*. Typhimurium (20). Additionally, we demonstrate that

307 the family 2 *gtr* is required for acetylation of the rhamnose, as was suggested previously (21).

308 Thus, family 2 GtrC is not a glycosyltransferase but an acyltransferase, and the *gtr* acryonym

309 thus only reflects proximity to (remnant) *gtrAB* genes (15). The galactose glucosylation in

310 the *S.* Typhi O-antigen was previously reported (33), but this is, to our knowledge, the first

Accepted Manuscript Posted Online

Infection and Immunity

IAI

311 report of rhamnose acetylation in *S.* Typhi. Acetylation of the family 3-dependent glucose
312 modification also may occur but the genes encoding this process remain to be identified (33,
313 34).
314
315 Acetylation of the rhamnose moiety will be heterogeneous in a bacterial population due to
316 phase variation of expression of family 2 *gtr*. This is consistent with a role in, and a
317 mechanism of, immune evasion (19). In contrast, the STy family 3 *gtr* regulatory region
318 deviates from the known phase variation signature sequence in one GATC sequence, which
319 caused abrogation of phase variation. Thus, glucosylation should be uniformly expressed
320 among cells in a population, likely contributing to the high degree of glucosylation observed
321 in *S.* Typhi O-antigen compared with other *Salmonella* serovars (34, 35). The sequence
322 variation associated with this lack of phase variation is present in both CT18 and Ty2 *S.*
323 Typhi genomes.
324
325 O-antigen glucosylation has implications for virulence in non-typhoidal serovars. In *S.*
326 Typhimurium, family 3-dependent glucosylation is associated with increased persistence in
327 the mouse intestine (20), and glucosylation of the *S.* Enteritidis O-antigen is associated with
328 an increase in virulence in a chicken-to-egg transmission model (35). The role we identified
329 for family 3-dependent glucosylation in serum resistance adds to the evidence that O-antigen
330 glucosylation can affect *Salmonella*–host interactions (14, 36). This one finding does not
331 exclude further benefits of this modification for *S.* Typhi.
332
333 Antibody recognition of a pathogen is an important feature for the clearance of infection. In
334 *S.* Typhi, the Vi capsular polysaccharide contributes to immune evasion (37) and antigen O9

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

335 antibodies can affect antibody mediated serum resistance. This role of O9 antibodies may be
336 relevant for antibody-mediated killing when there is reduced Vi capsule expression during
337 the infection cycle (38, 39). Our data highlight that both *gtr* mediated O-antigen
338 modifications can influence antibody recognition of the O-antigen. The trend from the
339 murine model was that immunization with a strain with modified O-antigen generated
340 antibodies that predominantly recognized the modified version of *Salmonella* O-antigen and
341 did not recognize the basal, unmodified O-antigen. These modifications thus could impact on
342 antibody-dependent killing mechanisms during *S.* Typhi infection.
343
344 In other *Salmonella* serovars, dominant epitopes induced by O-antigen modification have
345 been shown to be relevant for eliciting a protective immune response. For example, OafA-
346 dependent acetyl-modification of the abequose is required for protective antibodies against *S.*
347 Typhimurium (40, 41). A protective *S*. Paratyphi A glycoconjugate LPS vaccine required
348 acetylation, which is likely dependant on the described rhamnose acetylation modification
349 (42, 43). Based on the data presented here linking rhamnose acetylation to family 2 *gtr,* this
350 can now be attributed to the family 2 *gtr* encoded in the *S*. Paratyphi A genome (15).
351 Rhamnose acetylation was also implicated in the strain-specific dominant epitope of the
352 invasive *S.* Typhimurium D23580 isolate (41), which also can be attributed to the phage
353 encoded family 2 *gtr* that is expressed in this specific strain (21). However, detailed
354 analyses in context of the host, disease and serovars are needed to further clarify the impact
355 of O-antigen acetylation and glucosylation (44) (45).
356
357 Our results expand the body of evidence demonstrating that O-antigen composition in
358 *Salmonella* impacts upon host-pathogen interactions during infection. Strains within a

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

359 specific serovar may have a different repertoire of O-antigen modifying genes, and
360 expression may fluctuate by phase variation. Consequently, antibody generated by primary
361 infection or immunization may not wholly recognize subsequent infections by the same
362 serovar. Indeed, disease associated *S.* Typhi strains are not clonal and the genetic repertoire
363 of O-antigen modifying genes may vary (15, 46, 47). Furthermore, the response to the
364 Ty21a oral vaccine elicits a strong O-antigen antibody response, and thus any factors that
365 modify this response may impact serovar and strain cross-reactivity. In conclusion, we
366 suggest that O-antigen modification repertoires may need to be considered in vaccine design
367 to enhance efficacy against a broad range of *S.* Typhi isolates and phenotypes.
368
369
370 **Acknowledgements**
371 We thank Peter Roggentin for providing the $O12_2$ serum, Rob Kingsley for the BRD948 *S.*
372 Typhi strain, and Jane Dalton for assistance with the murine immunization experiments.
373
374 **Funding Statement**
375 This work was supported by the Wellcome Trust of the United Kingdom, through the project
376 grant WT094333MA awarded to MvdW. SB is a Sir Henry Dale Fellow, jointly funded by
377 the Wellcome Trust and the Royal Society (100087/Z/12/Z). Work carried out at the CCRC
378 was supported by U.S. Department of Energy grant (DE-SC0015662) to PA. The funders had
379 no role in study design, data collection and interpretation.
380
381
382

