Heterologous *Pseudomonas aeruginosa* O-antigen delivery using a *Salmonella enterica* serovar Typhimurium *wecA* mutant strain

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24 **ABSTRACT:**

25 There is a broad interest in adapting live vaccine strains (LVS) for use as recombinant vaccines
26 that can deliver heterologous antigens. The *Salmonella enterica* serovar Typhimurium SL1344
27 Δ *wecA* LVS contains a mutation in *wecA* that abrogates production of Enterobacterial common
28 antigen. This Δ *wecA* strain is attenuated *in vivo*, persistently colonizes the host, and protects
29 against both wild type and cross-*Salmonella* serovar lethal challenge in a murine model of
30 salmonellosis. Given these characteristics, we hypothesized that the SL1344 Δ *wecA* strain could
31 be used as a carrier for heterologous antigen expression. To test this hypothesis, SL1344 Δ *wecA*
32 was engineered to express the *Pseudomonas aeruginosa* O11 O-antigen gene cluster.
33 Intraperitoneal (IP) but not oral immunization of BALB/c mice with the heterologous expression
34 strain protected against lethal *P. aeruginosa* intranasal (IN) challenge. Furthermore, IP
35 immunization resulted in *P. aeruginosa* O11-specific Ig and IgG antibody production. Functional
36 analysis of sera collected from the IP immunized mice showed antibody-mediated agglutination
37 and opsonophagocytic activity against *P. aeruginosa*. *En masse*, these results indicate that the *S.*
38 Typhimurium SL1344 Δ *wecA* strain expressing the *P. aeruginosa* O11 O-antigen gene cluster is
39 able to induce a humoral immune response and to protect against lethal *P. aeruginosa* challenge.
40 As such, the *S. Typhimurium* SL1344 Δ *wecA* LVS can likely serve as a vehicle for expression of a
41 wide variety of heterologous antigens as a means to create recombinant vaccines.

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43 **Keywords:** *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *wecA*, heterologous antigen
44 presentation, vaccine, live-attenuated

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47 **ABBREVIATIONS**

48 ECA: Enterobacterial common antigen; gm: gentamicin; IN: intranasal; IP: intraperitoneal; LVS:
49 live-vaccine strain; MTD: mean time to death; ODU: OD₆₀₀ unit/mL; T3SS: type III secretion
50 system; Tet: tetracycline

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52 **INTRODUCTION**

53 Attenuated *Salmonella enterica* serovar Typhimurium strains have the ability to induce
54 protective immunity through induction of local and systemic immune responses (Kang *et al.*, 2002;
55 Lin *et al.*, 2015). Attenuation of these live vaccine strains (LVS) is achieved through mutations of
56 genes required for virulence and colonization such as *cya*, *crp*, *asd*, *phoP*, and *aro* (Galan and
57 Curtiss, 1989; Hoiseth and Stocker, 1981; Kang *et al.*, 2002; Nayak *et al.*, 1998; Pier *et al.*, 1995).
58 These LVS have also been used to construct recombinant vaccines that heterologously express
59 bacterial antigens: PspA and PspA fusion proteins from *Streptococcus pneumoniae* (Kang *et al.*,
60 2002; Nayak *et al.*, 1998; Xin *et al.*, 2009), the O11 O-antigen from *Pseudomonas aeruginosa*
61 (DiGiandomenico *et al.*, 2004; DiGiandomenico *et al.*, 2007; Pier *et al.*, 1995; Scarff and Goldberg,
62 2008), intimin gamma from *E. coli* O157:H7 (Oliveira *et al.*, 2012), CagA, VacA, and UreB fusion
63 proteins from *Helicobacter pylori* (Liu *et al.*, 2011), and cholera toxin B from *Vibrio cholerae*
64 (Vishwakarma *et al.*, 2015). Heterologous antigen expression by a *Salmonella* LVS has been
65 achieved using chromosomal and plasmid-based expression systems (Chatfield *et al.*, 1989; Kang
66 *et al.*, 2002; Lin *et al.*, 2015). Recently, a controlled bacterial lysis system for antigen expression
67 and DNA delivery has also been developed (Kong *et al.*, 2012; Kong *et al.*, 2008). Subsequent
68 immunization with the heterologous vaccines not only elicits an immune response towards

69 *Salmonella*, but also induces protection from the pathogen from which the antigen was derived
70 (Kang *et al.*, 2002; Lin *et al.*, 2015).

71 *wecA* is the first gene in the Enterobacterial common antigen (ECA) biosynthetic pathway
72 (Barr *et al.*, 1999), and loss of *wecA* in *S. Typhimurium* results in loss of ECA production,
73 decreased virulence in a murine model, and bile salt sensitivity (Gilbreath *et al.*, 2012; Ramos-
74 Morales *et al.*, 2003). Interestingly, despite evidence for a role for ECA in *S. Typhimurium* virulence
75 (Chaudhuri *et al.*, 2009; Gilbreath *et al.*, 2012; Ramos-Morales *et al.*, 2003; Valtonen *et al.*, 1976),
76 anti-ECA antibodies do not elicit passive protection against salmonellosis (Albertson *et al.*, 2003).
77 Our group previously conducted detailed characterization of *S. Typhimurium wecA* mutants and
78 found that they are severely attenuated *in vivo*, persistently colonize the spleen and liver at low
79 levels in a murine model, and induce protection against wild type (WT) *S. Typhimurium* challenge
80 (Gilbreath *et al.*, 2012). Moreover, both oral and intraperitoneal (IP) immunization with the *S.*
81 *Typhimurium* SL1344 *wecA* mutant is able to induce protection against non-parental *S.*
82 *Typhimurium* and *S. Enteritidis* challenge in a murine model of salmonellosis; IP immunization
83 offered the highest level of protection (Bridge *et al.*, 2015). Furthermore, immunization with SL1344
84 $\Delta wecA$ induced a *Salmonella*-specific, antibody-mediated immune response that produced cross-
85 reactive antibodies against both O4 and O9 serovar *Salmonella*. Functional antibody activity as
86 assessed by agglutination and opsonization assays showed activity against O4 serovar *Salmonella*
87 (Bridge *et al.*, 2015).

88 The opportunistic pathogen *P. aeruginosa* causes approximately 51,000 infections per year
89 with 440 associated deaths (CDC, 2013). Contributing to disease severity, approximately 6,700
90 (13%) of *P. aeruginosa* healthcare associated infections are due to multidrug resistant strains that
91 are resistant to three or more antibiotics; in 2004/2005 the first pan-resistant *P. aeruginosa*

92 infections were reported (CDC, 2013). As such, in 2013, the Centers for Disease Control and
93 Prevention (CDC) categorized *P. aeruginosa* as a serious threat. Moreover, they stated that
94 “threats will worsen and become urgent without public health monitoring and prevention” (CDC,
95 2013). The high patient mortality and disease severity associated with this bacterium make it a
96 major health concern and an ideal candidate for vaccine development. In this regard, previous
97 studies engineered an *S. Typhimurium* SL1344 Δ *aroA* derivative (SL3261) to express the *P.*
98 *aeruginosa* PA103 serogroup O11 O-antigen gene cluster (Goldberg *et al.*, 1992). This
99 heterologous expression system was tested in gastrointestinal, pneumonia, corneal, and burn
100 wound models of *P. aeruginosa* infection using oral, IP, and IN modes of immunization and was
101 shown to induce an O11-specific antibody response following immunization (DiGiandomenico *et*
102 *al.*, 2004; DiGiandomenico *et al.*, 2007; Pier *et al.*, 1995). Given that *P. aeruginosa* exacts a huge
103 burden on immunocompromised individuals, additional work investigated the ability of the
104 recombinant vaccine to provide protection in an immunocompromised mouse model (Scarff and
105 Goldberg, 2008). When administered as an intranasal (IN) immunization, the heterologous vaccine
106 strain provided protection against *P. aeruginosa* corneal and burn wound infections
107 (DiGiandomenico *et al.*, 2007).

