

The meningococcal vaccine antigen GNA2091 is an analogue of YraP and plays key roles in outer membrane stability and virulence.

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Short title: Role of GNA2091 in meningococcal membrane stability

31 **Nonstandard abbreviations**

32 4CMenB: Four component meningococcal serogroup B vaccine.

33 CFU: colony forming units

34 CI: competitive index

35 LOS: lipooligosaccharide

36 LPS: lipopolysaccharide

37 OMP: outer membrane protein

38 OMV: outer membrane vesicle

39 qRT-PCR: quantitative real time polymerase chain reaction

40 SBA: serum bactericidal activity

41

Abstract

GNA2091 is one of the components of the 4CMenB vaccine and is highly conserved in all meningococcal strains. However, its functional role has not been fully characterized. Here we show that *nmb2091* is part of an operon, and is co-transcribed with the *nmb2089*, *nmb2090* and *nmb2092* adjacent genes, and a similar but reduced operon arrangement is conserved in many other Gram negative bacteria. Deletion of the *nmb2091* gene causes an aggregative phenotype with a mild defect in cell separation; differences in the outer membrane composition and phospholipid profile, in particular in the phosphoethanolamine levels; an increased level of outer membrane vesicles; and deregulation of the zinc-responsive genes such as *znuD*. Finally, the $\Delta 2091$ strain is attenuated with respect to the wild type strain in competitive index experiments in the infant rat model of meningococcal infection. Altogether these data suggest that GNA2091 plays important roles in outer membrane architecture, biogenesis, homeostasis, and in meningococcal survival *in vivo* and a model for its role is discussed. These findings highlight the importance of GNA2091 as vaccine component.

Keywords: Bexsero, 4CMenB, outer membrane, phospholipid, zinc

Introduction

Neisseria meningitidis, a Gram-negative β -proteobacteria, is a leading cause of bacterial sepsis and meningitis worldwide (1). The meningococcal protein GNA2091 was first described during the *N. meningitidis* serogroup B (MenB) reverse vaccinology project as a lipoprotein predicted to be surface exposed in some meningococcal strains, which is able to induce passive protection in the

68 adult mouse model of meningococcal bacteraemia (2). The function of GNA2091 is still unknown
69 but due to its protective properties it was selected for inclusion in the 4CMenB vaccine (trade name
70 Bexsero) (3).

71

72 The 4CMenB vaccine is widely licensed and used to protect against invasive meningococcal
73 disease from MenB and has also been introduced in the UK for mass vaccination of infants (4).
74 4CMenB contains three recombinant proteins (fHbp, NHBA and NadA) and outer membrane
75 vesicles (OMVs) derived from New Zealand strain NZ 98/254 (2, 5). The immunogenicity and
76 stability of the recombinant antigens was optimized by generating protein-protein fusions of fHbp-
77 GNA2091 and NHBA-GNA1030, which induce higher serum bactericidal activity (SBA) titers than
78 those induced by the individual antigens alone (2). fHbp, NadA and NHBA have been extensively
79 characterised and shown to be involved in meningococcal virulence (6-11). The accessory protein
80 GNA1030 has recently been characterised as a Neisseria ubiquinone binding protein (NUbp) (12).
81 However, the role of GNA2091 has not yet been characterized in detail.

82

83 GNA2091 has been shown to be localized at the periplasmic side of the outer membrane, where it is
84 proposed to be required for the efficient assembly of a subset of outer membrane proteins (OMPs),
85 including PorA, PorB, PilQ and the Bam complex, with accumulation of misassembled monomeric
86 proteins seen in a *gna2091* mutant strain (13). The *gna2091* mutant is also sensitive to detergent
87 stress, indicating compromised membrane integrity (14). Here we further characterise the
88 expression and functional role of GNA2091 *in vitro* and in the *in vivo* infant rat model of
89 meningococcal bacteraemia.

90

91

92 **Materials and Methods**

93

94 ***Strains and culture conditions***

95 The MC58 wild type, the MC58 Δ 2091 mutant strain and the MC58 Δ 2091_C complemented strain,
96 have been previously described (14). Strains 2996 Δ 2091 and 2996 Δ 2091_C were generated by
97 transformation of 2996 with the plasmids pBSUD936::Erm and pComP_{RBS}936::Cm, as previously
98 described (14).

99

100 *N. meningitidis* strains were routinely grown on GC agar or Mueller-Hinton (MH) agar at 37°C and
101 5% CO₂ overnight. Columbia agar was used for growth of bacteria recovered from infant rat
102 experiments. For liquid cultures, overnight growth was used to inoculate GC or MH broth.
103 *Escherichia coli* strains used for cloning were cultured in Luria-Bertani (LB) broth or on LB agar.
104 When required, erythromycin and/or chloramphenicol was added to achieve a final concentration of
105 5 µg ml⁻¹. Growth rate experiments were performed as previously described by following the
106 optical density at 600 nm (OD₆₀₀) (14). Bacterial aggregation experiments were performed by
107 selecting at least six single colonies and suspending them independently in GC broth, or taking
108 samples of equal OD from liquid growth, then the number of viable colony-forming units (CFU)
109 was determined by plating serial dilutions onto MH agar. All experiments were performed in
110 triplicate and on at least three occasions. Statistical analysis was performed using the Student's t-
111 test.

112

113 ***General molecular biology and bioinformatic techniques***

114 Techniques including PCR, cloning, SDS-PAGE, Western Blot and flow cytometry analysis were
115 performed as previously described (14, 15). The NCBI Basic Local Alignment Search Tool
116 (BLASTn or BLASTp) was used to identify nucleotide and protein sequence homologues,
117 respectively. Sequence alignments were performed using ClustalW and were exported into Jalview
118 to generate the alignment figure.

119

120 ***Reverse transcription PCR (RT-PCR)***

121 RNA extraction was performed as previously described and 2µg of total RNA was reverse
122 transcribed with random primers and SuperScript II reverse transcriptase (Invitrogen). The resulting
123 cDNA was amplified using primer pairs (Table S1) specific for *gna2091* (2091rtF2 and 2091rtR2)
124 and for the intergenic regions of the genes from *gna2089* to *gna2093* (2089rtF and 2090rtR;
125 2090rtF and 2091rtR2; 2091rtF2 and 2092rtR; 2092rtF and 2093rtR). For each primer pair, an
126 RNA-containing reaction in which the reverse transcriptase step was omitted was used as a negative
127 control for DNA contamination, and genomic DNA was used as a positive control.

128

129 ***Microarray analysis and quantitative real time PCR (qRT-PCR)***

130 Triplicate cultures of MC58 wild type and Δ2091 mutant strains were grown to early-exponential
131 phase (OD₆₀₀ 0.2) in MH broth. RNA extraction, cDNA preparation and microarray analysis was
132 performed as previously described (16). Experiments were performed with cDNA from three pools
133 (*i.e.*, RNA extracted from nine-independent cultures for each strain), with a dye-swap. Relative
134 gene expression between the wild type and mutant strains was confirmed for a selection of genes
135 using quantitative real time PCR (qRT-PCR) with cDNA from triplicate RNA samples and primers
136 shown in Table S1, as previously described (16).

137

138 ***Scanning electron microscopy and thin section transmission electron microscopy (TEM)***

139 *N. meningitidis* strains were harvested into phosphate buffered saline (PBS) and washed three times
140 (5 min, 2000 × *g* centrifugation). Samples containing ~1x10⁶ CFU were fixed on plastic cover slips
141 with 2% glutaraldehyde / 5% formaldehyde solution for 10 min at room temperature. Cover
142 slips were washed three times with H₂O, dehydrated in ethanol (15, 30, 50, 75, 90 and 100%) and
143 hexamethyldisilazane (50% ethanol/HMDS then 100% HMDS). Samples were coated with ~6nm
144 gold prior to analysis on Jeol 5000 Scanning Electron Microscope.

145 For thin section TEM, Samples were fixed with a mixture of 0.5% glutaraldehyde and 4%
146 paraformaldehyde in 0.1 M phosphate buffer, pH 6.9 for 20 min at room temperature. After rinsing
147 in the same buffer for 10 min, samples were dehydrated in a graded ethanol series and embedded in
148 medium grade LR white resin. The resin was polymerised in tightly capped gelatine capsules for 24
149 h at 50°C. Ultrathin sections were obtained using a Reichert Ultracut ultramicrotome with a
150 diamond knife, and collected on nickel grids. Sections were subsequently stained with uranyl
151 acetate and lead citrate and observed with a Jeol JEM EX II transmission electron microscope at
152 100 kV.

153

154 ***Outer membrane vesicle (OMV) preparation and outer membrane protein (OMP) quantification***

155 OMVs were isolated from bacterial culture supernatants as described previously, with the following
156 modifications (17). Briefly, bacteria were inoculated in 500 ml baffled flasks containing 150 ml
157 Meningitidis Chemically Defined Medium I (MCDMI), grown with shaking at 180 rpm at 37 °C for
158 16 h and then centrifuged at $3200 \times g$ for 30 min at 4 °C. The supernatants were filtered through
159 0.2 µm pore size Stericup filters (Millipore Express Plus) followed by ultra-centrifugation at
160 $100,000 \times g$ for 2 h at 4 °C (rotor 45 Ti, Beckman). Pellets were washed with PBS and
161 ultracentrifugation was repeated. The final pellets were re-suspended in 500 µl PBS and protein
162 quantified using the DC Protein Assay (Bio-Rad) according to manufacturer's instructions.