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

383 **References**

384 1. Crump JA, Luby SP, Mintz ED. 2004. The global burden of typhoid fever. Bull World
385 Health Organ 82:346-53.
386 2. Snyder MJ, Hornick RB, McCrumb FR, Jr., Morse LJ, Woodward TE. 1963. Asymptomatic
387 Typhoidal Bacteremia in Volunteers. Antimicrob Agents Chemother (Bethesda)
388 161:604-7.
389 3. Martin LB. 2012. Vaccines for typhoid fever and other salmonelloses. Curr Opin Infect
390 Dis 25:489-99.
391 4. MacLennan CA, Martin LB, Micoli F. 2014. Vaccines against invasive Salmonella disease:
392 current status and future directions. Hum Vaccin Immunother 10:1478-93.
393 5. Baker S, Sarwar Y, Aziz H, Haque A, Ali A, Dougan G, Wain J. 2005. Detection of Vi-
394 negative Salmonella enterica serovar typhi in the peripheral blood of patients with
395 typhoid fever in the Faisalabad region of Pakistan. J Clin Microbiol 43:4418-25.
396 6. Janis C, Grant AJ, McKinley TJ, Morgan FJ, John VF, Houghton J, Kingsley RA, Dougan G,
397 Mastroeni P. 2011. In vivo regulation of the Vi antigen in Salmonella and induction of
398 immune responses with an in vivo-inducible promoter. Infect Immun 79:2481-8.
399 7. Germanier R, Fuer E. 1975. Isolation and characterization of Gal E mutant Ty 21a of
400 Salmonella typhi: a candidate strain for a live, oral typhoid vaccine. J Infect Dis 131:553-
401 8.
402 8. Murray GL, Attridge SR, Morona R. 2006. Altering the length of the lipopolysaccharide O
403 antigen has an impact on the interaction of Salmonella enterica serovar Typhimurium
404 with macrophages and complement. J Bacteriol 188:2735-9.
405 9. MacLennan CA. 2014. Antibodies and protection against invasive salmonella disease.
406 Front Immunol 5:635.

Accepted Manuscript Posted Online

407 10. Nagy G, Pal T. 2008. Lipopolysaccharide: a tool and target in enterobacterial vaccine
408 development. Biol Chem 389:513-20.
409 11. Grimont PAD, Weill F-X. 2007. Antigenic formulae of the Salmonella serovars.
410 9th edition, *on* WHO Collaborating Centre for Reference and Research on
411 Salmonella, Institut Pasteur, Paris. Accessed
412 12. House D, Wain J, Ho VA, Diep TS, Chinh NT, Bay PV, Vinh H, Duc M, Parry CM, Dougan G,
413 White NJ, Hien TT, Farrar JJ. 2001. Serology of typhoid fever in an area of endemicity and
414 its relevance to diagnosis. J Clin Microbiol 39:1002-7.
415 13. Kantele A, Pakkanen SH, Siitonen A, Karttunen R, Kantele JM. 2012. Live oral typhoid
416 vaccine Salmonella Typhi Ty21a - a surrogate vaccine against non-typhoid salmonella?
417 Vaccine 30:7238-45.
418 14. Maldonado RF, Sa-Correia I, Valvano MA. 2016. Lipopolysaccharide modification in
419 Gram-negative bacteria during chronic infection. FEMS Microbiol Rev 40:480-93.
420 15. Davies MR, Broadbent SE, Harris SR, Thomson NR, van der Woude MW. 2013.
421 Horizontally acquired glycosyltransferase operons drive salmonellae lipopolysaccharide
422 diversity. PLoS Genet 9:e1003568.
423 16. Broadbent SE, Davies MR, van der Woude MW. 2010. Phase variation controls
424 expression of Salmonella lipopolysaccharide modification genes by a DNA methylation-
425 dependent mechanism. Mol Microbiol.
426 17. Hone DM, Harris AM, Chatfield S, Dougan G, Levine MM. 1991. Construction of
427 genetically defined double aro mutants of Salmonella typhi. Vaccine 9:810-6.
428 18. van der Woude MW. 2006. Re-examining the role and random nature of phase variation.
429 FEMS Microbiol Lett 254:190-7.
430 19. van der Woude MW, Baumler AJ. 2004. Phase and antigenic variation in bacteria. Clinical
431 Microbiology Reviews 17:581-+.

