

1 **Heterogeneity of the group B streptococcal type VII secretion system and influence on**  
2 **colonization of the female genital tract**

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27 **Running title:** GBS T7SS diversity impacts colonization

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52 **SUMMARY:**

53 Type VIIb secretion systems (T7SSb) in Gram-positive bacteria facilitate physiology, interbacterial  
54 competition, and/or virulence via EssC ATPase-driven secretion of small  $\alpha$ -helical proteins and toxins.  
55 Recently, we characterized T7SSb in group B *Streptococcus* (GBS), a leading cause of infection in  
56 newborns and immunocompromised adults. GBS T7SS comprises four subtypes based on variation in  
57 the C-terminus of EssC and the repertoire of downstream effectors; however, the intra-species diversity  
58 of GBS T7SS and impact on GBS-host interactions remains unknown. Bioinformatic analysis indicates  
59 that GBS T7SS loci encode subtype-specific putative effectors, which have low inter-species and inter-  
60 subtype homology but contain similar domains/motifs and therefore may serve similar functions. We  
61 further identify orphaned GBS WXG100 proteins. Functionally, we show that GBS T7SS subtype I and  
62 III strains secrete EsxA *in vitro* and that in subtype I strain CJB111, *esxA1* appears to be differentially  
63 transcribed from the T7SS operon. Further, we observe subtype-specific effects of GBS T7SS on host  
64 colonization, as subtype I but not subtype III T7SS promotes GBS vaginal persistence. Finally, we  
65 observe that T7SS subtypes I and II are the predominant subtypes in clinical GBS isolates. This study  
66 highlights the potential impact of T7SS heterogeneity on host-GBS interactions.

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68 **Keywords:** *Streptococcus agalactiae*, group B *Streptococcus*, GBS, Type VII Secretion System, genetic  
69 diversity, colonization, operon, effectors

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## 78 1 | INTRODUCTION

79 Type VIIb secretion systems (T7SSb) in Gram-positive organisms contribute to interbacterial competition  
80 as well as virulence by damaging host cells and modulating immune responses (Unnikrishnan *et al.*,  
81 2017, Tran *et al.*, 2021, Bowman & Palmer, 2021). While T7SSb machinery varies in sequence and  
82 genomic arrangement across species, common components are cytoplasmic protein EsaB and  
83 membrane proteins EsaA, EssA, EssB, and EssC, an ATPase that powers secretion of substrates across  
84 the bacterial membrane (Bowman & Palmer, 2021). Another distinguished feature of T7SSb is the  
85 presence of canonical T7SS substrate WXG100 protein EsxA (named for its 100 amino acid sequence  
86 and central Trp-X-Gly [WXG] motif), other small  $\alpha$ -helical proteins, and T7SS-associated toxins (Warne  
87 *et al.*, 2016, Bowran & Palmer, 2021). These toxins, which sometimes contain an N-terminal LXG motif,  
88 encode unique C-terminal toxin domains and therefore have biochemically diverse functions. Some T7SS  
89 toxins function in interbacterial competition and are frequently co-transcribed with chaperones that  
90 facilitate their secretion and immunity factors that prevent self-toxicity. These functions have been  
91 described in *Staphylococcus aureus*, *Streptococcus intermedius*, *Bacillus subtilis*, *Enterococcus faecalis*,  
92 and recently *Streptococcus gallolyticus* (Cao *et al.*, 2016, Whitney *et al.*, 2017, Klein *et al.*, 2018, Ulhuq  
93 *et al.*, 2020, Kobayashi, 2021, Chatterjee *et al.*, 2021, Teh *et al.*, 2022, Klein *et al.*, 2022). Interestingly,  
94 in some cases, these toxins also promote virulence and modulate immune responses within the host (Dai  
95 *et al.*, 2017, Ohr *et al.*, 2017, Ulhuq *et al.*, 2020).