163

164 ***Lipid extraction and thin layer chromatography (TLC) analysis***

165 OMVs (20 µg based on protein content) were used to extract lipids as described previously (18),
166 with the following modifications. Briefly, one volume of methanol was added to the OMVs and
167 samples were gently mixed using a thermal shaker for 15 min at 25°C to complete protein
168 denaturation. One volume of chloroform was then added, and samples were incubated for 15 min at
169 25°C under gentle agitation. The samples were then centrifuged at $2000 \times g$ for 10 min to obtain
170 complete partitioning of lipids into the organic phase, which was collected into a new tube and

171 dried by applying nitrogen flow. Lipids were resuspended in 100 µl of chloroform and stored at -
172 80°C until use. For TLC separation, lipids were loaded on aluminium backed 50*75 mm TLC Silica
173 gel 60 F254 plates (layer thickness 200 µm) and resolved in vertical developing TLC chamber using
174 a running buffer composed by chloroform, ethanol, water and trimethylamine (35:35:7:35, v/v).
175 Lipids were visualized using a solution of primulin (0.05% w/v in a solution of acetone and water
176 8:2) (19) and visualised under UV light.

177 178 ***Phospholipid preparation and mass spectrophotometry (MS) analysis***

179 Bacteria from overnight growth were used to extract phospholipids as described previously (20).
180 Briefly, bacteria were collected and washed once in HPLC grade water, freeze dried, resuspended
181 in 1 mL of chloroform/methanol solvent (2:1 v/v), incubated for 30 min at 25°C, then centrifuged
182 and the supernatant collected. Extraction was repeated one more time. The supernatant was then
183 mixed with 2 volumes of Folch upper phase reagent (chloroform/methanol/water containing 0.74%
184 potassium chloride, 3:48:47 by volume) and vortexed. The lower phase was collected and the
185 extraction repeated three times, before being dried by applying nitrogen flow, weighed and stored at
186 -80°C.

187
188 Lipids were diluted to 1 µg/µl in chloroform/methanol (2:1). Samples were infused on a TripleTof
189 5600 mass spectrometer (SCIEX) at a flow rate of 200 nl/min using a Nanospray III source.
190 Ionisation conditions included: Gas 1 = 6 psi, Cur = 30 psi, heater = 150 °C, ISFV = 4000 V. Data
191 was acquired using the MSMS^{all} script. MS was performed across 200 – 2000 m/z for 10 s, followed
192 by MS/MS across 200 – 1250 m/z in 1 Da steps, acquired for 500 ms per step. For MS/MS, CE was
193 set to 50 with spread +/- 30V. Analysis was performed separately in both positive and negative
194 modes.

195 196 ***In vivo infant rat model of meningococcal infection***

197 The infant rat model competitive index (CI) was used as previously described (14). Briefly, bacteria
198 were grown to early-exponential phase (OD₆₀₀ 0.2) in GC medium, washed, and resuspended in
199 PBS. Five to six-day-old pups from litters of outbred Wistar rats (Charles River) were challenged
200 intraperitoneally with a 1:1 mix of the 2996 wild type and 2996Δ2091 mutant strains, or the
201 2996Δ2091_C complemented and 2996Δ2091 mutant strains, at an infectious dose of 1x10³, 1x10⁴
202 or 1x10⁵ CFU. A control group of infant rats was injected with PBS. Eighteen hours after the
203 bacterial challenge, blood samples were obtained by cheek puncture, and aliquots were plated onto
204 columbia agar + 5% horse blood and columbia agar + 5% horse blood plus erythromycin or
205 chloramphenicol (to select for 2996Δ2091 or 2996Δ2091_C bacteria, respectively) for viable cell
206 counting. The numbers of CFU/ml of blood were determined after overnight incubation and the CI
207 ratio calculated using the following formula: CI = (wild type CFU recovered / mutant CFU
208 recovered) / (wild type inoculum / mutant inoculum). Statistical analysis was performed using the
209 two-tailed Wilcoxon Signed-Ranks Test.

210

211

212 **Results**

213

214 **Characterisation of the *GNA2091* gene locus in *N. meningitidis***

215 GNA2091 is a lipoprotein of 202 amino acids (predicted MW ~22kDa) (Fig. 1A), BLASTp search
216 revealed matches (≥64% identity) to GNA2091 homologues not only in *Neisseria meningitidis* and
217 *Neisseria gonorrhoeae*, but also in other *Neisseria* species, as well as matches (≤55% identity) in
218 several other bacterial species. There is 24-28% identity (43-49% similarity) between GNA2091
219 and its orthologues, including YraP of *E. coli* (Fig. 1D). GNA2091 has been described as containing
220 a single BON (bacterial OsmY and nodulation) domain (Pfam; PF04972) (13). However, our
221 analysis of PROSITE indicates the presence of two BON domains, from amino acid 53-123 and
222 133-202 (Fig. 1A). BON domains have an unknown role but are postulated to be involved in

223 phospholipid-binding (21). GNA2091 also has a significant match from amino acid 1-200 with the
224 OsmY (predicted periplasmic or secreted lipoprotein) NCBI Conserved Domain family [COG2823].
225
226 GNA2091 is encoded by the 609 bp gene *nmb2091* in *N. meningitidis* strain MC58 (22) (Fig. 1B).
227 Reverse transcriptase PCR (RT-PCR) analysis on total RNA from strain MC58 revealed an
228 amplification product across the intergenic regions between the genes *nmb2089*, *nmb2090*,
229 *nmb2091* and *nmb2092* (Fig. 1C), suggesting that the *nmb2091* gene is included in an operon and is
230 co-transcribed with two upstream and one downstream gene. The genes adjacent to *nmb2091* are
231 annotated as follows: *nmb2089*- a conserved hypothetical protein that has putative conserved
232 domains matching COG0792 (predicted endonuclease domain); *nmb2090*- a homologue of
233 phosphoheptose isomerase *gmhA* that is involved in the first step of the biosynthesis of Gram-
234 negative bacteria inner core lipooligosaccharide (LOS) precursor, L-glycero-D-mannoheptose (23);
235 and *nmb2092*- a hypothetical protein. Interestingly, further analysis of this genomic region revealed
236 that several Gram-negative bacteria have a similar three gene arrangement, matching *nmb2089*,
237 *nmb2090*, *nmb2091* genes, while *nmb2092*, with an unknown function, appears to be *Neisseria*
238 specific.

239

240 **The GNA2091 mutant strain displays increased aggregation, likely due to a mild cell** 241 **separation deficiency**

242 To evaluate the influence of GNA2091 on bacterial growth *in vitro*, the wild-type, the $\Delta 2091$
243 mutant strain and a complemented strain were grown in GC broth and agar, and analysed for growth
244 rate, bacterial count and by electron microscopy. During the time course of these experiments, we
245 observed that the growth of the three strains was identical in terms of OD (Fig. 2A), but that the
246 mutant strain yielded fewer CFU ml⁻¹ than the wild type or the $\Delta 2091_C$ strains, when comparing
247 CFU from single colonies (Fig. 2B) or from growth in liquid media at equivalent ODs (data not
248 shown). Cell aggregation was not visible to the eye during liquid culture, however scanning

249 electron microscopy (SEM) revealed that while the wild type and complemented strains were
250 typically visualised as cocci or diplococci, the $\Delta 2091$ mutant formed larger aggregates of bacteria
251 (Fig. 2C), suggesting that the deletion of the *gna2091* gene may cause an alteration of the bacterial
252 surface or cell separation. Thin-section transmission electron microscopy (TEM) revealed that the
253 knockout has a mild defect in cell separation and while both wild type and complemented strains
254 clearly are present as single cocci or diplococci, there is a tendency for the mutant to form tetrads or
255 triads, and on occasions larger clusters with formed septa but which may fail to separate (Fig. 2D).

256

257 **GNA2091 is involved in outer membrane homeostasis, with altered OMP, OMV and**
258 **phospholipid levels seen in the GNA2091 mutant strain**

259 We analysed the composition of membrane LOS, phospholipids, integral membrane proteins and
260 lipoproteins in *N. meningitidis* MC58, $\Delta 2091$ and $\Delta 2091_C$ strains grown to mid-log in GC broth.
261 Whole-cell lysates and OMVs of wild type, mutant and complemented strains were prepared and
262 analysed by SDS-PAGE and Western blot with polyclonal mouse sera raised to OMVs. No
263 differences were seen in the overall banding pattern from whole cell lysates of the three strains, on
264 either the Coomassie stained SDS-PAGE or Western blot (Fig. 3A). However, several differences
265 were evident in the OMV preparations of the $\Delta 2091$ mutant strain compared to the wild type and
266 complemented strains (Fig. 3B). The three major bands detected on the Coomassie stained SDS-
267 PAGE (corresponding to PorB, and Opa/Opc proteins) were present at a higher level in the $\Delta 2091$
268 mutant strain, and Western blot analysis of a duplicate gel probed with anti-OMV antisera indicated
269 a significantly different antibody recognition pattern in the $\Delta 2091$ mutant, with increased
270 expression of several bands compared to the wild type and $\Delta 2091_C$ strains (Fig. 3B). The yield of
271 OMVs released, measured as total protein ml^{-1} of culture supernatant processed, was also found to
272 be approximately 10-fold higher in the $\Delta 2091$ mutant relative to wild type and complemented
273 cultures (Fig. 3C). Hypervesiculation of the $\Delta 2091$ mutant strain was confirmed by scanning
274 electron micrography (Fig. 3D)

275

276 GNA2091 contains two BON domains (Fig. 1A). While the function of BON domains is unknown,
277 they are proposed to play a role in phospholipid binding (21). To investigate whether the
278 phospholipid content of the outer membrane of *N. meningitidis* was altered in the absence of
279 GNA2091, phospholipids were isolated from wild type, Δ 2091 and Δ 2091_C whole cells and OMV
280 preparations and analysed by thin-layer chromatography and mass spectrometry. Using identical
281 quantities of OMV phospholipid extract, we observed that the phospholipid composition of the
282 Δ 2091 mutant was altered, with an upper band missing and an increase in a lower band relative to
283 the wild type and complemented strains (Fig. 4A). MS/MS on whole cell samples confirmed an
284 altered phospholipid composition between the strains, with an increased abundance of 1-(9Z-
285 hexadecenoyl)-2-hexadecanoyl-glycero-3-phosphoethanolamine PE(16:1(9Z)/16:0) in the Δ 2091
286 mutant strain relative to the wild type and complemented strains (Fig. 4B-D). Together, these data
287 indicate that GNA2091 affects OMP, OMV and phospholipid composition in *N. meningitidis*.