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

432 20. Bogomolnaya LM, Santiviago CA, Yang HJ, Baumler AJ, Andrews-Polymenis HL. 2008.
433 'Form variation' of the O12 antigen is critical for persistence of Salmonella
434 Typhimurium in the murine intestine. Mol Microbiol 70:1105-19.
435 21. Kintz E, Davies M, Hammarlof DL, Canals R, Hinton JC, van der Woude M. 2015. A BTP1
436 prophage gene present in invasive non-typhoidal Salmonella determines composition
437 and length of the O-antigen of the LPS. Mol Microbiol doi:10.1111/mmi.12933.
438 22. Sambrook J, Fritsch E, Maniatis T. 1987. Molecular Cloning: A Laboratory Manual.
439 Cold Spring Harbor Laboratory Press, Plain View.
440 23. Haldimann A, Wanner BL. 2001. Conditional-replication, integration, excision,
441 and retrieval plasmid-host systems for gene structure-function studies of
442 bacteria. J Bacteriol 183:6384-93.
443 24. Kaminska R, van der Woude MW. 2010. Establishing and maintaining sequestration of
444 Dam target sites for phase variation of agn43 in Escherichia coli. J Bacteriol 192:1937-
445 45.
446 25. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in
447 Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.
448 26. Cherepanov PP, Wackernagel W. 1995. Gene disruption in Escherichia coli: TcR
449 and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-
450 resistance determinant. Gene 158:9-14.
451 27. Miller J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory,
452 Cold Spring Harbor, N. Y.
453 28. Blyn LB, Braaten BA, White-Ziegler CA, Rolfson DH, Low DA. 1989. Phase-variation of
454 pyelonephritis-associated pili in Escherichia coli: evidence for transcriptional regulation.
455 EMBO J 8:613-20.

Infection and Immunity
IAI
Infection and Immunity
Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

456 29. MacLennan CA, Gondwe EN, Msefula CL, Kingsley RA, Thomson NR, White SA, Goodall M,
457 Pickard DJ, Graham SM, Dougan G, Hart CA, Molyneux ME, Drayson MT. 2008. The
458 neglected role of antibody in protection against bacteremia caused by nontyphoidal
459 strains of Salmonella in African children. J Clin Invest 118:1553-62.
460 30. Ong SY, Pratap CB, Wan X, Hou S, Abdul Rahman AY, Saito JA, Nath G, Alam M. 2012.
461 Complete genome sequence of Salmonella enterica subsp. enterica serovar Typhi P-stx-
462 12. J Bacteriol 194:2115-6.
463 31. Makela PH. 1973. Glucosylation of lipopolysaccharide in Salmonella: mutants
464 negative for O antigen factor 1221. J Bacteriol 116:847-56.
465 32. Reicher F, Gorin PAJ, Sierakowski MR, Correa JBC. 1989. Highly Uneven Distribution of
466 O-Acetyl Groups in the Acidic D-Xylan of Mimosa-Scabrella (Bracatinga). Carbohydrate
467 Research 193:23-31.
468 33. Reeves P. 1993. Evolution of Salmonella O antigen variation by interspecific gene
469 transfer on a large scale. Trends Genet 9:17-22.
470 34. Hellerqvist CG, Lindberg B, Svensson S, Holme T, Lindberg AA. 1969. Structural studies
471 on the O-specific side chains of the cell wall lipopolysaccharides from Salmonella typhi
472 and S. enteritidis. Acta Chem Scand 23:1588-96.
473 35. Rahman MM, Guard-Petter J, Carlson RW. 1997. A virulent isolate of Salmonella
474 enteritidis produces a Salmonella typhi-like lipopolysaccharide. J Bacteriol
475 179:2126-31.
476 36. Lerouge I, Vanderleyden J. 2002. O-antigen structural variation: mechanisms and
477 possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev 26:17-47.
478 37. Crawford RW, Wangdi T, Spees AM, Xavier MN, Tsolis RM, Baumler AJ. 2013. Loss of
479 very-long O-antigen chains optimizes capsule-mediated immune evasion by Salmonella
480 enterica serovar Typhi. MBio 4.