96  
97 Despite these common traits, the T7SSb encodes largely unique effectors across species. Extensive  
98 intra-species T7SS diversity has also been characterized in *S. aureus*, *Listeria monocytogenes*, and  
99 *Staphylococcus lugdunensis* T7SSb based on EssC C-terminus variants and downstream effectors  
100 (Warne *et al.*, 2016, Lebeurre *et al.*, 2019, Bowran & Palmer, 2021). These EssC variants are thought to  
101 determine specificity for substrate recognition and secretion. In *S. aureus*, although all four EssC variants  
102 are capable of secreting common T7SS substrate EsxA, the EssC2, EssC3 and EssC4 variants are  
103 incapable of secreting non-cognate substrate EsxC (which is encoded for downstream of *essC1* only)

104 (Jager *et al.*, 2018). We recently showed intra-species diversity in T7SS downstream effectors in group  
105 B *Streptococcus* (GBS) (Spencer *et al.*, 2021b), and hypothesized that variant-specific T7SSb effectors  
106 may promote differing bacterial interactions with the host or other microbes (Spencer & Doran, 2022).

107

108 As an opportunistic pathogen, GBS (or *Streptococcus agalactiae*) asymptotically resides in the  
109 gastrointestinal and/or female genital tract of 25-30% of healthy adults (Wilkinson, 1978, Regan *et al.*,  
110 1991) but can cause severe infections in some individuals, such as pregnant people and newborns, the  
111 elderly, and patients living with cancer or diabetes (Nandyal, 2008, Pimentel *et al.*, 2016, Russell *et al.*,  
112 2017, Patras & Nizet, 2018, van Kassel *et al.*, 2019, Navarro-Torne *et al.*, 2021). Within the female genital  
113 tract, GBS coexists and/or competes with the vaginal microbiota and, therefore, has evolved mechanisms  
114 to survive these encounters while also avoiding immune clearance (Okumura & Nizet, 2014, Vrbanac *et*  
115 *al.*, 2018, Coleman *et al.*, 2021). We showed previously that GBS T7SS subtype I plays a role in virulence,  
116 cytotoxicity, and pore formation via the secreted effector EsxA (Spencer *et al.*, 2021b), and that four  
117 different GBS T7SS subtypes can be delineated based on differing number of copies of *esxA*, a unique  
118 EssC ATPase C-terminus, and a unique repertoire of downstream genes/putative effectors. However,  
119 the full heterogeneity of the GBS T7SS operon and the functions of the putative T7SS effectors in GBS-  
120 host and GBS-microbe interactions has not yet been investigated.

121

122 We hypothesized that GBS T7SS subtypes encode unique downstream effectors that differ from  
123 previously studied T7SS substrates in other species and may modulate GBS fitness within the host. Using  
124 bioinformatic analyses of the T7SS locus across the four subtypes, we found that the GBS T7SS encodes  
125 putative effector proteins with high genetic variability but with similar domains and motifs, suggesting  
126 some conserved functions. Functionally, we confirmed that GBS T7SS subtype I and III isolates secrete  
127 EsxA *in vitro* and we observed variable impacts of *essC* deficiency across T7SS subtypes *in vivo* using  
128 a murine model of female genital tract colonization. This study highlights T7SSb diversity across GBS

129 strains and indicates that the T7SS subtype-specific effector repertoire may differentially modulate host  
130 phenotypes.