108 The studies described herein sought to determine if the SL1344 Δ *wecA* mutant strain could
109 be used as a heterologous antigen carrier; the strain was engineered to express the *P. aeruginosa*
110 O11 O-antigen as described above. We investigated the ability of the heterologous expression
111 strain to provide protection in a *Pseudomonas* pneumonia model after oral and IP immunization.
112 We found that only IP immunization resulted in significant protection. Analysis of the production
113 and functionality of antibodies produced as a result of immunization indicated production of *P.*
114 *aeruginosa* O11-specific Ig and IgG antibody that could mediate opsonophagocytic and

115 agglutination activity against *P. aeruginosa*. Together, our results highlight the potential use of the
116 *S. Typhimurium* SL1344 *wecA* mutant as a LVS that can serve as a carrier for heterologous
117 antigens.

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138 MATERIALS AND METHODS

139 **Bacterial strains, plasmids, and growth conditions.** Table 1 lists the bacterial strains and
140 plasmids used in this study. Bacterial stocks were maintained in Trypticase Soy Broth (TSB; BD
141 Biosciences; Franklin Lakes, NJ) or Luria Broth (LB; MoBio; Carlsbad, CA) containing 40% glycerol
142 at -80⁰ C. Bacteria were cultured at 37⁰C in TSB or LB containing 20 µg/ml gentamicin (gm) or 10
143 µg/ml tetracycline (tet) where indicated. Plasmids were isolated and purified using Qiagen reagents
144 (Qiagen; Valencia, CA).

145
146 **Eukaryotic cells.** J774A.1 murine macrophage cells (ATCC TIB-67) were provided by Joan C.
147 Olson (West Virginia University School of Medicine, Morgantown, WV) and were cultured at 37⁰ C
148 in 5% CO₂ according to ATCC specifications in Dulbecco's modified Eagle medium (DMEM)
149 containing 4.5 g/L D-glucose, 584 g/L L-glutamine, and 110 mg/L sodium pyruvate (Quality
150 Biological, Inc.; Gaithersburg, MD). Media was supplemented with 10% fetal bovine serum (FBS;
151 Gibco, Grand Island, NY).

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153 **Mice.** Five-seven week old female BALB/c mice (Jackson laboratories; Bar Harbor, ME) were used
154 as indicated below. All animal protocols were conducted in accordance with National Institutes of
155 Health guidelines and were approved by the Uniformed Services University of the Health Sciences
156 Institutional Animal Care and Use Committee (Protocol number MIC-11-815). Intranasal instillation
157 was performed under anesthesia as detailed below. To minimize suffering, all animals that
158 displayed extreme signs of morbidity, including hunched posture and shallow breathing, were
159 euthanized.

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161 **Construction of *S. Typhimurium* Δ wecA heterologous O-antigen expression strains.** *S.*
162 *Typhimurium* strain SL3261 carrying the cloning vector, pLAFR1, or the plasmid encoding the
163 PA103 serogroup O11 O-antigen, pLPS2, were kindly provided by Joanna A. Goldberg (Emory
164 University, Atlanta, GA) (DiGiandomenico *et al.*, 2004; Goldberg *et al.*, 1992; Pier *et al.*, 1995).
165 pLAFR1 and pLPS2 were independently purified and electroporated into SL1344 *wecA*::gm
166 (SL1344 Δ *wecA*), and transformants were selected on LB agar (MoBio) containing 10 µg/ml tet at
167 37°C to yield SL1344 Δ *wecA*/pLAFR1 (Δ *wecA*/pLAFR1) and SL1344 Δ *wecA*/pLPS2
168 (Δ *wecA*/pLPS2).
169
170 **ELISA analysis of *P. aeruginosa* lipopolysaccharide (LPS) expression from *S. Typhimurium*.**
171 Production of *P. aeruginosa* LPS from the heterologous expression strains was tested via ELISA.
172 PA103, provided by Joanna A. Goldberg (Emory University, Atlanta, GA), and SL1344 Δ *wecA*
173 served as positive and negative controls, respectively. Liquid overnight cultures of each strain were
174 pelleted and re-suspended in phosphate buffered saline (PBS) to a density of approximately 1 OD
175 unit/mL (ODU). After re-suspension, PA103 was serially diluted in 1:2 increments for dilutions
176 ranging from 1:25-1:25,600. Immulon 4HBX (Thermo Scientific; Waltham, MA) ELISA plates were
177 coated in triplicate with 100 µl of the heterologous expression strain cell suspensions, SL1344
178 Δ *wecA*, or PA103, and incubated overnight at 4°C, followed by blocking with PBS containing 10%
179 FBS (Gibco) for 1 h at room temperature. For detection of the PA103 O11 O-antigen, plates were
180 incubated for 1 h at room temperature with 100 µl of a 1:1000 dilution of *P. aeruginosa* serogroup
181 O11-specific polyclonal anti-sera (Group E; Cat #YCC310-717; Accurate Chemical and Scientific
182 Chemical Corp., Westbury, NY) (DiGiandomenico *et al.*, 2004) and detected with 100 µl of a
183 1:4000 dilution of goat anti-rabbit IgG-HRP (Cat #SC-2004, Lot F2215; Santa Cruz Biotechnology,

184 Inc., Dallas, TX) in blocking buffer. The anti-rabbit HRP secondary was detected with 100 µl Ultra
185 TMB-ELISA Solution (Thermo Scientific) at 370 nm on a SpectraMax M2 plate reader (Molecular
186 Devices, Downingtown, PA).

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188 **Immunization with the *S. Typhimurium* Δ wecA heterologous expression strains and lethal *P.***
189 ***aeruginosa* IN challenge.** SL1344 Δ wecA/pLAFR1 and Δ wecA/pLPS2 were cultured in LB with 10
190 µg/ml tet and then diluted and utilized for immunization of BALB/c mice as previously described
191 (Bridge *et al.*, 2015). Briefly, BALB/c mice were immunized with the heterologous expression
192 strains by oral gavage with 1.3×10^6 colony forming units (CFU) or IP injection with 8.0×10^2 CFU
193 in 0.2 mL of isotonic saline. Control mice were mock immunized with 0.2 mL of isotonic saline.
194 Mice receiving oral immunizations were fasted for 4 h prior to and 1 h post-immunization. Serial
195 dilution and plating were used to determine immunization doses. Disease symptoms and mortality
196 were monitored for 28 days in the vaccinated animals.

197 For lethal challenge studies, PA103 was grown for 12 h on TSA, re-suspended in isotonic
198 saline, and diluted to a density of 0.5 ODU in isotonic saline. Mice were anesthetized by IP
199 injection with 65 mg/kg ketamine and 10 mg/kg xylazine (AnaSed; Shenandoah, IA) in 0.2 mL
200 isotonic saline and then challenged IN with 2.6×10^5 to 3.4×10^6 CFU of PA103 (reported LD₅₀ is
201 $\sim 1.0 \times 10^5$ CFU) (DiGiandomenico *et al.*, 2007). Each mouse received 10 µl of PA103 per nostril.
202 As signs of morbidity and mortality are observed within hours of *Pseudomonas* inoculation, in the
203 first experiment, the mice were monitored hourly for the initial 13 h then hourly from 20 to 33 h. In
204 the second experiment, mice were monitored hourly for the first 48-55 h; differences in the hourly
205 monitoring period between experiments were due to differences in the challenge doses utilized
206 between experiments. For both experiments, following the initial hourly monitoring period, the mice

207 were monitored daily for an additional 10 days for signs of further morbidity or mortality. All animals
208 that displayed extreme signs of morbidity, including hunched posture and shallow breathing, were
209 euthanized.