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

481 38. Bravo D, Silva C, Carter JA, Hoare A, Alvarez SA, Blondel CJ, Zaldivar M, Valvano MA,
482 Contreras I. 2008. Growth-phase regulation of lipopolysaccharide O-antigen chain
483 length influences serum resistance in serovars of Salmonella. J Med Microbiol 57:938-46.
484 39. Hart PJ, O'Shaughnessy CM, Siggins MK, Bobat S, Kingsley RA, Goulding DA, Crump JA,
485 Reyburn H, Micoli F, Dougan G, Cunningham AF, MacLennan CA. 2016. Differential
486 Killing of Salmonella enterica Serovar Typhi by Antibodies Targeting Vi and
487 Lipopolysaccharide O:9 Antigen. PLoS One 11:e0145945.
488 40. Slauch JM, Mahan MJ, Michetti P, Neutra MR, Mekalanos JJ. 1995. Acetylation (O-factor 5)
489 affects the structural and immunological properties of Salmonella typhimurium
490 lipopolysaccharide O antigen. Infect Immun 63:437-41.
491 41. Lanzilao L, Stefanetti G, Saul A, MacLennan CA, Micoli F, Rondini S. 2015. Strain Selection
492 for Generation of O-Antigen-Based Glycoconjugate Vaccines against Invasive
493 Nontyphoidal Salmonella Disease. PLoS One 10:e0139847.
494 42. Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC. 1996. Synthesis, characterization,
495 and immunological properties in mice of conjugates composed of detoxified
496 lipopolysaccharide of Salmonella paratyphi A bound to tetanus toxoid with emphasis on
497 the role of O acetyls. Infect Immun 64:2709-15.
498 43. Ravenscroft N, Cescutti P, Gavini M, Stefanetti G, MacLennan CA, Martin LB, Micoli F.
499 2015. Structural analysis of the O-acetylated O-polysaccharide isolated from Salmonella
500 paratyphi A and used for vaccine preparation. Carbohydr Res 404:108-16.
501 44. Rondini S, Micoli F, Lanzilao L, Gavini M, Alfini R, Brandt C, Clare S, Mastroeni P, Saul A,
502 MacLennan CA. 2015. Design of Glycoconjugate Vaccines against Invasive African
503 Salmonella enterica Serovar Typhimurium. Infect Immun 83:996-1007.
504 45. Onsare RS, Micoli F, Lanzilao L, Alfini R, Okoro CK, Muigai AW, Revathi G, Saul A, Kariuki
505 S, MacLennan CA, Rondini S. 2015. Relationship between Antibody Susceptibility and

IAI Infection and Immunity

Accepted Manuscript Posted Online

506 Lipopolysaccharide O-Antigen Characteristics of Invasive and Gastrointestinal
507 Nontyphoidal Salmonellae Isolates from Kenya. Plos Neglected Tropical Diseases 9.
508 46. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, Murphy N, Holliman R, Sefton
509 A, Millar M, Dyson ZA, Dougan G, Holt KE. 2016. An extended genotyping framework for
510 Salmonella enterica serovar Typhi, the cause of human typhoid. Nat Commun 7:1287.
511 47. Roumagnac P, Weill FX, Dolecek C, Baker S, Brisse S, Chinh NT, Le TA, Acosta CJ, Farrar J,
512 Dougan G, Achtman M. 2006. Evolutionary history of Salmonella typhi. Science
513 314:1301-4.
514

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

Infection and Immunity

IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

515 **Table 1.** Methylation analysis of the three LPS samples.

No.	**Glycosyl Linkage**	**Mole per cent**		
		BRD948	**STy-Gluc**	**STy-FM**
a	t-Tyv	0.2	2.3	3.0
b	4-Rha	13.2	15.4	17.2
c	t-Glc	17.6	15.2	19.4
d	2-Man or 3-Man	1.8	3.0	3.2
e	3-Gal	6.8	3.6	3.3
f	4-Glc	24.7	20.4	19.7
g	2,3-Man	15.6	16.2	15.9
h	3,4-Gal	11.3	16.8	15.6
i	4,6-Glc	1.5	1.6	0.8
DG[a]		62.3	82.4	82.5

516 [a]DG (degree of glucosylation)=(h/(h+e)*100)

517

518

519 **Table 2.** Expression of S. Typhi *gtr* operons

Strain	Lac phenotype	Miller Units[a]	ON to OFF switch frequency	OFF to ON switch frequency
LT2$^{F2reg\text{-}lacZ}$	Lac+/Lac-	1116 (42)	3.0×10^{-3}	3.3×10^{-3}
LT2$^{F3reg\text{-}lacZ}$	Lac+	763 (28)	n/a	n/a

520 a. Standard deviations are shown in parenthesis. Miller units were calculated for 100%
521 ON cells for phase varying isolates.

522

Accepted Manuscript Posted Online
Infection and Immunity
IAI
Infection and Immunity
Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

523 **Figure Legends**

524 **Figure 1. Effect of *gtr* modifications on *S*. Typhi O-antigen.**

525 (A) Expression of family 3 *gtr* leads to recognition by $O12_2$ sera. LPS was prepared as

526 described in the methods and run on TSDS-PAGE. Blots were probed with commercial OMA

527 sera (top panel) and $O12_2$ sera (bottom panel). B = STy-Basal; WT = parent strain BRD948

528 ("wild-type") *S*. Typhi; Ac = STy-acetyl; G = STy-gluc; FM = STy-FM.

529 (B) Summary of results from the chemical analysis of *S*. Typhi BRD948 O-antigen.

530 (C) Schematic of the O-antigen of engineered strains used in these studies. See Figure S1 for

531 additional silver stain analysis of the *S*. Typhi O-antigen.