131

## 132 **2 | RESULTS**

### 133 **2.1 | Bioinformatic analysis of GBS T7SS subtypes I-IV**

134 In our previous analysis of GBS whole-genome sequences, we identified four GBS T7SS subtypes based  
135 on variation in the EssC C-terminus, T7SS substrate EsxA, and several genes encoded downstream of  
136 *essC* (Spencer *et al.*, 2021b). However, in other species, T7SS loci can encode 10-20 or more genes  
137 following *essC* (Bowran & Palmer, 2021, Lebeurre *et al.*, 2019, Warne *et al.*, 2016). To investigate the  
138 full range of GBS T7SS diversity, we extended bioinformatic analysis to the entire T7SS region encoded  
139 by 80 T7SSb<sup>+</sup> GBS genomes deposited in GenBank. Using CJB111, 2603V/R, CNCTC 10/84, and COH1  
140 as example strains for GBS T7SS subtypes I, II, III, and IV respectively, we analyzed each gene within  
141 the putative T7SS locus for conserved domains [via InterPro (Blum *et al.*, 2021) and Conserved Domain  
142 Architecture Retrieval Tool [CDART] analysis (Geer *et al.*, 2002)], for predicted protein topology [Protter  
143 analysis (Omasits *et al.*, 2014)], and for T7SS-associated motifs [(W/F/L)xG and [Y/F]xxxD/E] (**Supp.**  
144 **Table 1**). As discussed in detail below, we found high conservation of GBS T7SS genomic location and  
145 machinery between subtypes, but variability in the copy number of locus-associated and orphaned *esxA*  
146 genes and heterogeneity in the EssC ATPase and associated putative effector repertoire (**Fig. 1**). We  
147 further assessed homology of the T7SS locus to eight other Gram-positive T7SSb-containing species  
148 (**Supp. Fig. 1** and **Supp. Table 2**).

149

#### 150 *WXG100 proteins and putative GBS T7SS machinery encoding genes*

151 Similar to several other Gram-positive bacteria, most GBS T7SS loci contain putative core component  
152 genes (*esxA*, *esaA*, *essA*, *esaB*, *essB*, and *essC*) followed by putative effector and chaperone-encoding  
153 genes (Warne *et al.*, 2016, Cao *et al.*, 2016, Lai *et al.*, 2017, Taylor *et al.*, 2021, Chatterjee *et al.*, 2021).  
154 In GBS subtypes I-III, the T7SS locus begins with gene(s) encoding the canonical substrate, WXG100

155 protein EsxA, which has been hypothesized to also function as a core machinery component of the  
156 T7SSb (Sundaramoorthy *et al.*, 2008, Kneuper *et al.*, 2014). GBS subtype I and III isolates encode two  
157 copies of *esxA* (Spencer *et al.*, 2021b), while subtype II encodes one copy, and subtype IV strains do not  
158 encode a locus-associated *esxA* (**Fig. 1**). The EsxA sequence is highly conserved across GBS T7SS  
159 subtypes ( $\geq 94\%$  identity) and both EsxA1 and EsxA2 contain the canonical central WYG motif (Spencer  
160 *et al.*, 2021b) (**Supp. Table 2**).

161

162 Downstream of *esxA* (or an WxcM-domain protein encoding gene in subtype IV), putative GBS T7SS  
163 machinery genes include *esaA*, *essA*, *esaB*, *essB*, and ATPase-encoding *essC*. These first four core  
164 components are highly conserved across GBS T7SS subtypes I-IV, with 97-100% identity at the protein  
165 level (**Supp. Table 2**) and appear in the same genomic arrangement as *S. aureus* and *L. monocytogenes*  
166 T7SS loci (Kneuper *et al.*, 2014, Bowran & Palmer, 2021). Despite this similarity in arrangement, T7SS  
167 core machinery sequences exhibit extremely low homology across a panel of Gram-positive species. For  
168 example, GBS *EsaA* is only ~15-20% identical to *EsaA* homologs in *Bacillus*, *Enterococcus*,  
169 *Staphylococcus*, and *Listeria spp.*, and only 30-40% identical to streptococcal *EsaA* homologs in *S.*  
170 *gallolyticus*, *S. intermedius*, and *S. suis*. Similarly, GBS *EssA*, *EsaB*, and *EssB* proteins exhibited only  
171 11-34% identity to homologs in other genera and 21-54% identity to homologs in other streptococci. The  
172 *EssC* ATPase exhibited the highest inter-species identity, but the lowest intra-species protein sequence  
173 identity of the T7SS core proteins. For example, GBS *EssC* variants were 34-48% identical to non-  
174 streptococcal *EssC* proteins, and 56-80% identical to other streptococcal *EssC* proteins (**Supp. Table**  
175 **2**). Within GBS, *EssC* variants shared 89-98% identity, with sequence variation primarily restricted to the  
176 *EssC* C-terminal 225 amino acids (Spencer *et al.*, 2021b). As these variants are associated with unique  
177 effector repertoires, it is likely that a given GBS *EssC* may only export substrates encoded by their  
178 cognate subtype, as has been demonstrated in *S. aureus* (Jager *et al.*, 2018).