210 To investigate the possible role of non-specific, *Salmonella*-induced protection against
211 PA103, 5-7 week old female BALB/c mice were immunized IP with 2.0×10^2 , 5.0×10^2 , or 8.0×10^2
212 CFU of SL1344 Δ *wecA* as previously described (Bridge *et al.*, 2015). Mice were then challenged IN
213 with 6.4×10^5 CFU of PA103 and monitored as described above.

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215 **Sera collection.** To evaluate the presence of Ig and IgG antibodies, pre-challenge sera were
216 collected 25-27 days post-immunization via tail-vein bleeds. Additionally, terminal sera were
217 collected 12 days post-challenge via cardiac puncture; the sera were collected and processed as
218 previously described (Bridge *et al.*, 2015).

219

220 **Isolation of *P. aeruginosa* LPS for ELISA.** PA103 LPS was extracted using the hot-phenol-water
221 protocol as described in (Iwashkiw *et al.*, 2012; Westphal, 1965). Briefly, liquid overnight grown
222 PA103 was harvested by centrifugation (8,000 rpm) for 10 min at 4° C and the cell pellet was
223 washed with Buffer A (50 mM phosphate, 150 mM NaCl, pH 7.4). Following re-suspension in
224 phenol-chloroform-petroleum ether (1:2.5:4; 2g per 50 ml), the cells were lysed, and centrifuged at
225 7,000 rpm for 10 min at 4° C. To evaporate the chloroform-ether phase, the supernatant fraction
226 was incubated at 55° C. LPS was then precipitated from the phenolic phase with 1 volume of
227 distilled, deionized H₂O and centrifuged at 7,000 rpm for 10 min at 4° C. The phenol phase was
228 removed with a Pasteur pipet and the precipitation was repeated. The combined LPS phases were
229 centrifuged at 12,000 rpm at 4° C. The LPS pellet was then washed with 20 mL of methanol,

230 centrifuged at 12,000 rpm, and vacuum dried; the dried pellet was resuspended in 10 ml 0.1 mM
231 MgCl₂ and stored at 4⁰ C until use. LPS was quantified using a Pierce LAL Chromogenic Endotoxin
232 Quantification Kit (Thermo Scientific).

233

234 **ELISA analysis of serum antibodies.** To determine anti-*Pseudomonas* LPS serum Ig and IgG
235 responses, one set of Immulon 4HBX ELISA plates (Thermo Scientific) were coated with 150 µl of
236 1 ODU/mL SL1344 Δ*wecA* suspension prepared as described in (Bridge *et al.*, 2015). A second
237 set of ELISA plates were coated with 1 µg/mL purified PA103 LPS in a 100mM
238 carbonate/bicarbonate buffer (Na₂CO₃ and NaHCO₃; pH 9.6) and were incubated at 37⁰ C for 3 h.
239 SL1344 Δ*wecA* coated plates were blocked for 2 h at room temperature and PA103 LPS coated
240 plates were blocked overnight at 4⁰ C with PBS containing 1% bovine serum albumin (BSA; PBS-
241 B; Calbiochem, Billerica, MA). Serum samples from each animal were diluted in PBS-B 1:20 and
242 then serially diluted 1:4. To pre-absorb *Salmonella*-specific antibodies, sera were incubated in
243 duplicate on the SL1344 Δ*wecA* coated ELISA plates for 4 h at room temperature and then
244 transferred to the PA103 LPS coated ELISA plates and incubated overnight at 4⁰C.

245 For *Pseudomonas* LPS-specific antibody responses, total Ig was detected with a 1:2000
246 dilution of biotin labeled goat anti-mouse Ig primary (Cat #553999; Lot 16325; BD Biosciences)
247 followed by a 1:3000 dilution of Streptavidin-AKP secondary (Cat #554065; Lot 08661; BD
248 Biosciences) (Bridge *et al.*, 2015). IgG antibodies were detected using a 1:2000 dilution of biotin
249 labeled goat anti-mouse IgG primary (Cat #1030-08, Lot D5012-T342C; Southern Biotech;
250 Birmingham, AL) followed by a 1:3000 dilution of Streptavidin-AKP secondary (Cat #554065; Lot
251 08661; BD Biosciences). Antibodies were detected with 4-nitrophenyl phosphate (PNP; Sigma-
252 Aldrich) in TM buffer (0.5M Tris-base, 300 µM MgCl₂, pH 9.8) at 1 mg PNP/mL TM buffer (Bridge *et*

253 *al.*, 2015). ELISA plates were read at an absorbance of 405 nm on a SpectraMax M2 plate reader
254 (Molecular Devices). Ig and IgG titers are reported as titer units and were calculated based on a
255 standard titer curve. Determination of anti-*Salmonella* Ig and IgG antibody responses was
256 performed as previously described (Bridge *et al.*, 2015).

257
258 **Analysis of antibody functionality.** Following individual sample ELISA analysis, 5 or 10 µl of
259 sera from each animal (15 to 16 animals/group) that had been mock-immunized, immunized IP
260 with 6.2×10^2 CFU of $\Delta wecA/pLAFR1$, or immunized IP with 7.6×10^2 CFU of $\Delta wecA/pLPS2$ as
261 described above were pooled for assessment of antibody functionality.

262
263 **i. Serum agglutination.** PA103 was cultured overnight in TSB and 3 µl was mixed with 9 µL of
264 PBS or pooled sera from animals that had been mock-immunized or immunized IP with
265 $\Delta wecA/pLAFR1$ or $\Delta wecA/pLPS2$ (Bridge *et al.*, 2015). As positive and negative serotype controls,
266 strains were also incubated with either *P. aeruginosa* serogroup O11 (Accurate Chemical) or
267 *Salmonella* O4 (Cat #23839, Lot 876E-H20; Statens Serum Institute, Denmark) diagnostic anti-
268 sera, respectively. Agglutination was monitored at 40X using differential interference contrast (DIC)
269 microscopy on a Leica AF6000 microscope (Leica Microsystems, Inc., Buffalo Grove, IL). Images
270 were exported as TIFF files using the Leica LAS X software (Leica Microsystems, Inc.) and
271 imported into Adobe Photoshop CC (Adobe Systems Inc., San Jose, CA) where they were cropped
272 and adjusted using the levels function for contrast and brightness. Images are representative of
273 three-independent experiments.

274

275 **ii. Opsonophagocytic uptake.** Serum-mediated uptake by macrophages was examined by an
276 opsonophagocytic uptake assay as described in (Bridge *et al.*, 2015; Tennant *et al.*, 2011). Briefly,
277 J774A.1 mouse macrophages were seeded (1×10^5) for 2 days in 24-well plates (Costar, Corning
278 Inc.; Corning, NY). Liquid overnight cultures of PA103 or PA103 Δ exoU Δ exoT::Tc pUCP
279 (PA103 Δ UT; kindly provided by Dara Frank, Medical College of Wisconsin, Milwaukee, WI) were
280 diluted 1:1000 in TSB and incubated until they reached an OD₆₀₀ of 0.3-0.5. The 0.3-0.5 OD₆₀₀
281 cultures were then diluted 1:20 and incubated with heat-inactivated (55°C for 20 min) serum from
282 IP mock-immunized animals or from Δ wecA/pLAFR1 or from Δ wecA/pLPS2 IP immunized animals
283 as indicated above. As controls, the strains were also incubated with PBS or *P. aeruginosa*
284 serogroup O11 (Accurate Chemical) and O4 (Statens Serum Institute) anti-sera (Bridge *et al.*,
285 2015). These cultures (1.0×10^5 to 8.0×10^5 CFU/mL) were next added to the J774A.1 cells and
286 incubated for 1 h at 37°C, 5% CO₂ (Bridge *et al.*, 2015). Following gentamicin treatment (100
287 µg/mL for 1 h at 37°C, 5% CO₂) to kill extracellular bacteria, the cells were washed and lysed in
288 0.5% (wt./vol) Triton X-100 in PBS. Intracellular bacteria were enumerated in duplicate for each
289 strain from four to five independent experiments (Bridge *et al.*, 2015).