288

289 GNA2091 is co-transcribed with *nmb2090* (encodes GmhA), an enzyme involved in the
290 biosynthesis of the LOS inner core, suggesting the GNA2091 may be involved in LOS synthesis.
291 However, SDS-PAGE and silver staining of LOS extracted from the wild type, mutant and
292 complemented strains (during early exponential growth, with all strains in the same growth phase)
293 showed consistent LOS profiles between these strains (data not shown). No difference in capsule
294 was detected between the three strains, as determined by flow cytometry with anticapsular
295 monoclonal antibody SEAM12 (data not shown).

296

297 **Microarray analysis reveals induction of the zinc regulon in the GNA2091 mutant strain**

298 Although the *N. meningitidis* MC58 Δ 2091 mutant strain has normal growth in GC (0.4% glucose
299 w/v), it has previously been shown that the mutant strains decreased growth in MH (no glucose
300 added) relative to the wild type strain, and that this growth defect can be relieved by addition of

301 glucose (14). To gain further insight into the role of GNA2091 and the basis of these growth
302 phenotypes, the MC58 wild type and Δ 2091 strains were compared by microarray analysis using a
303 meningococcal genome array. The strains were grown in MH broth to an OD₆₀₀ of 0.2, which is just
304 before the growth defect of Δ 2091 is evident (Fig. 5A), and RNA extracted. A total of 20 genes had
305 ≥ 2 fold altered expression between the wild type and Δ 2091 mutant strains; 15 genes had increased
306 expression in Δ 2091, while five genes had decreased expression in Δ 2091 (Table S2). Microarray
307 results were validated by qRT-PCR of a selection of regulated genes, or by Western blot analysis or
308 flow cytometry (Table S2). The majority of regulated genes are involved in zinc or iron
309 homeostasis, or metabolism (Fig. 5B). Figure 5B shows the overlap of regulated genes with nine
310 members of the *N. meningitidis* Zur (Zn uptake regulator) regulon (24) or the *N. gonorrhoeae* Zur
311 regulon (originally described as the Mn uptake regulator PerR) (25), and five members of the Fur
312 (ferric uptake regulator) regulated genes (26, 27). Almost the complete Zur regulon has altered
313 expression in the Δ 2091 strain, suggesting that there is a decreased intracellular zinc concentration
314 in the absence of GNA2091.

315

316 **Decreased growth in the GNA2091 mutant strain can be rescued by zinc**

317 Based on the microarray results obtained, we further investigated the growth of the wild type,
318 Δ 2091 mutant and Δ 2091_C complemented strains in MH media supplemented with zinc. Figure
319 5A shows that addition of zinc to MH broth is able rescue the growth defect of the Δ 2091 mutant
320 strain. The ability of zinc to restore growth was also confirmed in the Δ 2091 mutant of *N.*
321 *meningitidis* strain 2996 (data not shown).

322

323 Expression of *znuD*, which encodes the zinc uptake protein ZnuD that is upregulated under low Zn
324 conditions (28), was investigated as a marker of intracellular zinc concentrations in the wild type,
325 Δ 2091 mutant and Δ 2091_C complemented strains. qRT-PCR analysis revealed a 12-fold increase
326 in expression of *znuD* in the Δ 2091 strain grown in MH compared to the wild type strain, with

327 normal *znuD* expression restored in the complemented strain (Fig. 5C). When zinc is added to the
328 media, *znuD* expression is repressed in all 3 strains, with restoration of the normal wild type *znuD*
329 expression profile in the mutant strain. These findings suggest that GNA2091 plays a role in the
330 efficient uptake of zinc into the cell during growth in low zinc conditions.

331

332 **GNA2091 is required for survival in the infant rat model of meningococcal infection**

333 Changes in the bacterial cell membrane and efficiency of nutrient uptake often affect how bacteria
334 can interact with their host. In order to examine the role of GNA2091 *in vivo*, the fitness of the *N.*
335 *meningitidis* 2996 (a strain adapted for infant rat infection) was investigated in the infant rat model
336 of meningococcal infection using a competitive index (CI) assay. *N. meningitidis* 2996 wild type
337 and Δ 2091 mutant bacteria were injected intraperitoneally at a ratio of 1:1, at infectious doses of
338 1×10^3 , 1×10^4 or 1×10^5 CFU, to determine if the wild type bacteria could out-compete the Δ 2091
339 bacteria. Bacterial CFU in the blood were determined after 18 hrs and a CI of <1 was seen for
340 almost all rats in each group indicating that the mutant Δ 2091 bacteria had decreased survival with
341 respect to the wild type (Fig. 6; mean CI of 0.42, 0.36 and 0.32 with an infectious dose of 1×10^3 ,
342 1×10^4 and 1×10^5 CFU, respectively). Furthermore, the wild type phenotype was partially restored in
343 the complemented strain. This suggests that a functional GNA2091 is required for optimal growth
344 and survival of *N. meningitidis in vivo*.

345

346

347 **Discussion**

348

349 The *N. meningitidis* protein GNA2091 is a component of the serogroup B meningococcal vaccine
350 4CMenB (Bexsero), and is present as a fusion protein with fHbp (2, 5). The fHbp-GNA2091 fusion
351 protein has increased immunogenicity and stability relative to fHbp alone (2). Previously it has been
352 shown that GNA2091 is required for optimal growth in minimal media and for resistance to

353 membrane stress (14). GNA2091 has also been proposed to be required for the efficient assembly of
354 a subset of OMPs, including PorA, PorB, PilQ and others (13). Here we expand the characterization
355 of the function of GNA2019 and suggest that it plays a broader role in outer membrane biogenesis
356 and/or homeostasis than previously described.

357 The Gram-negative bacterial outer membrane is a complex biological barrier that is composed of an
358 asymmetric lipid bilayer, with LOS or lipopolysaccharide (LPS) in the outer leaflet and
359 phospholipids in the inner leaflet, as well as integral membrane proteins and lipoproteins. Outer
360 membrane biogenesis is complex and involves several pathways including the Lpt complex for LOS
361 or LPS, the Lol and Slam pathways for lipoproteins, the Bam and Tam complexes for OMPs, and a
362 network of chaperones (reviewed in 29, 30, 31). Phospholipid transport to the outer membrane
363 remains relatively poorly understood, but involves the Mla pathway genes, so called initially for
364 their role in maintenance of OM lipid asymmetry (32) and recently described as a lipid transport
365 system and also known as the MCE protein superfamily (33). Here we show that the deletion of
366 GNA2091 results in several phenotypes in *N. meningitidis* that indicate it is involved in OM
367 biogenesis and/or homeostasis, and that result in reduced fitness in an infant rat infection model.

368 While GNA2091 is not an essential protein for the growth or survival of *N. meningitidis* (13, 14),
369 the importance of its functional role is supported by the fact that the gene encoding GNA2091 is
370 well conserved and always in frame in the genus *Neisseria* (34). Specifically, it is highly conserved
371 in *N. meningitidis* (34, 35) and *N. gonorrhoeae* (34, 36) and the gene is present in all commensal
372 *Neisseria* species investigated, including *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria*
373 *flavescens*, *Neisseria cinerea* (34) and *Neisseria weaveri* (34; unpublished GenBank search).

374 GNA2091 is constitutively expressed during growth in broth (14), but is upregulated during growth
375 at 32°C versus 37°C (37) and during growth in blood (38), consistent with conditions seen during
376 nasopharyngeal colonization and invasive disease, respectively. Here we show that GNA2091 is
377 expressed as part of a four gene operon (nmb2089-nmb2092) conserved in *Neisseria* spp. The first
378 three genes are conserved across many Gram negative bacteria and may play a role in envelope

379 biogenesis across the genera. The gene *nmb2090* encodes a homologue of phosphoheptose
380 isomerase GmhA that is involved in the biosynthesis of LOS (23). The genes *nmb2089* and
381 *nmb2092* are hypothetical proteins, the latter specific to *Neisseria*, and elucidation of their functions
382 may reveal additional details of the role of GNA2091.