179

180 Putative T7SS effectors, chaperones, and immunity factors:

181 As many coding sequences within the hypervariable T7SS effector region are annotated as hypothetical,  
182 we compared these proteins across GBS subtypes using InterPRO, NCBI CDART, and Protter  
183 transmembrane analysis (**Supp. Table 1**). Subtypes I-III encode SACOL2603 T7SS effector proteins  
184 (DUF3130-containing or TIGR04197 family proteins), putative LXG toxins, DUF4176-containing proteins,  
185 and transmembrane proteins. In addition, amidase domain-containing proteins, C-terminal fragments of  
186 EssC, putative toxin fragments, and lipoproteins are also found in some, but not all, GBS T7SS loci (**Fig.**  
187 **1, Supp. Table 1**). Although similar domains and motifs are detected in different GBS T7SS loci, many  
188 proteins differ significantly in sequence homology across subtypes I-III (**Fig. 1B**), suggesting they are  
189 distinct proteins with possibly biochemically diverse activities. Interestingly, subtype IV strains do not  
190 encode locus associated *esxA* or many common T7SS effectors (SACOL2603, DUF4176, etc.) and  
191 encode the shortest GBS T7SS locus, with just 5 hypothetical genes downstream of *essC* that share the  
192 highest homology with the subtype II locus (**Fig. 1B**).

193

194 Within GBS subtypes I-III variable regions, a commonly occurring module consists of two adjacent  
195 WXG100-like proteins (the first containing DUF3130 and the second predicted to contain coil domains),  
196 a putative LXG toxin, and one or two hypothetical protein(s), followed by a DUF4176-domain containing  
197 protein – hereafter termed an “LXG module” (**Fig. 1C**). A similar gene cluster was recently described in  
198 *S. intermedius* where the DUF3130 gene and the adjacent gene encode accessory proteins/chaperones  
199 required for secretion of the LXG toxins TelC and TelD (Klein *et al.*, 2022). Consequently, these  
200 chaperones were named LXG-associated  $\alpha$ -helical proteins (Lap). Genes encoding DUF4176 proteins  
201 were also observed near this LXG module in *S. intermedius*, although a role for these proteins in T7SS  
202 has yet to be shown in any species. A second commonly occurring module in GBS loci consists of a  
203 fragment of the subtype IV *essC*, followed by lipoprotein and  $\alpha\beta$  hydrolase encoding genes (**Fig. 1D**).

204

205 The first two GBS WXG100-like proteins encoded within the LXG module, hereafter termed putative Lap1  
206 and Lap2, are small and  $\alpha$ -helical but lack the canonical central WXG motif; however, Lap1 does display



207 a central FXG motif and a FxxxD/E motif in many GBS strains (**Supp. Fig. 2**). GBS Lap1 proteins exhibit  
208 some homology across subtypes I - III (33-75% identity; **Supp. Fig. 2A**) but generally low homology to  
209 their Gram-positive counterparts, with ~40% identity to *S. gallolyticus*, *S. intermedius*, and *S. suis* (**Supp.**  
210 **Fig. 1**). While neither CDART nor InterPro analyses indicate any putative function for Lap2, these GBS  
211 proteins are predicted to have coil domains and to be  $\alpha$ -helical in nature (**Fig. 2A, Supp. Table 1**), similar  
212 to those described in *S. intermedius* (Klein *et al.*, 2022). GBS Lap2 proteins are not well conserved across  
213 subtypes I-III, with  $\leq 41\%$  identity between any two (**Supp. Fig. 2D-F**). This indicates that specific Lap/LXG  
214 protein pairs may also be required for GBS LXG protein secretion. Although it is currently unknown  
215 whether GBS Laps associate with the putative downstream LXG toxin as chaperones, using Alpha Fold  
216 modeling and predictions, putative Lap1 and Lap2 across GBS T7SS subtypes seem likely to interact  
217 with their cognate LXG protein (**Fig. 2A**) as their counterparts do in *S. intermedius*.