291 **Data analysis and statistics.** GraphPad Prism 6 (GraphPad Software, Inc.; La Jolla, California)
292 was used to create all graphs. Significance was determined by a two-sided α level (0.05) for all
293 data. A Kaplan-Meier analysis was used to determine differences in percent survival using SPSS
294 Statistics Version 20 (IBM; Armonk, NY). Differences in ELISA antibody titers were determined by
295 a one-way analysis of variance (ANOVA; GraphPad Software, Inc.). Multiple comparisons were
296 then evaluated with a Tukey's multiple comparison test with a single pooled variance (GraphPad
297 Software, Inc.). An unpaired Student's *t*-test with Welch's correction was used to determine

298 differences in antibody titers between non-survivors and survivors (GraphPad Software, Inc.). For
299 groups of three or more animals, a paired Student's *t*-test was used to assess differences in pre-
300 challenge vs. terminal antibody titers (GraphPad Software, Inc.). All ELISA data are displayed as
301 the geometric mean + 95% confidence interval (Bland and Altman, 1996). Percent uptake and
302 opsonization of PA103 and PA103ΔUT by the control sera were assessed by a regular two-way
303 ANOVA while the percent uptake of opsonized PA103ΔUT mediated by immune sera were
304 evaluated using a one-way ANOVA; Tukey's multiple comparisons test or Tukey's multiple
305 comparisons test with a single pooled variance was used for the two and one-way ANOVAs,
306 respectively (GraphPad Software, Inc.). Opsonophagocytic uptake is shown as the mean percent
307 uptake of opsonized bacteria + the standard error of the mean (SEM) for four to five experiments.

321 RESULTS

322 **SL1344 Δ wecA can serve as a carrier for heterologous antigens.** We previously showed that
323 an ECA mutant of *S. Typhimurium* SL1344 was attenuated *in vivo* and could protect against WT
324 and cross-*Salmonella* serovar challenge (Bridge *et al.*, 2015; Gilbreath *et al.*, 2012). As several
325 studies have shown that live-attenuated *S. Typhimurium* vaccine strains can also be used as
326 carriers for heterologous antigens (Chatfield *et al.*, 1989; DiGiandomenico *et al.*, 2004;
327 DiGiandomenico *et al.*, 2007; Kang *et al.*, 2002; Liu *et al.*, 2011; Nayak *et al.*, 1998; Oliveira *et al.*,
328 2012; Pier *et al.*, 1995; Scarff and Goldberg, 2008; Xin *et al.*, 2009), we sought to determine if the
329 *S. Typhimurium* SL1344 Δ wecA mutant could also serve in this capacity. To this end, the broad
330 host-range cosmid pLAFR1 harboring the entire O11 O-antigen gene locus from PA103
331 (designated as pLPS2) (Goldberg *et al.*, 1992) was transformed into the SL1344 Δ wecA strain. As
332 a vector control, a SL1344 Δ wecA strain that contained only the pLAFR1 backbone was also
333 constructed. Expression of the O11 O-antigen by the vaccine strain Δ wecA/pLPS2, but not the
334 vector control strain Δ wecA/pLAFR1 was verified by ELISA (Fig. 1). PA103 and SL1344 Δ wecA
335 served as positive and negative controls, respectively. LPS expression levels were determined for
336 several Δ wecA/pLPS2 transformants (data not shown), and the clone that expressed the highest
337 amount of PA103 LPS (Fig. 1) was used for subsequent immunization studies.

338
339 **IP immunization with SL1344 Δ wecA/pLPS2 protects against *P. aeruginosa* challenge.** To
340 determine if SL1344 Δ wecA/pLPS2 could protect against challenge with an O11 *P. aeruginosa*
341 strain, BALB/c mice were either mock-immunized with isotonic saline, or were immunized with the
342 Δ wecA/pLAFR1 vector control, or with the Δ wecA/pLPS2 vaccine strain orally or by IP injection
343 (Table 2). In the first experiment, mice were then challenged IN with either 6.4×10^5 CFU or $3.4 \times$

10⁶ CFU of PA103 to test the boundaries of any observed protection (Table 2). Oral immunization with the Δ wecA/pLPS2 vaccine strain did not offer significant protection compared to immunization with mock or Δ wecA/pLAFR1 vector immunized controls (Table 2). In contrast, IP immunization with the Δ wecA/pLPS2 vaccine conferred significant protection compared to both mock and Δ wecA/pLAFR1 vector immunized mice when challenged with the lower PA103 challenge dose (Table 2). In mice challenged with 3.4×10^6 CFU of *P. aeruginosa*, IP immunization with the Δ wecA/pLPS2 vaccine conferred significant protection compared to mock-immunized mice (Table 2). As has been previously observed in immunizations with *Salmonella* LVS carrying empty vectors (DiGiandomenico *et al.*, 2004; Nayak *et al.*, 1998), a number of mice immunized with the Δ wecA/pLAFR1 vector control strain survived challenge (Table 2); thus at the high challenge dose, protection mediated by IP immunization with the Δ wecA/pLPS2 vaccine was not significant when compared to Δ wecA/pLAFR1 vector control immunized mice (Table 2).

In a repeat experiment, again utilizing two challenge doses to test boundaries of protection, oral immunization with the Δ wecA/pLPS2 vaccine again did not offer improved protection from lethal PA103 challenge (2.6×10^5 or 5.2×10^5 CFU) compared to mock or Δ wecA/pLAFR1 vector control immunized mice (Table 2). When the PA103 challenge dose (2.6×10^5 CFU) approached the reported LD₅₀ of PA103 (1.0×10^5 CFU) (DiGiandomenico *et al.*, 2007), a number of IP mock-immunized and Δ wecA/pLAFR1 vector immunized controls survived challenge (Table 2), resulting in a lack of significant protection. However, similar to the previous experiment, when the PA103 challenge dose was increased to 5.2×10^5 CFU, mice immunized IP with the Δ wecA/pLPS2 vaccine were protected from lethal PA103 challenge in contrast to mock and Δ wecA/pLAFR1 vector immunized controls (Table 2). Together these results indicate that IP immunization with the

366 Δ wecA/pLPS2 vaccine, but not oral immunization, confers protection against lethal challenge with
367 an O11 *P. aeruginosa* strain.

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369 **Immunization with SL1344 Δ wecA/pLPS2 generates a *P. aeruginosa* LPS-specific antibody**

370 **response.** Previous studies have shown induction of *Pseudomonas* O11 O-antigen-specific

371 antibody titers in response to immunization with pLPS2 (DiGiandomenico *et al.*, 2004;

372 DiGiandomenico *et al.*, 2007; Pier *et al.*, 1995). Therefore, we next sought to determine if

373 immunization with the Δ wecA/pLPS2 vaccine strain resulted in an O11-specific antibody response.

374 As there was no protection in mice that were orally immunized with the Δ wecA/pLPS2 strain, we

375 focused our analysis on sera obtained from IP immunized mice; we first chose those animals

376 immunized IP with mock, 6.2×10^2 CFU of Δ wecA/pLAFR1, or 7.6×10^2 CFU of Δ wecA/pLPS2

377 (Table 2 and Fig. 2). These groupings were analyzed irrespective of the PA103 challenge dose

378 and therefore include mice challenged IN with 6.4×10^5 or 3.4×10^6 CFU of PA103. To reduce

379 cross-reactive antibody titers in both the mock and Δ wecA/pLAFR1 vector control mice, sera were

380 pre-absorbed using the SL1344 Δ wecA strain prior to *Pseudomonas* LPS-specific ELISA analysis.