383 GNA2091 contains two bacterial OsmY and nodulation (BON) domains, which have an unknown
384 role but are considered to have a structural rather than a catalytic function, in particular they are
385 believed to be involved in phospholipid binding (21). The BON domain is typically ~60 residues
386 long with an alpha/beta predicted fold, a conserved glycine residue and several hydrophobic
387 regions. BON domains are found in a family of osmotic shock protection proteins (21) that includes
388 the OsmY protein of *E. coli*, which is expressed in response to various stress conditions and is
389 involved in protection against osmotic shock (39). The GNA2091 homolog in *E. coli*, YraP, also
390 contains BON domains and a *yraP* knockout strain has a detergent-sensitive phenotype (40), similar
391 to the *N. meningitidis gna2091* knockout (14). In *E. coli*, YraP is believed to be involved in a
392 periplasmic protein folding pathway that functions in parallel to the chaperone protein SurA, since
393 there is synthetic lethality between *yraP* and *surA* mutations (40). However, in *N. meningitidis*, the
394 *surA* single mutant is not noticeably affected in OMP assembly (41), and no synthetic defects were
395 seen in the *surA* / GNA2091 double mutant (13). Although there were higher levels of unassembled
396 porins in a double mutant of GNA2091 and the chaperone Skp (13). YraP of *E. coli* is also involved
397 in cell division, and is believed to be recruited to the divisome following cell constriction where it is
398 involved in NlpD and AmiC activation by an as yet unknown mechanism (42). The divisome is a
399 ring-shaped cytokinetic apparatus that contains dozens of proteins involved in cell division, and
400 while a single $\Delta yraP$ mutant divided normally and a single $\Delta envC$ mutant had a mild separation
401 phenotype, a $\Delta envC \Delta yraP$ mutant resulted in a severe chaining defect (42). Here we show that the
402 GNA2091 mutant has a mild cell separation phenotype resembling that of *Neisseria* spp. with
403 defects in AmiC amidase protein (43). Interestingly, while *E. coli* have three amidase proteins,

404 AmiC is the unique cell separation amidase in *Neisseria*. Therefore both YraP from *E. coli* and
405 GNA2091 have an affect on cell separation that may be through lack of AmiC activation.

406 The GNA2019 mutant exhibits an increased blebbing or yield of OMVs as well as an altered level
407 and/or altered immune recognition of several OMV proteins. This is consistent with the increased
408 protein level seen by Bos *et al.* in the culture supernatant of the GNA2091 mutant (13). The
409 increased blebbing may be a consequence of the cell separation phenotype as defects in cell
410 separation for other bacteria result in hypervesiculation (44, 45). On the other hand, an increase in
411 the relative proportion of PE may also induce stress and disrupt the OM bilayer, as PE is able to
412 form non-bilayer structures due to its curvature (46) and essentially result in hypervesiculation (47).

413 The role of YraP has also recently been investigated in *Salmonella enterica* Serovar Typhimurium,
414 where a *yraP* mutant had increased sensitivity to anionic compounds and attenuated virulence in an
415 oral infection model and during the early stages of systemic infection (48). The YraP mutation is
416 proposed to result in a defective outer membrane barrier, and since levels of lipopolysaccharide, O
417 antigen and major OMPs were not affected in *S. enterica* it was suggested that other membrane
418 components, such as phospholipids, may be affected (48). As of May 2019 the BON family
419 contains 13009 sequences from 3394 species and there are 64 different Bon domain architectures,
420 and 1983 sequences in the Pfam database with a BONx2 architecture similar to that seen in
421 GNA2091. It has been reported that most proteobacteria have one or two BON containing proteins
422 (49) and our recent searches indicate that GNA2091 is the only BON containing protein in *N.*
423 *meningitidis* presently in the Pfam database. Due to the sequence and functional similarities
424 between GNA2091 and YraP of *E. coli*, we propose that GNA2091 be named YraP from herein
425 also in *Neisseria* spp.

426 The phenotypes seen for the meningococcal YraP mutant described herein and previously (13, 14),
427 may be due to changes in the phospholipid composition of the membrane resulting from the absence
428 of YraP and BON domain-mediated phospholipid binding. Although the specific mechanism of
429 YraP in outer membrane biogenesis remains unknown, the phenotype of the mutant holds much in

430 common with the phenotype described for many Gram negative bacteria lacking a functional
431 phospholipid transport system (32, 47). Mutants of a functional Mla system were shown to result in
432 hypervesiculation of OM vesicles with increased PE (47) similar to what we describe here for the
433 YraP mutant. We show also that there is an interplay between YraP, phospholipids, OMVs, OMPs,
434 and zinc. The phospholipid composition of the YraP mutant is altered, with an increased level of
435 phosphoethanolamine (PE(16:1(9Z)/16:0)). In *N. meningitidis*, phosphatidylethanolamine (PE) is
436 the major phospholipid, followed by phosphatidylglycerol (PG), and there are minor amounts of
437 phosphatidic acid (PA) and trace levels of cardiolipin (DPG) (20). The altered phospholipid content
438 of the OM of the mutant may explain the report by Bos *et al.* that there are a subset of proteins in
439 the YraP mutant which are misassembled in the OM (13). Several properties of phospholipids affect
440 membrane protein assembly and function, including their hydrophobicity, charge, rigidity and
441 radius of curvature (50). The ratio of PE, PG and phosphatidylcholine (PC) in vesicles can affect
442 the rate of protein folding and membrane insertion (50, 51) and protein topology (52). For example,
443 the absence of PE in *E. coli* affects cell division resulting in filamentous cells (46), and also causes
444 the misfolding of sugar and amino acid transporters (46), including lactose permease (LacY) (53,
445 54). The YraP mutant shows upregulation of several genes, including the majority of genes in the
446 zinc regulon, suggesting low internal Zn concentrations leading also to decreased growth of the
447 mutant in low zinc conditions. Furthermore, the induction of the zinc regulon can be reversed by the
448 addition of zinc to the media, suggesting that zinc uptake remains intact in the mutant, but that it is
449 inefficient. This is consistent with a decreased efficiency of zinc-transporter functions in the mutant
450 strain. While there are many direct links between zinc and phospholipids (55-57), the model we
451 propose for the role of YraP is that in *N. meningitidis* wild type cells that express YraP,
452 phospholipids are correctly incorporated into the outer membrane and enable normal recruitment,
453 formation, stabilization and/or functional activity of outer membrane proteins, including ZnuD.
454 However, in the absence of YraP in the $\Delta 2091$ mutant strains, the outer membrane phospholipid
455 composition of *N. meningitidis* is altered and outer membrane protein assembly and/or function is

456 altered for a number of proteins including ZnuD which in turn effects efficient Zinc uptake.
457 Interestingly, the cell separation AmiC protein uses a zinc cofactor (43). The zinc transport
458 deficiency that we observed in the YraP mutant may therefore result in reduced function of AmiC
459 and thereby give one plausible explanation for the cell separation phenotype of the mutant.
460 In summary, the outer membrane lipoprotein GNA2091, herein called YraP, is involved in outer
461 membrane biogenesis and/or homeostasis, as indicated by the altered outer membrane protein
462 profile, reduced zinc uptake, altered cell separation and increased levels of outer membrane vesicles
463 likely through direct effect on the phospholipid content of the OM and increased
464 phosphoethanolamine levels seen for the YraP mutant strain relative to the wild type strain. YraP is
465 also important for meningococcal survival in the infant rat model of meningococcal infection.
466 Given the presence of two BON domains in YraP that are proposed to be involved in phospholipid
467 binding, we propose that YraP is involved in maintaining phospholipid homeostasis in the outer
468 membrane. These findings suggest that antibodies raised by GNA2091 could interfere with outer
469 membrane homeostasis during bacterial replication *in vivo*, a mechanism that could be the basis for
470 the “*in vivo*” protective activity induced by YraP in the mouse model of infection (2). Therefore,
471 these data further support the use of YraP as vaccine antigen and suggest that YraP and its
472 homologues could be an ideal target for novel antibiotics to disrupt the outer membrane of *N.*
473 *meningitidis* or closely related bacteria.

474

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Authors Contribution

K. L. Seib and I. Delany designed research; K. L. Seib, A. F. Haag, F. Oriente, L. Fantappiè, S. Borghi, E. A. Semchenko, B. L. Schulz, F. Ferlicca and A. Taddei performed research; K. L. Seib, B. L. Schulz, M. Giuliani, M. Pizza, I. Delany analyzed data; K. L. Seib, M. Pizza, I. Delany wrote the paper.

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681

Figure Legends

Figure 1: Characterisation of the GNA2091 locus. (A) Representation of the GNA2091 protein. The leader peptide and predicted LipoP signal are shown, as well as the BON and OsmY protein domain predictions from Pfam and NCBI Conserved Domain database. (B) Representation of the genome locus of NMB2091 in *N. meningitidis* strain MC58. The open reading frames are shown by arrows, with the NMB locus number inside the arrow, and the annotated gene function above the arrow. (C) Investigation of the *nmb2089-2092* operon. Reverse transcriptase (RT)-PCR of the *NMB2089-NMB2092* region in meningococcal strain MC58, demonstrated that *nmb2091* is co-transcribed with adjacent genes. The locations of PCR primers are shown, as well as the PCR products from cDNA, and the negative (no reverse transcriptase (no RT) and water (H₂O)) and positive (genomic DNA (gDNA)) controls. (D) Representative organization of homologues of NMB2089-2091 from various Gram-negative organisms. Protein sequences were aligned in ClustalW and visualized with JalView [identical nucleotides are shown as vertical lines, with identity over a run of nucleotides shown as dark gray (>80% identity), light gray (<50%), or white (<50% identity or a gap)]. The genome loci of aligned proteins is indicated on the left, and is from NMB, *Neisseria meningitidis* MC58; ECP, *Escherichia coli* 536; STY, *Salmonella enterica* subsp. enterica serovar Typhi str. CT18; VC, *Vibrio cholerae* O1 biovar El Tor str. N16961; PM, *Pasteurella multocida* subsp. multocida str. Pm70; LP, *Legionella pneumophila* subsp. pneumophila str. Philadelphia 1; HI, *Haemophilus influenzae* Rd KW20.