532

533

534 **Figure 2. Alignment of *S.* Typhi *gtr* regulatory regions to the phase varying regulatory**

535 **region of the *S.* Typhimurium family 3 *gtr* operon.**

536 The GATC sites are underlined and the OxyR binding sites are outlined in light gray boxes.

537 The -35/-10 sigma sites and the +1 transcriptional start site are indicated. Alignment was

538 performed in TCOFFEE.

539

540 **Figure 3. Serum sensitivity of *S.* Typhi strains with different *gtr* modifications.**

541 (A) Results from the serum survival assay for strains grown to stationary phase and

542 incubated in 50% serum. Different symbols represent the data obtained from individual

543 experiments. One-way ANOVA and Tukey post-analysis were performed in GraphPad Prism

544 (version 5.0d). * $p < 0.05$. (B) Binding of C3 complement protein to *S*. Typhi strains with

545 different O-antigen composition. Strains were incubated in human serum followed by anti-C3

546 FITC-conjugated antibody and surface-bound fluorescence measured with flow cytometry.

Accepted Manuscript Posted Online

Infection and Immunity

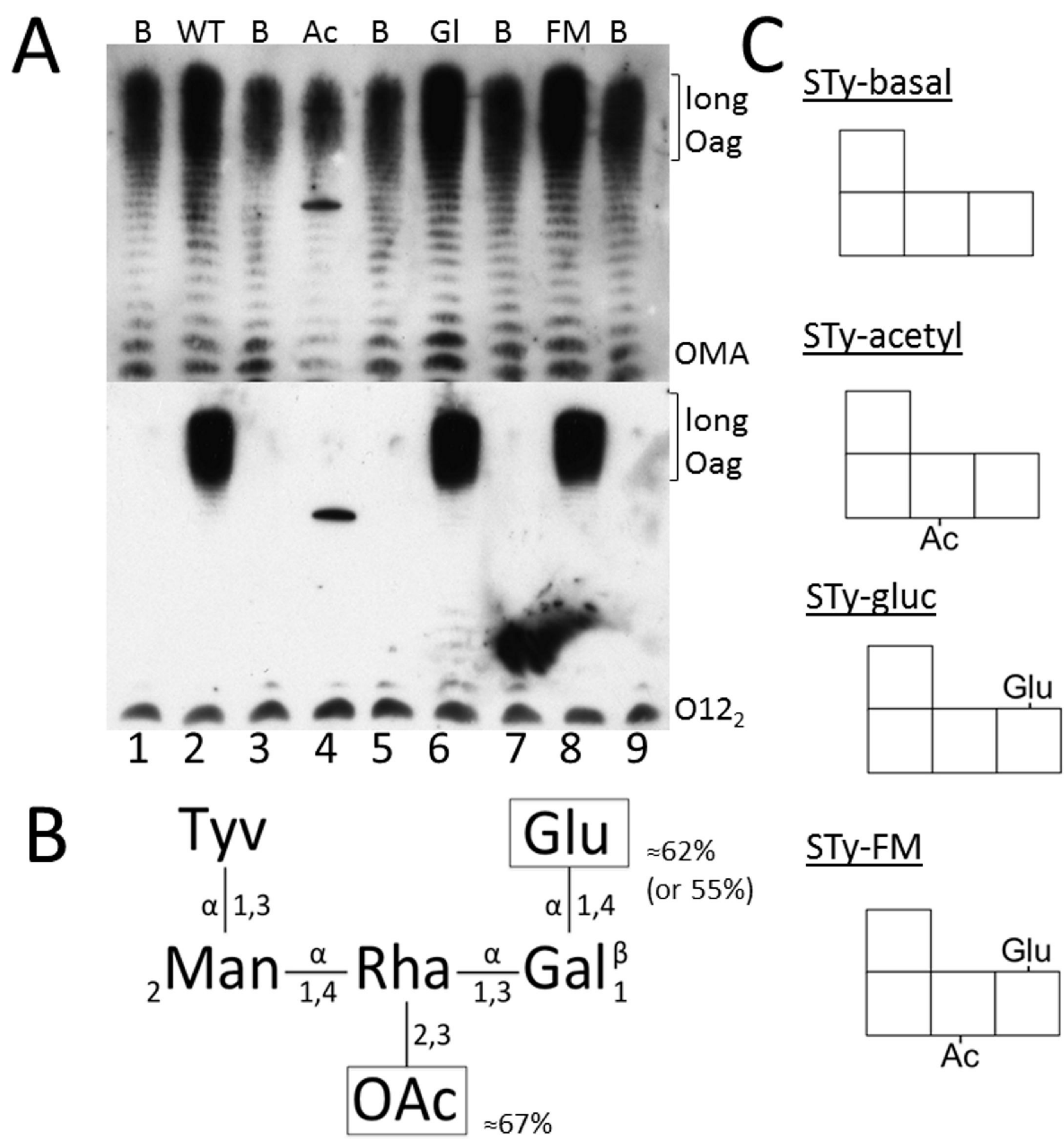
IAI

Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

547 Data is combined from four experiments. MESF = molecules of equivalent soluble
548 fluorochrome. One-way ANOVA and Tukey post-analysis were performed in GraphPad
549 Prism (version 5.0d). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate mean ± SEM.
550
551 **Figure 4. Recognition of *S.* Typhi O-antigen by serum in a murine immunization model**
552 **depends on the O-antigen composition of the immunizing strain.**
553 In a LPS Western blot, LPS from different strains was probed with serum from mice
554 immunized either with Sty-Basal (A) or STy-FM (B). In (A), panel #1 (numbered in lower
555 left hand corner) shows reactivity of pooled serum from the PBS-immunized control group,
556 and each following panel (2-10) represents the serum from an individual mouse. In (B) panel
557 #1 shows reactivity of OMA commercial serum, which recognizes several *Salmonella*
558 serovars; each following panel represents the serum from an individual mouse. Lanes 1-4 in
559 each panel have LPS from the following strains: as follows 1) STy-Basal; 2) STy-Acetyl; 3)
560 STy-Gluc 4) STy-FM.
561

Accepted Manuscript Posted Online
Infection and Immunity
IAI
Infection and Immunity



Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online

IAI Infection and Immunity

Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

```
STm-F3  GATCGGTAACAACGATCAATTAATAAGATAACAATAACTTTAAACTATTGAATAC
STy-F2  GATCGATAACATCGATCAATATATTAAAACTCAATAGCTTAAAACTATTAAAAAT
STy-F3  GATCGGTAACAACGATCAATTAATAAGACAACAATAAGTTCAAGCTATCAAATAT
        ***** ***** ********  ** * *   *****  ** ** ****  ** *

                                     -35                  -10     +1
STm-F3  CACATTATTGATCGTTTATATCGATCAAAGCAATTTGTAGTGCTACACTCCAGAC
STy-F2  ACAATTATTGATCGCTTATATCGATCAAACCAATTTGTAGTGCTACACTCCAGAC
STy-F3  AACATTATTGATCGTTTATATCGAAAAAAGCAATTTGTAGTACTATACTCCAGAC
           ******* **** *********  *** *********** *** *********
```