381 Despite pre-absorbance of the sera, we still observed a significant increase in both serum Ig and

382 IgG titers in animals vaccinated with the Δ wecA/pLAFR1 vector control (Fig. 2A and B). In spite of

383 this observation, the increase in pre-challenge Ig and IgG titers in animals immunized with the

384 Δ wecA/pLPS2 vaccine was significantly higher than in mock-immunized or Δ wecA/pLAFR1-

385 immunized animals (Fig. 2A and B). To determine whether the pre-challenge Ig and IgG antibody

386 titers correlated with survival, we grouped pre-challenge Ig and IgG titers irrespective of the

387 immunization group. We observed that pre-challenge serum from animal that survived PA103

388 challenge displayed significantly elevated Ig and IgG titers (Fig. 2C and D). Lastly, we compared

pre-challenge Ig and IgG titers to titers found in animals that survived PA103 challenge. While we observed a significant increase in *P. aeruginosa* LPS-specific Ig titers in terminal sera as compared to pre-challenge sera in mice immunized with the $\Delta wecA$ /pLPS2 vaccine (Fig. 2E), the increase in the IgG terminal sera titers failed to reach significance (Fig. 2F). We note that while we also observed an increase in terminal sera Ig and IgG titers in mice immunized with the $\Delta wecA$ /pLAFR1 vector control as compared to pre-challenge titers (Fig. 2E), we were unable to assess statistical significance due to an insufficient number of animals in the data set. While these increases are likely a result of the generation of *Pseudomonas*-specific antibodies post-PA103 challenge, the increase seen in terminal titers with the $\Delta wecA$ /pLPS2-immunized mice may suggest a possible memory response. To extend our findings, we repeated the serum ELISA analysis on the second group of IP immunized mice: mock, 4.2×10^2 CFU of $\Delta wecA$ /pLAFR1, or 5.8×10^2 CFU of $\Delta wecA$ /pLPS2 (Table 2 and Fig. S1). In general, the results were comparable to the first experiment (Fig. S1). Overall, the data indicate that immunization with the $\Delta wecA$ /pLPS2 vaccine induces a *Pseudomonas* LPS-specific Ig and IgG antibody response that correlates with survival following challenge with PA103.

Immunization with the SL1344 $\Delta wecA$ heterologous expression strains induces a *Salmonella*-specific antibody response. We previously observed a *Salmonella*-specific antibody response that correlated with survival when mice were immunized with SL1344 $\Delta wecA$ (Bridge *et al.*, 2015). As such, we wondered if these *Salmonella*-specific responses would be preserved with the heterologous expression strains. To evaluate this, we again analyzed pre-challenge sera based on IP immunization groups as described above; data include mice from the first survival experiment that were immunized with mock, 6.2×10^2 CFU of $\Delta wecA$ /pLAFR1, or 7.6×10^2 CFU of

412 Δ wecA/pLPS2 (Table 2 and Fig. 3). Consistent with our previous results (Bridge *et al.*, 2015),
413 serum Ig and IgG titers were significantly increased in the immunized animals as compared to the
414 mock-immunized controls (Fig. 3A and B). As expected, there were no differences in *Salmonella*-
415 specific antibody titers between mice immunized with Δ wecA/pLAFR1 and Δ wecA/pLPS2 (Fig. 3A
416 and B). Analysis of the pre-challenge Ig and IgG antibody titers in relation to animal survival post-
417 lethal PA103 challenge showed that animals that survived PA103 challenge displayed significantly
418 elevated *Salmonella*-specific Ig and IgG titers (Fig. 3C and D). Moreover, these Ig and IgG
419 antibody levels were further increased in terminal sera (Fig. 3E and F). Similar assays were also
420 conducted on IP immunized animals challenged with 5.2×10^5 or 2.6×10^5 CFU PA103 from the
421 second survival experiment (Table 2 and Fig. S2). Overall, these results were reproducible from
422 the first experiment (Fig. S2).

423 *En masse*, the ELISA analyses indicate that IP immunization with Δ wecA/pLPS2 induces a
424 *P. aeruginosa* LPS-specific humoral response that correlates with survival in a pneumonia model
425 of infection. Furthermore, immunization with the heterologous strains preserves the previously
426 observed *Salmonella*-specific immune response.

427

428 **SL1344 Δ wecA immunization alone does not protect against lethal *P. aeruginosa* challenge.**

429 Given that we observed a level of protection against lethal PA103 challenge in animals immunized
430 with the Δ wecA/pLAFR1 vector control (Table 2), and because we observed that the *Salmonella*-
431 specific Ig and IgG antibody titers correlated with survival against PA103 (Fig. 3 and Fig. S2), we
432 next sought to determine if IP immunization with SL1344 Δ wecA alone could provide non-specific
433 protection against PA103 challenge. To this end, mice were mock-immunized IP or were
434 immunized IP with increasing doses of SL1344 Δ wecA (Table 3). Twenty-eight days post-

immunization, mice were challenged IN with 6.4×10^5 CFU of PA103; none of the mice immunized with SL1344 Δ wecA were protected from PA103 challenge (Table 3). These results indicate that SL1344 Δ wecA alone is insufficient to protect against *P. aeruginosa* challenge despite the apparent production of cross-reactive antibodies.

Immunization with SL1344 Δ wecA/pLPS2 induces production of functional antibodies. Prior studies have shown that immunization with a *Salmonella* LVS carrying pLPS2 results in the production of functional *Pseudomonas* O11 O-antigen-specific antibodies that can induce opsonophagocytosis (DiGiandomenico *et al.*, 2004; DiGiandomenico *et al.*, 2007). Therefore, we sought to determine if the antibodies produced after immunization with Δ wecA/pLPS2 were also functional; sera from animals that were mock-immunized or immunized with Δ wecA/pLAFR1 were similarly analyzed. Initially, we tested the ability of serum antibodies to mediate bacterial agglutination. To this end, PA103 was incubated with PBS, immune sera from IP immunized animals, or diagnostic anti-sera; *P. aeruginosa* O11 and *Salmonella* O4 anti-sera served as positive and negative controls, respectively. PA103 was agglutinated by sera from mice immunized with the Δ wecA/pLPS2 vaccine strain, but not by sera from mock or Δ wecA/pLAFR1 vector control immunized animals (Fig. 4A-C). Furthermore, PA103 was agglutinated by *P. aeruginosa* O11 anti-sera but not by PBS or the *Salmonella* O4 anti-sera (Fig. 4D-F). These results suggest that sera from mice IP immunized with Δ wecA/pLPS2 contain antibodies that can mediate PA103 agglutination. Furthermore, the lack of agglutination seen with the *Salmonella* O4 anti-sera further supports the notation that any cross-reactive antibodies produced in response to the presence of *S. Typhimurium* are not functionally responsible for the observed protection against *P. aeruginosa* challenge.

458 As a second readout of antibody functionality, we also evaluated serum-mediated bacterial
459 opsonization of PA103 and uptake by J774A.1 macrophages. Initial opsonization and uptake
460 studies using PA103 and the *P. aeruginosa* O11-specific anti-sera, revealed a significant, but low
461 level of bacterial uptake as compared to PA103 pre-incubated with PBS or *Salmonella* O4 anti-
462 sera, both of which served as negative controls (Fig. 5A). Given that PA103 expresses the type III
463 secretion (T3S) effectors, ExoU and ExoT (Finck-Barbancon *et al.*, 1997; Fleiszig *et al.*, 1997), and
464 because ExoT has been shown to inhibit bacterial phagocytosis (Garrity-Ryan *et al.*, 2000), we
465 also assessed opsonization and uptake with PA103 Δ UT; the ExoU and ExoT effectors are deleted
466 in this strain (Vallis *et al.*, 1999). O11 anti-sera-mediated uptake of PA103 Δ UT by J774A.1
467 macrophages was significantly increased compared to uptake of WT PA103 (Fig. 5A). Based on
468 this increase, we utilized the PA103 Δ UT strain to test opsonization-mediated uptake of bacteria by
469 antibodies contained in the immune sera.