Figure 2. The Δ 2091 mutant strain displays increased bacterial aggregation. Bacterial growth in GC broth (A) and formation of bacterial aggregates were analyzed by (B) colony counts and (C) scanning electron microscopy. (A) Growth rates of MC58 wild type, the Δ 2091mutant and C-2091 complemented strains were assessed by optical density (OD) at 600 nm. (B) Single colonies of the wild type, mutant and complemented strains were selected, serially diluted and plated to determine CFU per colony. Student's t-test p-value; 0.00003 for wild type vs. mutant, 0.191 for wild type vs. complemented strain. Experiments were performed with six colonies on at least three occasions and representative results are shown. (C) Scanning electron micrographs of the wild type, mutant and complemented strains, showing mainly diplococci for the wild type and complement strains, while several aggregates are seen for the Δ 2091 mutant strain. (D) Thin section transmission electron micrographs of the wild type, mutant and complemented strains, showing mainly individual or diplococci for the wild type and complement strains while the GNA2091 mutant shows a mild cell separation phenotype and can manifest as triple and quadruple cells per group. Images are representative of multiple fields at x 5,600 magnification, whereas the different coccoid multimers that can be present in each strain are highlighted at x 15,000 magnification.

Figure 3. The Δ 2091 mutant strain has altered outer membrane protein and outer membrane vesicle (OMV) formation. Coomassie stained SDS-PAGE and Western blot analysis with polyclonal mouse sera raised to OMVs of (A) whole-cell lysates and (B) OMVs of MC58 wild type, the Δ 2091mutant and C-2091 complemented strains. (C) The OMV yields of overnight cultures of the wild type, mutant and complemented strains, based on total protein concentration in OMV preparations. (D) Scanning electron micrographs of the wild type, mutant and complemented strains, showing hypervesiculation of the Δ 2091 mutant strain.

Figure 4. The Δ 2091 mutant strain has altered phospholipid formation as shown by thin layer chromatography (TLC) and mass spectrometry (MS). (A) TLC analysis of phospholipid extracts from OMV preparations of the defined strains. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid. (B) Negative ion mode MS/MS all total ion trace for lipids extracted from wild type (black), Δ 2091mutant (red), and C-2091 complemented (black dash) strains. Inset shows *m/z* region around the 688.5 Da peak. (C) MS/MS of species at an *m/z* of 688.5 Da. (D) Cartoon structure of PE(16:1(9Z)/16:0), consistent with MS/MS in (C).

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Figure 5: GNA2091 is involved in zinc homeostasis. (A) Growth rates of MC58 wild type, the $\Delta 2091$ mutant and C-2091 complemented strains in Mueller Hinton (MH) broth (left) and MH supplemented with zinc (right). Growth rates were assessed by optical density (OD) at 600 nm over 7 hours. (B) Summary of DNA microarray results, showing altered gene expression between the $\Delta 2091$ and wild type strains and the overlap of regulated genes that are present in the *Neisseria* Zur and Fur regulons, and other genes involved in metabolism. The list of genes with ≥ 2 fold altered expression between the MC58 wild type, the $\Delta 2091$ mutant strains is shown in Table S2. (C) Quantitative real-time (qRT)-PCR of *znuD* expression MC58 wild type, $\Delta 2091$ mutant and C-2091 complemented strains in MH broth +/- zinc.

Figure 6. GNA2091 is required for optimal survival in an infant rat model of infection.

Infant rats were infected intraperitoneally with an infectious dose of (A) 1×10^3 , 1×10^4 , or 1×10^5 colony forming units (CFU) of *N. meningitidis* 2996 wild type and $\Delta 2091$ mutant strains, or (B) 1×10^4 CFU C-2091 complemented and $\Delta 2091$ mutant strains, at a 1:1 ratio. At 18 h post-injection blood was collected and serial dilutions were plated and bacterial colonies were counted. The competitive index (CI) for individual rats are shown as well as the mean and median for each infectious dose. The CI was calculated as follows, $CI = (\text{wild type CFU recovered} / \text{mutant CFU recovered}) / (\text{wild type inoculum} / \text{mutant inoculum})$. The p-values using the Wilcoxon Signed-Ranks Test for the survival of the mutant strain with respect to the isogenic wild type parent strain are 0.0047, <0.0001 and 0.0001 for the dose of 1×10^3 , 1×10^4 , and 1×10^5 , respectively. The p-value for the mutant strain with respect to the complemented strain is 0.0434.

757 **Table S1 Oligonucleotides used in this study.**

Name	Sequence
2089rtF	CGGCATGATTCTGTTTGTTG
2090rtF	ACGACTACGGTTTCGACCAC
2090rtR	GTGGAAATGCCGACCAATAC
2091rtF2	ACGTTATGGCGTTGCGTAT
2091rtR2	TGTAGCCTTTGGTTTGTTG
2092rtF	TGACCCCCAAACTCCTGATA
2092rtR	GGTTGAATGTGCTGACGATG
2093rtF	ATGAACGGCATCATCATCAA
2093rtR	CGAATTGTCCGATGTAGTCG
2093rtF	CACTGGGCGACGTAGGTTAC
1475rtF	ACGGCAGTTACCTCGTCATC
1475rtR	ATAGCTTGCGTCAGGCATTT
0941rtF	TCAAAACCGCCAAACAACG
0941rtR	GCGCTTTAAAACGCGGATTG
znuDrtF	GCATCCACGCTTCGCAATA
znuDrtR	TTTCGCCGTGATGGTTCAA
0634rtF	TCTATCCGATACGCACTGCT
0634rtR	TGTGCTGCTTCTTTGTGTTG
16SRT_F	ACGGAGGGTGCGAGCGTTAATC
16SRT_R	CTGCCTTCGCCTTCGGTATTCCT

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759 **Table S2. Differentially expressed genes from microarray studies of MC58 wild type and**
760 **Δ 2091 mutant strains.**

Regulated Genes		DNA Microarray Fold change ^a		qRT-PCR Fold change ^b	
MC58 Gene	Gene Function, Name	Δ 2091 vs. WT	P-val	Δ 2091 vs WT	Δ 2091 vs C2091
Up regulated in Δ2091					
NMB1475 ^c	conserved hypothetical protein, <i>hyp</i> .	8.8	2.1E-06	4.4 *	0.5
NMB0942 ^c	50S ribosomal protein L31, <i>rpmE2</i>	5.7	1.5E-05		
NMB0941 ^c	50S ribosomal protein L36, <i>rpmJ2</i>	5.5	7.2E-04	8.0	1.0
NMB0964 ^c	TonB-dependent Zn receptor, <i>znuD</i>	5.4	1.6E-03	6.8 *	1.0
NMB2092 ^c	hypothetical protein	3.6	4.9E-07	3.2	4.9
NMB2093 ^c	methionine aminopeptidase, <i>map</i>	2.8	1.8E-05	2.0	3.0
NMB2039	porin, <i>porB</i>	2.5	1.3E-03		
NMB1988 ^d	Fe transporter, <i>fetA /frpB</i>	2.4	3.8E-04	*	
NMB0028	hypothetical protein	2.4	9.3E-03		
NMB0744 ^d	hypothetical protein	2.3	8.5E-05		
NMB0634 ^d	Fe ABC transporter, <i>fbpA</i>	2.2	1.8E-04	1.7	1.0
NMB1377 ^d	L-lactate dehydrogenase, <i>lldA</i>	2.1	1.6E-04		
NMB1458 ^d	fumarate hydratase, class II, aerobic, <i>fumC</i>	2.1	0.0E+0 0		
NMB0317 ^c	conserved hypothetical protein	2.1	2.3E-02		
NMB0586 ^c	Mn/Zn ABC transporter, periplasmic binding protein, <i>mntC</i>	1.9	1.2E-03	*	
Down regulated in Δ2091					
NMB0378	putative phosphate permease	2.0	5.8E-03		
NMB1938	ATP synthase F0, <i>atpF</i>	2.1	3.1E-03		
NMB0431	methylcitrate synthase, <i>prpC</i>	2.5	2.6E-03		
NMB0430	putative carboxyphosphoenol-pyruvate phosphonmutase, <i>prpB</i>	2.6	1.4E-03		
NMB2091	GNA2091	59.2	0.0E+0 0	*	

761 ^a Average fold change from three separate microarray experiments including a dye swap experiment
762 (cut off, an average of ≥ 2 fold change and p-value ≤ 0.05).

763 ^b Average fold change from three separate experiments. * Differential expression confirmed by
764 Western blot or flow cytometry analysis (data not shown).

765 ^c Members of the *N. meningitidis* (24) or *N. gonorrhoeae* (25) Zur regulons. NB, other genes from
766 the *N. meningitidis* Zur regulon (24) were also altered but below the cut-off threshold used;
767 NMB0588 (*mntA*, 1.5 fold increased expression in GNA2091, P value 0.012); NMB0587 (*mntB*,
768 1.4, P value 0.08) and NMB0820 (*hyp*, 1.5, P value 0.017); NMB0546 (*adhP*, 1.7 fold decreased
769 expression in GNA2091; P value 0.013).