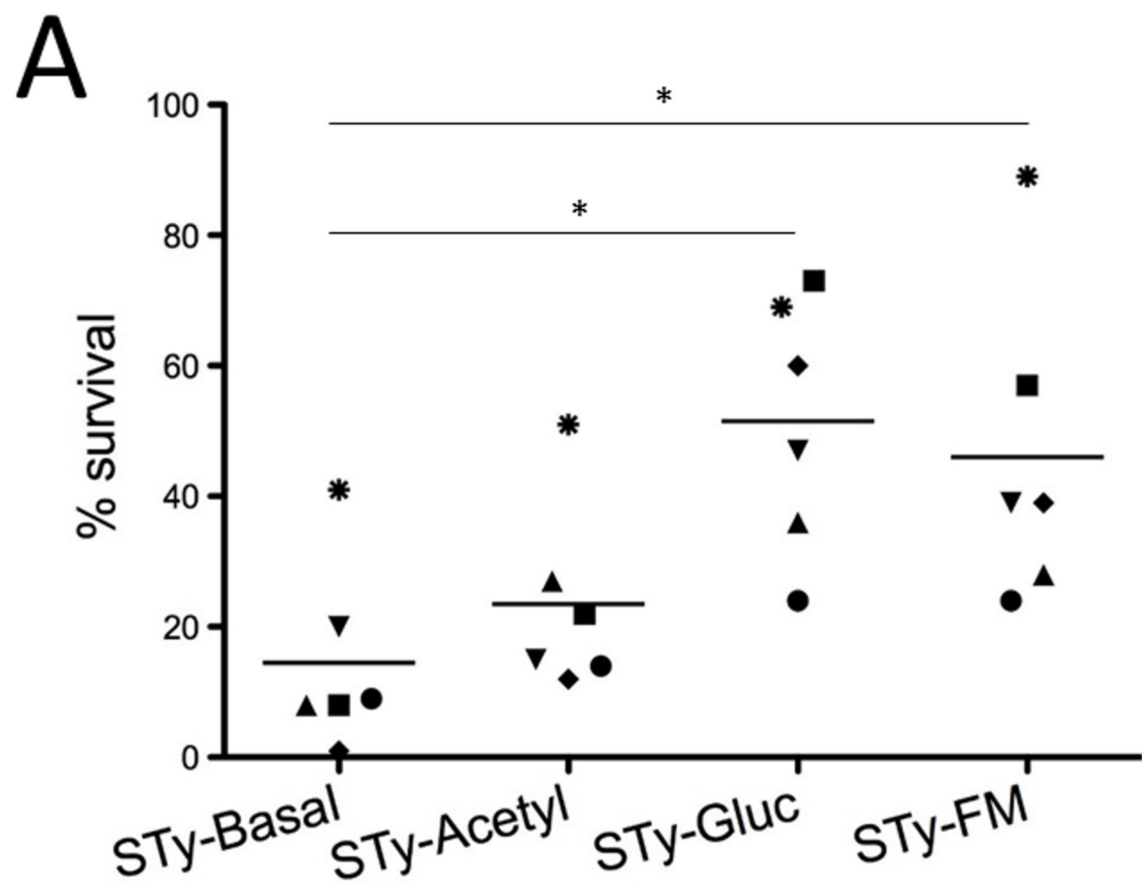
Infection and Immunity

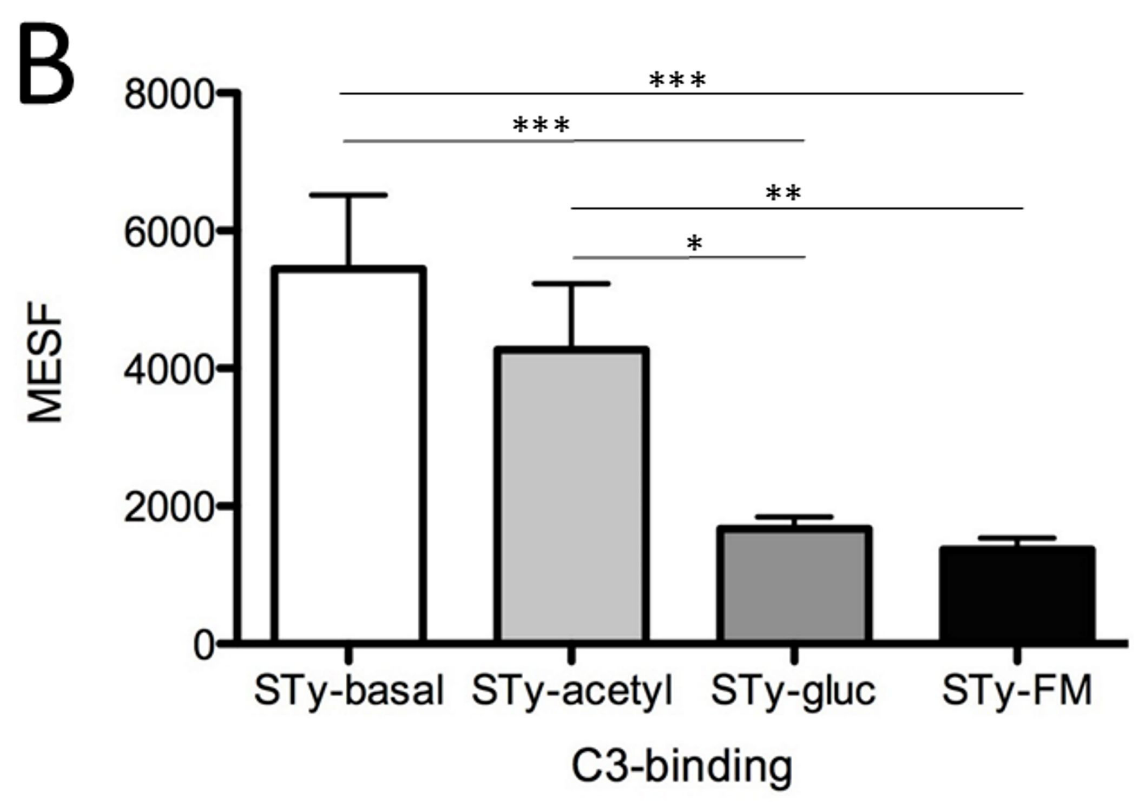
Accepted Manuscript Posted Online

Infection and Immunity

IAI