470 Incubation of PA103 Δ UT with sera from mock-immunized animals or Δ wecA/pLAFR1 IP
471 immunized mice resulted in minimal uptake by J774A.1 cells (Fig. 5B). In contrast, uptake of
472 PA103 Δ UT was significantly increased by sera from animals immunized with the Δ wecA/pLPS2
473 vaccine (Fig. 5B). Given the significant increases in Ig and IgG seen in animals that survived *P.*
474 *aeruginosa* challenge, we also assessed the uptake of bacteria mediated by sera from these
475 animals. As compared to the pre-challenge sera, uptake was significantly increased for both the
476 Δ wecA/pLAFR1 and Δ wecA/pLPS vaccinated survivors (Fig. 5B). Furthermore, the increase in
477 uptake seen with terminal sera from Δ wecA/pLPS2-immunized animals was the most profound
478 (Fig. 5B). Taken together, these results indicate that immunization with the Δ wecA/pLPS2 is able
479 to induce production of functional antibodies that mediate agglutination and uptake of PA103.
480 Thus, SL1344 Δ wecA can indeed serve as a carrier for heterologous antigen expression.

481 DISCUSSION

482 Numerous studies have investigated the utility of *Salmonella* LVS to serve as carriers of
483 heterologous antigens (Chatfield *et al.*, 1989; Kang *et al.*, 2002; Lin *et al.*, 2015). Indeed,
484 immunization with several of these recombinant vaccine strains has been shown to result in
485 protection from challenge with the WT strain from which the antigen originated (DiGiandomenico *et*
486 *al.*, 2004; DiGiandomenico *et al.*, 2007; Kang *et al.*, 2002; Liu *et al.*, 2011; Nayak *et al.*, 1998;
487 Oliveira *et al.*, 2012; Pier *et al.*, 1995; Scarff and Goldberg, 2008; Vishwakarma *et al.*, 2015; Xin *et*
488 *al.*, 2009). Based on our previous characterization of an ECA deficient *S. Typhimurium* LVS
489 (Bridge *et al.*, 2015; Gilbreath *et al.*, 2012), herein we investigated the ability of the strain to serve
490 as a carrier for heterologous antigen expression. Our results indicate that IP immunization with
491 SL1344 Δ *wecA* engineered to express the PA103 O11 O-antigen gene cluster can provide
492 protection against lethal IN *P. aeruginosa* challenge; protection was seen at 5.2, 6.4, and 34 times
493 the reported LD₅₀. Moreover, immunization resulted in production of functional antibodies that
494 could opsonize and agglutinate *P. aeruginosa*.

495 Other *Salmonella* LVS carrying the pLPS2 expression system or pLAFR1 vector control
496 have been extensively characterized in various models of *P. aeruginosa* infection using oral, IP,
497 and IN modes of immunization (DiGiandomenico *et al.*, 2004; DiGiandomenico *et al.*, 2007; Pier *et*
498 *al.*, 1995). Of these prior studies, the work that characterized the ability of oral and IP immunization
499 with SL3261/pLPS2 to protect against IN challenge with *P. aeruginosa* (DiGiandomenico *et al.*,
500 2004) serves as a good comparison point for our work since the same expression vectors were
501 used in both studies. Of note, SL3261 is a *S. Typhimurium* Δ *aroA*, non-revertant derivative of *S.*
502 *Typhimurium* SL1344 (Hoiseth and Stocker, 1981). Similarly to the work with SL3261/pLPS2
503 (DiGiandomenico *et al.*, 2004), oral immunization with the Δ *wecA*/pLPS2 vaccine strain did not

increase survival in mice challenged with *P. aeruginosa* (Table 2). However, in contrast to the SL3261/pLPS2 study, we did not observe any increases in the mean time to death (MTD) in the orally immunized mice (data not shown). As the expression level of LPS by the Δ wecA/pLPS2 strain was comparable to the expression level from the SL3261/pLPS2 strain (data not shown), the differences in the oral immunization results between studies may be due to several key points. First, the lack of an increase in the MTD in mice orally immunized with Δ wecA/pLPS2 may be due to the virulence of the *P. aeruginosa* challenge strain. While a simple model of infection, IN instillation of *P. aeruginosa* results in a rapid, highly virulent infection that can be achieved with a low inoculum dose; higher inoculum doses can result in extrapulmonary spread within hours (Allewelt *et al.*, 2000). Previous studies in this model have indicated that the LD₅₀ for *P. aeruginosa* strains that express ExoU can range from 10⁵ to 10⁶ CFU (Allewelt *et al.*, 2000). Thus, the PA103 strain, which carries ExoU, that was utilized for our challenge study is approximately 200 times more lethal than the non-cytotoxic strain of *P. aeruginosa* utilized in the SL3261/pLPS2 studies (Allewelt *et al.*, 2000; DiGiandomenico *et al.*, 2004; DiGiandomenico *et al.*, 2007). As such, the ability to detect subtle differences in protection may have been lost due to challenge strain cytotoxicity. Second, as our previous studies showed that a single oral dose of SL1344 Δ wecA resulted in persistent colonization in a murine model and protected against lethal *Salmonella* challenge (Bridge *et al.*, 2015; Gilbreath *et al.*, 2012), we similarly utilized a single, oral immunization strategy to test protection from lethal heterologous challenge. While the level of protection induced by oral immunization with the SL1344 Δ wecA strain is sufficient to aid in protection against *Salmonella* (Bridge *et al.*, 2015; Gilbreath *et al.*, 2012), it appeared to be insufficient for oral immunization with the heterologous antigen expression system. Therefore, it is tempting to speculate that survival rates could be increased if the mice were orally boosted similar

527 to the oral immunization strategy used in the SL3261/pLPS2 study; mice were orally inoculated
528 once per week for four weeks prior to challenge (DiGiandomenico *et al.*, 2004). A potential pitfall to
529 this approach in our system is that neurological cytotoxicity consistent with meningitis (spinning,
530 head tilt, and unidirectional movement) was observed in some mice receiving high oral doses (10^6
531 - 10^8 CFU) of WT SL1344 or SL1344 Δ wecA (this study, and data not shown in (Bridge *et al.*,
532 2015; Wickham *et al.*, 2007), or greater than 10^8 CFU with a SL1344 Δ crp strain (Zhang *et al.*,
533 1997). Due to cytotoxicity with increased bacterial CFU, the SL1344 Δ wecA strain likely will require
534 additional attenuating mutations for boosting purposes. Another option for optimization of the
535 SL1344 Δ wecA strain for oral immunization for heterologous antigen delivery may be to express
536 the heterologous antigens using an *in vivo*-inducible promoter system (Chatfield *et al.*, 1992; Wang
537 *et al.*, 2010; Wang *et al.*, 2011) or to create a controlled bacterial lysis system using the SL1344
538 Δ wecA background (Kong *et al.*, 2008). In sum, the lack of survival in animals orally immunized
539 with the Δ wecA/pLPS2 vaccine strain is likely due to the cytotoxicity of the challenge strain and the
540 single dose oral immunization strategy.