770 ^d Members of the Fur regulon (repressed by Fur and Fe).

771 ^e The altered regulation of these genes cannot be directly attributed to the role of GNA2091.
772 NMB2092 and NMB2093 are downstream of, and co-transcribed with NMB2091, and their
773 expression is not restored to wild type level in the C-2091 complemented strain.

Figure 1

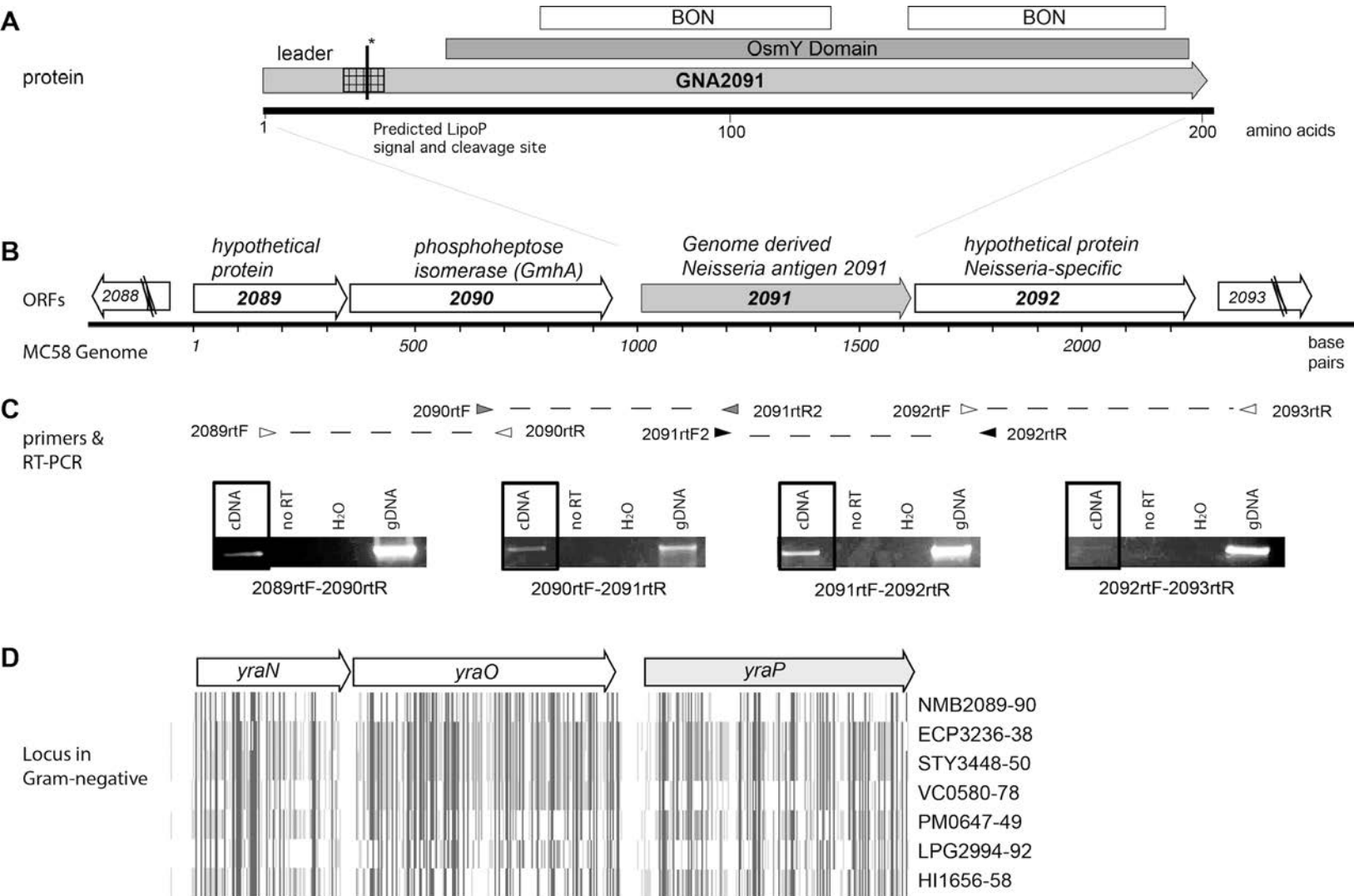


Figure 2

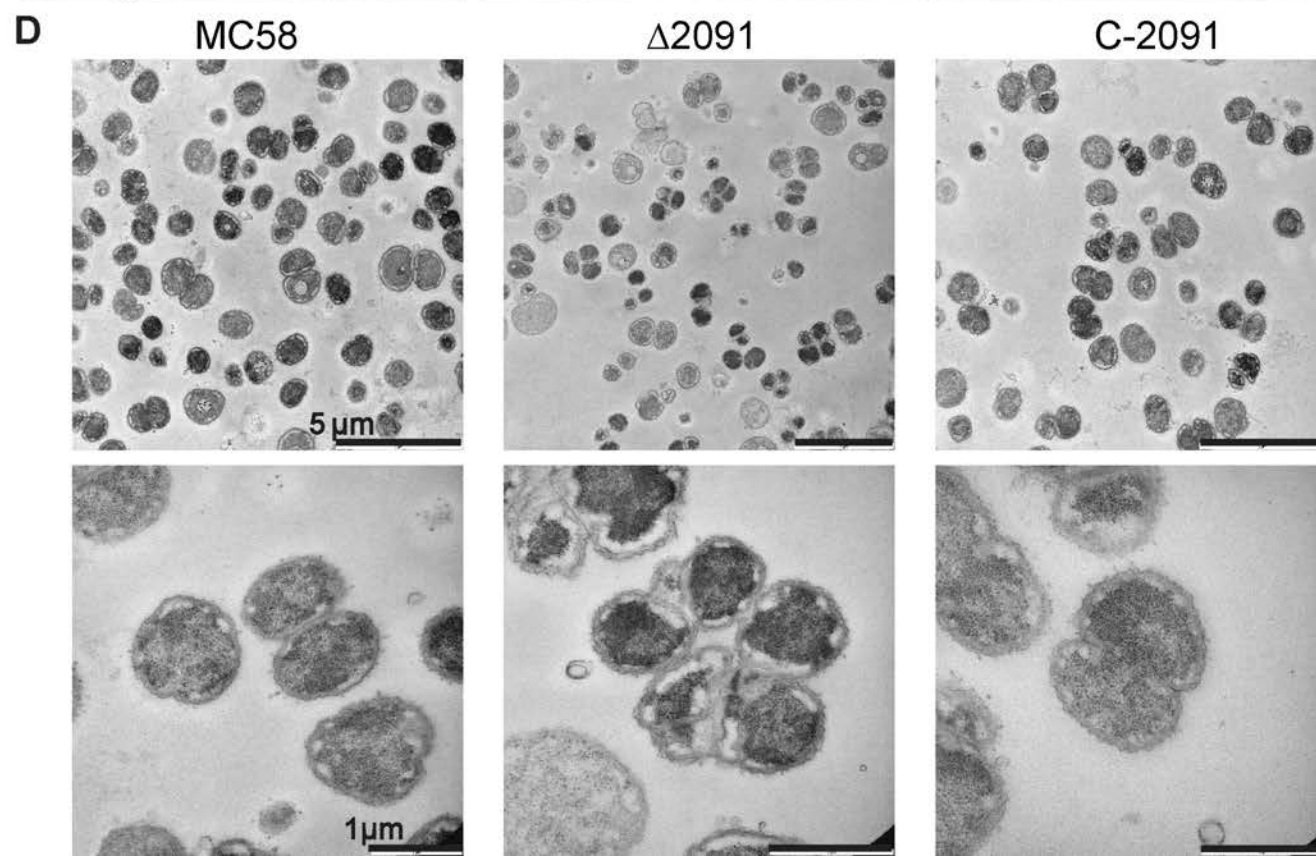
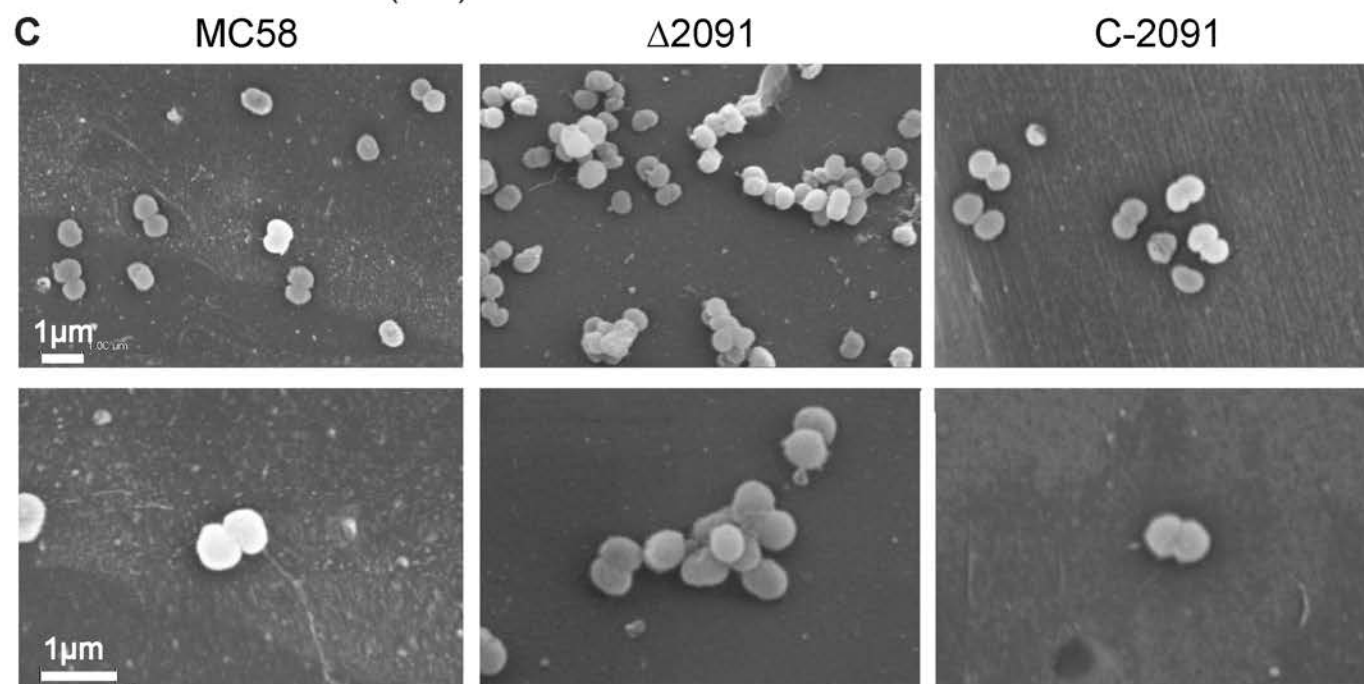
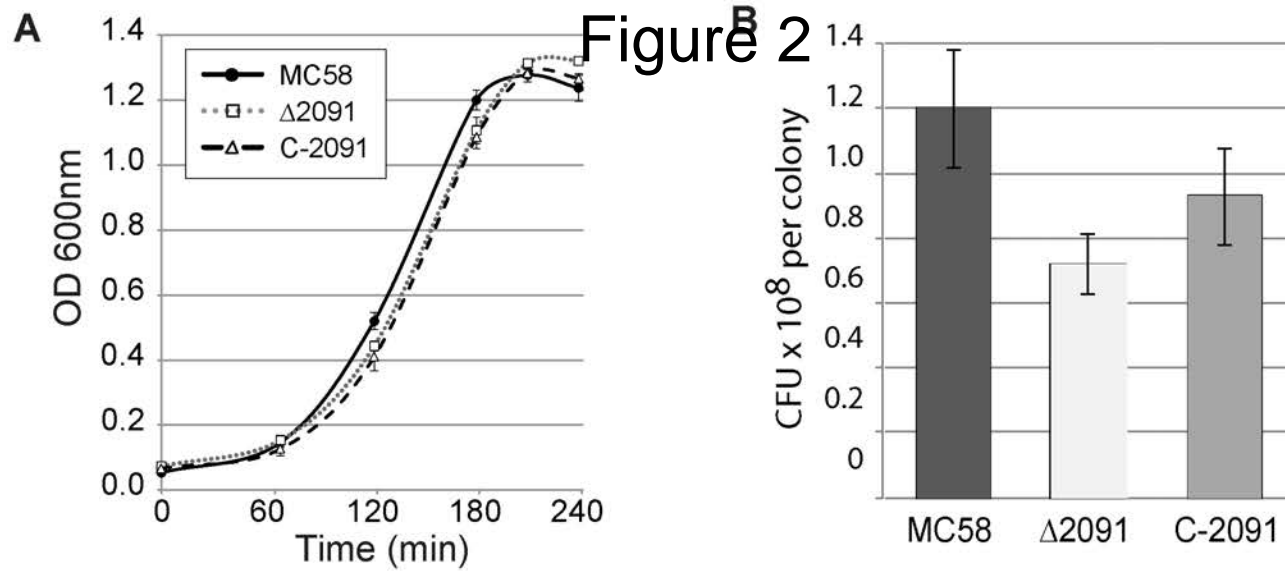
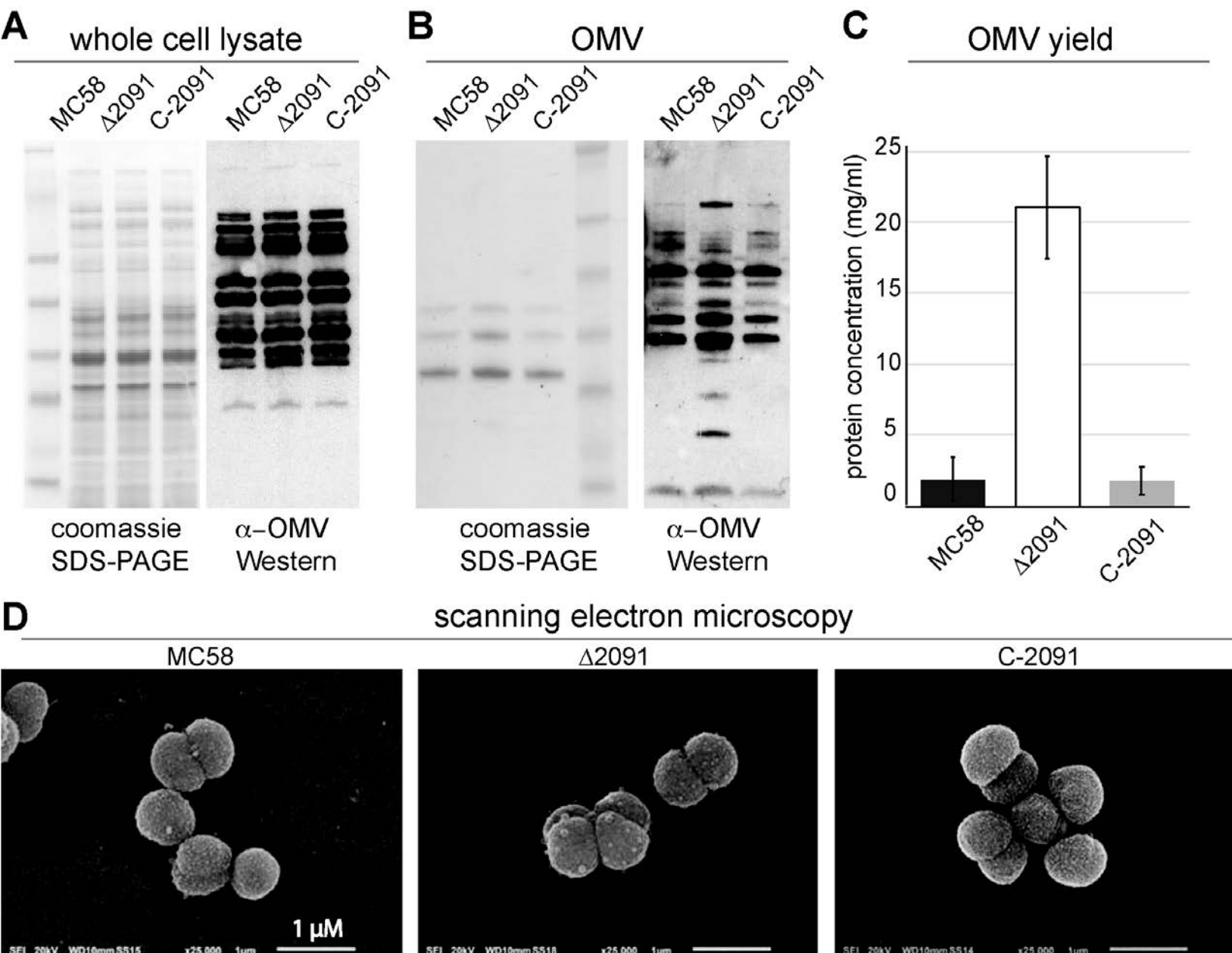