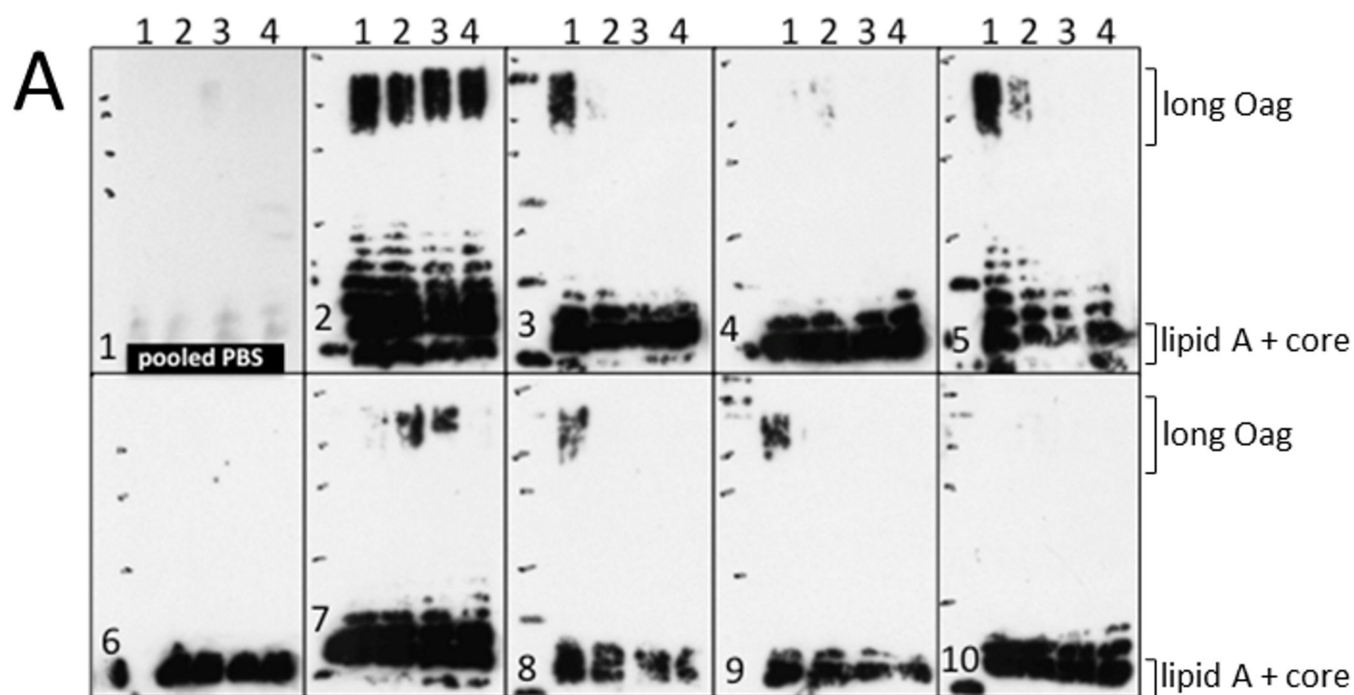
Infection and Immunity

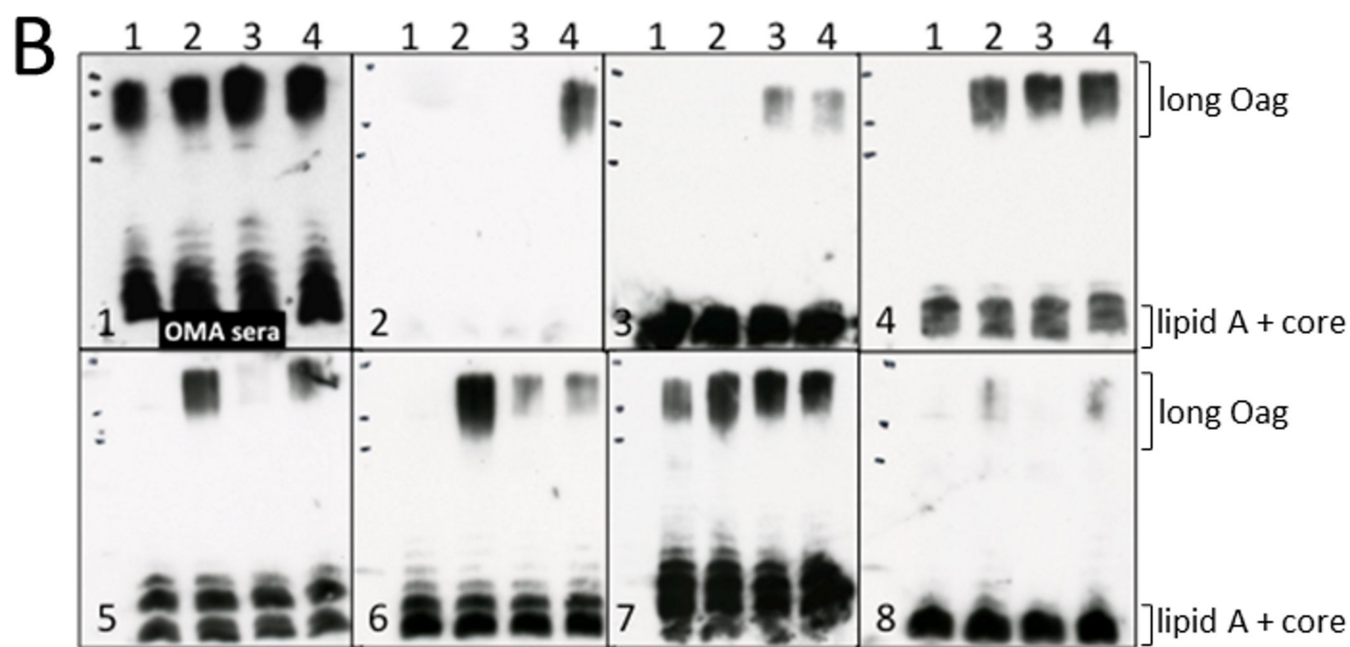




Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Accepted Manuscript Posted Online





Downloaded from http://iai.asm.org/ on February 28, 2017 by University of East Anglia

Infection and Immunity
IAI
Infection and Immunity