541 In contrast to the SL3261/pLPS2 study, we observed protection from PA103 challenge in
542 mice immunized IP with Δ wecA/pLPS2. Survival rates ranged from 22 to 89% and varied based on
543 the PA103 challenge dose; the lower survival rates were seen at challenge doses that were 34
544 times the reported LD₅₀. In parallel, we observed a significant increase in serum *P. aeruginosa*
545 O11-specific Ig and IgG titers in Δ wecA/pLPS2 immunized animals that increased following
546 challenge. We also detected a low level of survival in vector control immunized mice that ranged
547 from 12-45%. In spite of pre-absorbance of the sera of *Salmonella*-specific antibodies, we detected
548 a significant amount of non-specific antibodies in vector control mice that cross-reacted with the
549 O11 O-antigen (Fig. 2). These results are consistent with previous reports of non-specific

550 protection and non-specific antibody production induced in animals immunized with *Salmonella*
551 LVS carrying a vector control (DiGiandomenico *et al.*, 2004; Nayak *et al.*, 1998). In this regard,
552 SL3261/pLAFR1 vector control immunized animals showed cross-reactive antibodies detected by
553 ELISA that were not protective and did not mediate opsonophagocytic uptake (DiGiandomenico *et*
554 *al.*, 2004). Similarly, antibodies from mice that were immunized with a *Salmonella* host strain
555 carrying a vector control that survived pneumococci challenge were not protective when passively
556 transferred to naïve mice (Nayak *et al.*, 1998). Consistent with those results, the antibodies
557 contained within the pre-challenge sera of Δ wecA/pLAFR1 vector immunized controls were not
558 able to mediate agglutination or opsonization and uptake of *P. aeruginosa* when compared to sera
559 from Δ wecA/pLPS2 immunized mice (Figs. 4 and 5). Thus, the data suggest that immunization with
560 the *Salmonella* host strain results in the production of cross-reactive non-specific antibodies. The
561 increase in serum Ig and IgG antibody titers contained within terminal sera from vector control mice
562 that survived challenge is not surprising as these animals would have had exposure to PA103 and
563 adequate time to build a *P. aeruginosa* antibody response. Indeed, sera from these animals were
564 able to mediate *P. aeruginosa* opsonization and uptake, whereas pre-challenge sera were not. The
565 significant increase in opsonization and uptake of PA103 mediated by sera from animals
566 immunized with Δ wecA/pLPS2 as compared to sera from animals immunized with Δ wecA/pLAFR1
567 that survived challenge indicates a potential memory response. In sum, IP immunization with
568 Δ wecA/pLPS2 results in protection from *P. aeruginosa* challenge and generates functional
569 antibodies that can mediate agglutination and opsonization, while immunization with the vector
570 control results in non-specific survival and non-functional antibody production.

571 Lastly, IP immunization with the *Salmonella* heterologous expression strains also resulted in
572 a significant increase in *Salmonella*-specific antibody titers as has been reported (DiGiandomenico

573 *et al.*, 2004; DiGiandomenico *et al.*, 2007; Kang *et al.*, 2002; Lin *et al.*, 2015; Xin *et al.*, 2009).
574 Therefore, our LVS maintains the characteristics of other attenuated *Salmonella* LVS that can
575 induce both homologous and heterologous antibody responses (Kang *et al.*, 2002; Lin *et al.*, 2015).
576 When we evaluated pre-challenge *Salmonella*-specific Ig and IgG titers in regards to survival, we
577 noted a significant increase in *Salmonella*-specific antibodies in animals that ultimately survived
578 challenge and these pre-challenge titers increased upon PA103 challenge. These results were not
579 intuitive, as immunization with SL1344 Δ wecA alone did not protect against PA103 challenge
580 (Table 3). Upon further analysis the animals with the highest Ig and IgG titers in the survivor
581 analysis were those that were immunized with Δ wecA/pLPS2. Together these results may be
582 attributed to the observation that immunization with a *Salmonella* LVS is known to induce non-
583 specific immune responses against antigenically unrelated organisms; the exact mechanism
584 remains undefined (Hormaeche *et al.*, 1991; Nayak *et al.*, 1998).

585 In conclusion, we have shown that the *Salmonella* LVS, SL1344 Δ wecA, is able to serve as
586 a vehicle for heterologous antigen delivery. While we have shown that protection was only offered
587 by IP immunization, protection was mediated by the production of antibodies against the
588 heterologous *P. aeruginosa* O11 O-antigen. While we have identified a humoral antibody response
589 that underlies survival following immunization with the heterologous vaccine strain, further studies
590 will be required to identify any role for cell-mediated immunity in heterologous antigen mediated
591 protection. Future studies will also focus on optimization of the SL1344 Δ wecA strain for oral
592 immunization and in direct comparison of the SL1344 Δ wecA strain against previously developed
593 attenuated *Salmonella* LVS.

594

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712

713

714 **FIGURE LEGENDS**

715 **Figure 1. Expression of PA103 LPS from SL1344 Δ wecA.** Plasmids pLAFR1 and pLPS2 were
716 isolated from *S. Typhimurium* SL3261 and independently transformed into *S. Typhimurium* SL1344
717 Δ wecA to create Δ wecA/pLAFR1 and Δ wecA/pLPS2. Expression of the O11 O-antigen by
718 Δ wecA/pLPS2 but not Δ wecA/pLAFR1 was verified by ELISA. PA103 and SL1344 Δ wecA served
719 as positive and negative controls, respectively. Bars represent the mean + standard deviation of
720 three technical replicates

721 **Figure 2. Heterologous expression of *P. aeruginosa* LPS by SL1344 Δ wecA induces a**
722 **humoral immune response that protects against lethal *P. aeruginosa* challenge.** Pre-
723 challenge sera were collected 25-27 days post-immunization from animals immunized IP with $6.2 \times$
724 10^2 CFU of Δ wecA/pLAFR1 or 7.6×10^2 CFU of Δ wecA/pLPS2. Mice were also mock-immunized
725 with isotonic saline as a control. Prior to assessment of a *Pseudomonas* LPS-specific humoral
726 response, the sera were pre-absorbed using the SL1344 Δ wecA strain to remove cross-reactive
727 *Salmonella* antibodies. The pre-challenge sera were then analyzed for *P. aeruginosa* LPS-specific
728 (A) Ig and (B) IgG antibody titers (ANOVA; overall *P* value: $P < 0.0001$ for Ig and IgG). Pre-
729 challenge (C) Ig and (D) IgG antibody titers were then grouped based on survival status to
730 determine if titers correlated with survival following lethal PA103 challenge (unpaired Student's *t*-
731 test). Lastly, (E) Ig and (F) IgG antibody titers from animals that survived lethal challenge with
732 PA103 were then compared to pre-challenge titers to assess a memory immune response (paired
733 Student's *t*-test). Immunization with the Δ wecA/pLPS2 heterologous expression strain induced a
734 significant increase in Ig and IgG antibodies as compared to mock and Δ wecA/pLAFR1 vector
735 control immunized mice. Survival from lethal PA103 challenge correlated with the increase in Ig

736 and IgG titers. **PC**-pre-challenge sera, **T**-terminal sera, **#**-statistical analysis could not be
737 determined due to an insufficient number of animals in the data set, **NS**-not significant. ELISA data
738 are presented for each animal as individual data points. The bar represents the geometric mean +
739 95% confidence interval. No bar indicates zero survivors. In animals without detectable antibody
740 titers, a value of 1 was assigned for statistical analysis.

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742 **Figure 3. Immunization with the SL1344 Δ wecA heterologous expression strains induces a**
743 ***Salmonella*-specific humoral immune response.** The pre-challenge sera collected 25-27 days
744 post-immunization from mock IP immunized or animals immunized IP with 6.2×10^2 CFU of
745 Δ wecA/pLAFR1 or 7.6×10^2 CFU of Δ wecA/pLPS2 was also analyzed by ELISA for *S.*
746 Typhimurium-specific **(A)** Ig and **(B)** IgG antibody titers (ANOVA; overall *P* value: $P < 0.0001$ for Ig
747 and IgG). To determine if pre-challenge antibody titers corresponded to survival status, **(C)** Ig and
748 **(D)** IgG antibody titers were grouped based on survival status (unpaired Student's *t*-test). **(E)** Ig
749 and **(F)** IgG antibody titers from animals that survived lethal challenge with WT PA103 were then
750 compared to pre-challenge titers to assess a memory immune response (paired Student's *t*-test).
751 As previously observed, immunization with SL1344 Δ wecA induced a *S. Typhimurium*-specific Ig
752 and IgG antibody response. Furthermore, the presence of Ig and IgG antibodies correlated with
753 survival status post-challenge. **PC**-pre-challenge sera, **T**-terminal sera, **NS**-not significant. ELISA
754 data are presented for each animal as individual data points. The bar represents the geometric
755 mean + 95% confidence interval. No bar indicates zero survivors. In animals without detectable
756 antibody titers, a value of 1 was assigned for statistical analysis.