Figure 3



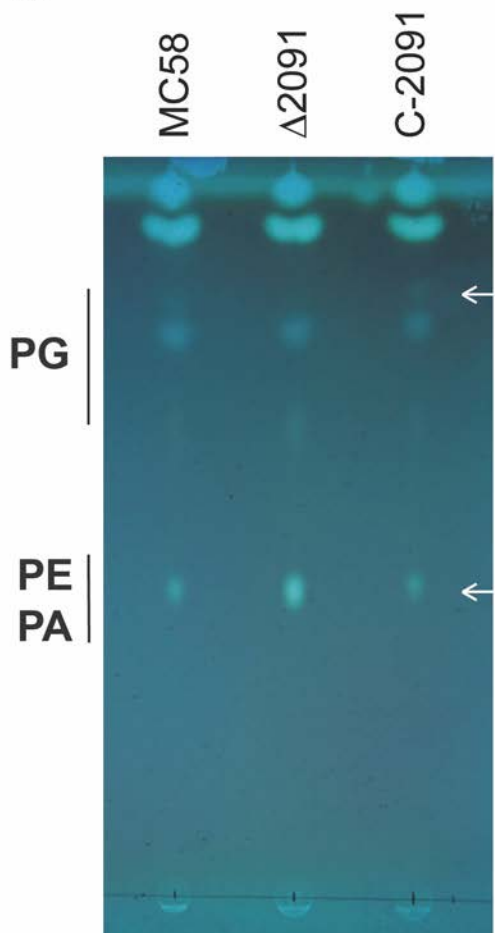
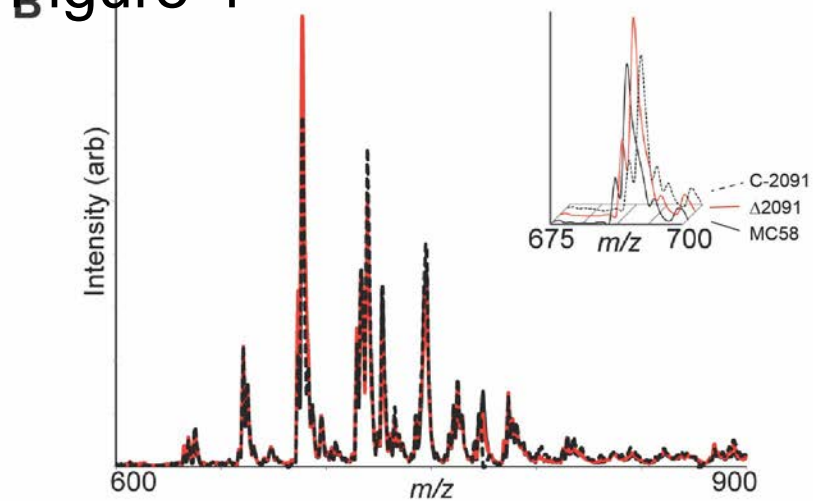
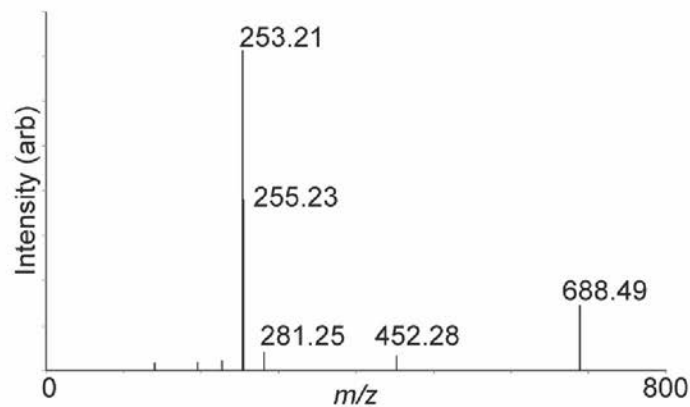
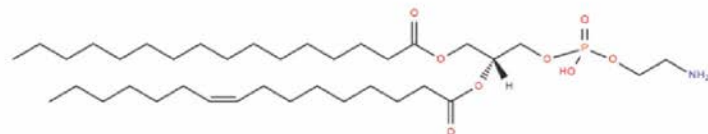
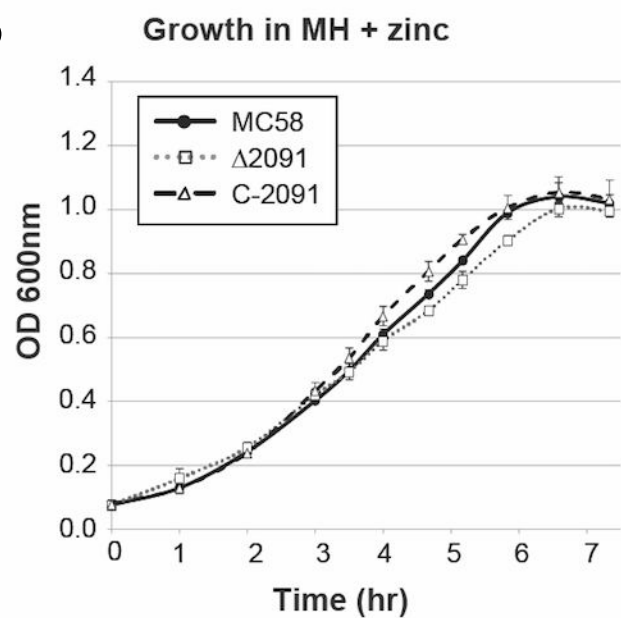
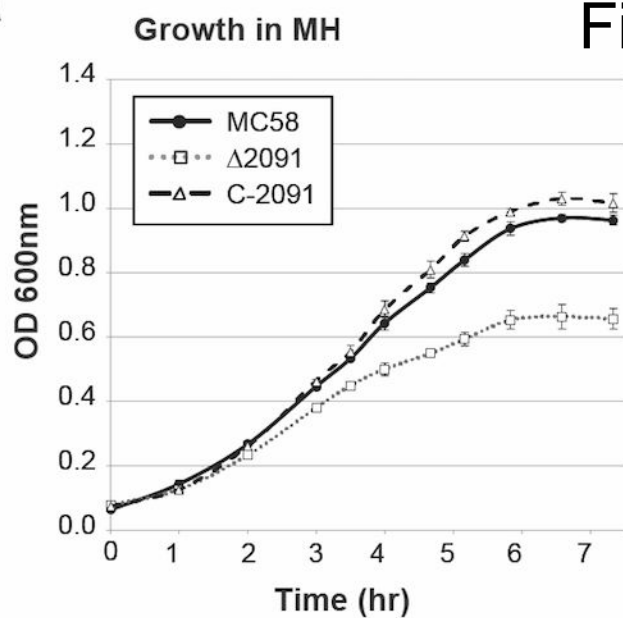
A**Figure 4****C****D**

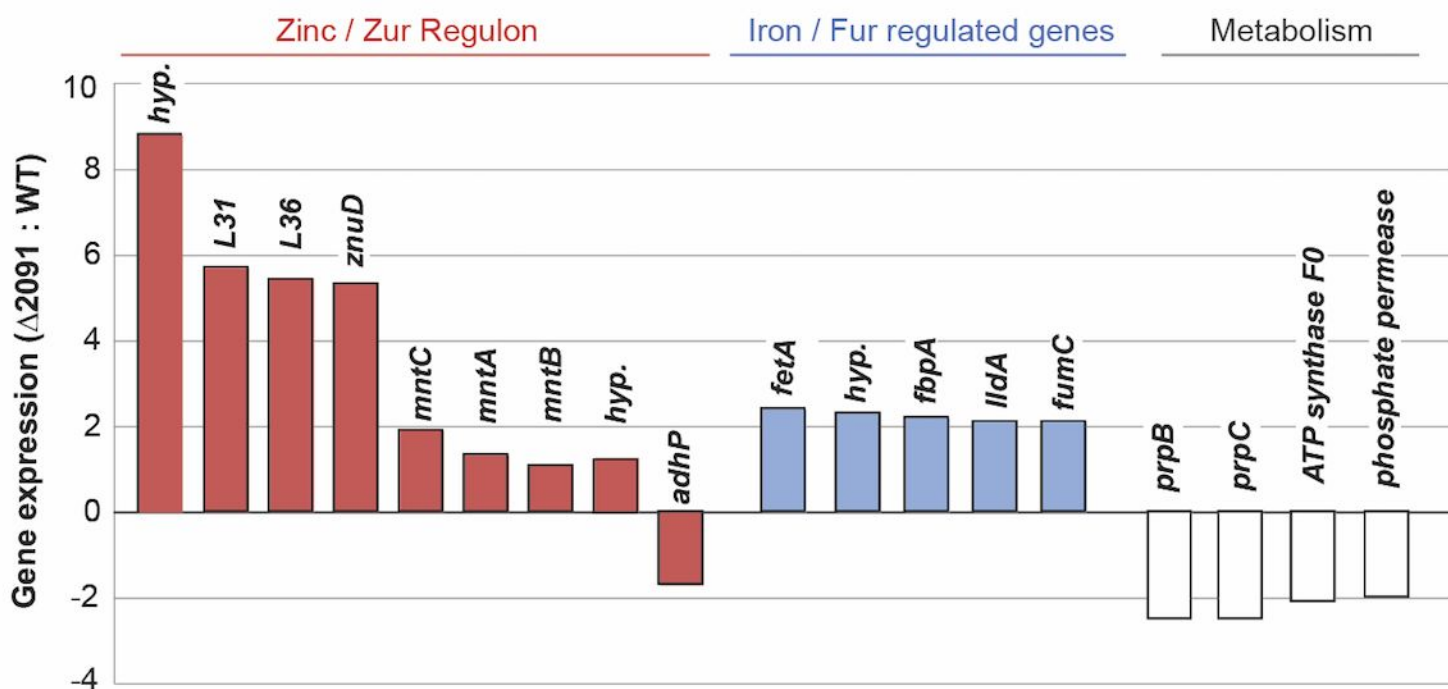
Figure 5

A



B

MC58 $\Delta 2091$ vs. WT transcriptome analysis (DNA microarray)



C

znuD expression (qRT-PCR)

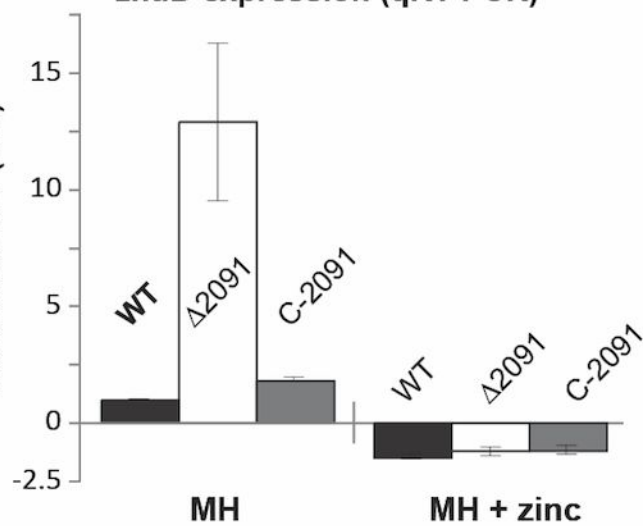


Figure 6