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Figure 4. Serum antibodies from SL1344 Δ wecA /pLPS2 immunized mice agglutinate WT PA103. To monitor the ability of antibodies produced following immunization with the heterologous strains to mediate bacterial agglutination, PA103 was incubated with pooled pre-challenge sera from (A) mock-immunized animals, (B) animals immunized IP with 6.2×10^2 CFU of Δ wecA/pLAFR1, or (C) animals immunized IP with 7.6×10^2 CFU of Δ wecA/pLPS2. As controls, PA103 was also incubated with (D) PBS, (E) *P. aeruginosa* O11 O-antigen anti-sera, or (F) *Salmonella* O4 anti-sera. PA103 was agglutinated in the presence of sera from Δ wecA/pLPS2-immunized animals and in the presence of the *P. aeruginosa* O11 O-antigen anti-sera. DIC images were taken at 40X and are representative of three-independent experiments.

Figure 5. Immunization with SL1344 Δ wecA/pLPS2 induces serum antibodies that mediate uptake of opsonized PA103. A) WT PA103 and the T3S system effector-less strain, PA103 Δ UT, were pre-incubated with *P. aeruginosa* O11-specific anti-sera prior to incubation with J774A.1 murine macrophage cells. Strains were also pre-incubated with PBS or *Salmonella* O4-specific anti-sera as negative controls. The percent uptake of opsonized bacteria was determined by comparing the number of bacteria taken up by the J774A.1 cells to the inoculum. Significance was determined by an ordinary two-way ANOVA (*P* value for the Row and Column Factors was <0.0001). Due to the efficiency of opsonization and uptake of the T3S effector-less strain, PA103 Δ UT was used to assess opsonization and uptake mediated by immune sera. B) PA103 Δ UT was pre-incubated with pooled sera from mock-immunized or mice immunized with 6.2×10^2 CFU of Δ wecA/pLAFR1 or 7.6×10^2 CFU of Δ wecA/pLPS2 prior to incubation with J774A.1 cells. Sera from animals immunized with Δ wecA/pLAFR1 or Δ wecA/pLPS2 that survived lethal challenge with PA103 were also evaluated. The percent uptake of opsonized bacteria was determined as

781 previously described (Bridge *et al.*, 2015). Significance was determined using a one-way ANOVA
782 (overall *P* value <0.0001). **PC**-pre-challenge sera, **T**-terminal sera, **NS**-not significant. Data are
783 presented as the mean + SEM for four to five independent experiments.

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804 **Table 1. Bacterial Strains and Plasmids**

Strain or Plasmid	Genotype or Relevant Characteristics	Source or Reference
Plasmids		
pLAFR1	Broad-host-range cosmid; Tet ^r	(Friedman <i>et al.</i> , 1982)
pLPS2	pLAFR1 containing a ~ 26.2 kb fragment encoding the O11 O-antigen gene cluster from <i>P. aeruginosa</i> PA103; Tet ^r	(Goldberg <i>et al.</i> , 1992)
<i>S. enterica</i> strains		
DSM754	SL1344 <i>wecA</i> ::gm; Gm ^r	(Gilbreath <i>et al.</i> , 2012)
DSM1175	SL1344 <i>wecA</i> ::gm/pLAFR1; Gm ^r , Tet ^r	This study
DSM1176	SL1344 <i>wecA</i> ::gm/pLPS2; Gm ^r , Tet ^r	This study
<i>P. aeruginosa</i> strains		
DSM1140	PA103	(Liu, 1973)
DSM1516	PA103Δ <i>exoU</i> Δ <i>exoT</i> ::Tc pUCP	(Vallis <i>et al.</i> , 1999)

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806 Tet^r-tetracycline resistant

807 Gm^r-gentamicin resistant

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817 **Table 2. Heterologous expression of PA103 O11 O-antigen by SL1344 Δ wecA/pLPS2 protects against *P. aeruginosa***
818 **challenge post-IP immunization, but not post-oral challenge.**

Immunization				IN <i>P. aeruginosa</i> Challenge			
Strain	Route	Dose (CFU)	No. survived/total	Dose (CFU)	No. survived/total	% survival	Log-Rank compared to Δ wecA/pLPS2 (P-value)
Mock	Oral	NA	8/8	6.4×10^5	0/8	0	0.390
Δ wecA/pLAFR1	Oral	2.0×10^6	7/8	6.4×10^5	0/7	0	0.088
Δ wecA/pLPS2	Oral	3.0×10^6	7/8	6.4×10^5	1/7	14.3	NA
Mock	Oral	NA	8/8	3.4×10^6	0/8	0	0.125
Δ wecA/pLAFR1	Oral	2.0×10^6	8/8	3.4×10^6	1/8	12.5	0.350
Δ wecA/pLPS2	Oral	3.0×10^6	7/10	3.4×10^6	0/7	0	NA
Mock	Oral	NA	8/8	2.6×10^5	4/8	50	0.225
Δ wecA/pLAFR1	Oral	6.8×10^5	7/8	2.6×10^5	3/7	42.9	0.156
Δ wecA/pLPS2	Oral	8.0×10^5	8/9	2.6×10^5	7/9	77.8	NA
Mock	Oral	NA	8/8	5.2×10^5	1/8	12.5	0.480
Δ wecA/pLAFR1	Oral	6.8×10^5	7/8	5.2×10^5	0/7	0	0.223
Δ wecA/pLPS2	Oral	8.0×10^5	9/9	5.2×10^5	1/9	11.1	NA
Mock	IP	NA	7/8*	6.4×10^5	0/7	0	0.001
Δ wecA/pLAFR1	IP	6.2×10^2	8/8	6.4×10^5	2/8	25	0.009
Δ wecA/pLPS2	IP	7.6×10^2	9/9	6.4×10^5	8/9	88.9	NA
Mock	IP	NA	8/8	3.4×10^6	0/8	0	0.000391
Δ wecA/pLAFR1	IP	6.2×10^2	8/8	3.4×10^6	3/8	37.5	0.802
Δ wecA/pLPS2	IP	7.6×10^2	9/9	3.4×10^6	2/9	22.2	NA
Mock	IP	NA	8/8	2.6×10^5	5/8	62.5	0.063
Δ wecA/pLAFR1	IP	4.2×10^2	8/8	2.6×10^5	7/8	87.5	0.317
Δ wecA/pLPS2	IP	5.8×10^2	8/9*	2.6×10^5	8/8	100	NA
Mock	IP	NA	8/8	5.2×10^5	0/8	0	<0.0001
Δ wecA/pLAFR1	IP	4.2×10^2	8/8	5.2×10^5	0/8	0	<0.0001
Δ wecA/pLPS2	IP	5.8×10^2	9/9	5.2×10^5	7/9	77.8	NA

819 NA-Not applicable; *One mouse succumbed post-tail-vein bleeds; Dashed line denotes Experiment 1 from Experiment 2; solid
820 line denotes oral immunization from IP immunization

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838 **Table 3. IP Immunization with SL1344 Δ wecA does not protect against lethal *P. aeruginosa* challenge**

IP Immunization			IN <i>P. aeruginosa</i> Challenge (6.4×10^5 CFU)		
Strain	Dose (CFU)	No. survived/total	No. survived/total	% survival	Log-Rank compared to Mock (<i>P</i> -value)
Mock	NA	9/10*	1/9	11.1	NA
SL1344 Δ wecA	3.4×10^2	10/10	0/10	0	0.088
SL1344 Δ wecA	5.2×10^2	10/10	0/10	0	0.374
SL1344 Δ wecA	9.6×10^2	10/10	0/10	0	0.205

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840 NA-Not applicable; *One saline-immunized mouse succumbed post-tail-vein bleeds

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5