218

219 Similar to the Lap-encoding genes, GBS putative LXG toxins exhibit minimal identity across T7SS  
220 subtypes, with homology concentrating towards the N-terminal LXG domain and sequences diverging in  
221 the C-terminal domains (**Fig. 2B-D**) indicating potential differing biochemical properties. We identified  
222 four putative full-length LXG toxins encoded within GBS T7SS loci: one in subtype I (CJB111,  
223 ID870\_4215), one in subtype II (2603V/R, SAG\_RS07880), and two within subtype III strains (CNCTC  
224 10/84, W903\_RS05440 and C001, GT95\_RS05840) (**Fig. 2B-D; Supp. Fig. 4A**). Upon intra-subtype  
225 comparisons, subtype I and II LXG proteins were highly conserved across strains. In 44 of the 46 subtype  
226 I strains, the LXG protein exhibited 99.8-100% identity, with just one amino acid substitution (A157E) in  
227 the N-terminal LXG domain in some strains. Similarly, the subtype II LXG protein demonstrated 99-100%  
228 identity across isolates, with just two of the 15 strains encoding a nonsense mutation at amino acid 218  
229 (G218\*) resulting in premature truncation after the LXG domain. As subtype III contains only five  
230 completely sequenced strains, further assessment of the two full-length LXG proteins was not possible.

231

232 GBS T7SS LXG toxins are unique from those encoded by other Gram-positive organisms and any limited  
233 homology is restricted to the N-terminal LXG domain within the first ~200 amino acids of the protein. An  
234 exception to this was ~40% homology observed between the CJB111 LXG C-terminus (ID870\_4215) and  
235 that of *S. intermedius* TeID (**Supp. Fig. 1**). As CDART/InterPro failed to identify domains within the C-  
236 terminus of these proteins (**Supp. Table 1**), the functions of GBS LXG proteins remain unknown and  
237 must be determined experimentally. Finally, we observed that the four full-length GBS LXG toxins  
238 commonly encode a longer LXG motif: LxGxAYxxAKxYA (**Fig. 2D**). This full motif was conserved across  
239 other streptococcal LXG proteins from *S. gallolyticus* (TX20005 JGX27\_RS02965) and *S. suis*  
240 (WUSS351 E8M06\_RS09920). Other Gram-positive LXG proteins contain a similar but slightly  
241 abbreviated/modified version of this motif (LxGxAYxxA[K/R]), including those encoded by *S. aureus*  
242 (TspA), *S. intermedius* (TeIC and TeID), *S. lugdunensis* (HKU09-01 SLGD\_RS02660), *S. suis* (WUSS351  
243 E8M06\_RS09940 and RS09970), and *S. gallolyticus* (JGX27\_RS04665, RS08265, RS03950, and  
244 RS11360). This longer and more specific conserved motif may facilitate easier identification of  
245 streptococcal LXG toxins in the future.

246

247 In several species, T7SS toxins are produced in tandem with an immunity factor to prevent self-toxicity.  
248 Downstream of the putative LXG toxins, GBS subtypes I and III encode one hypothetical gene followed  
249 by a DUF4176 gene and GBS subtype II encodes two hypothetical genes (a LXG protein fragment [7875]  
250 and a predicted transmembrane protein encoding gene [7870]) followed by a DUF4176 gene (**Fig. 1**).  
251 We hypothesize that one of these downstream genes may function as an immunity factor for each  
252 subtype's unique LXG protein. In support of this, these hypothetical proteins are highly subtype specific  
253 and have low homology to their counterparts across GBS T7SS subtypes (**Supp. Fig. 4B**) or to any  
254 proteins found in other Gram-positive T7SSb (CJB111 ID870\_4220, **Supp. Fig. 1**). Further, no functional  
255 domains were identified in these proteins by cDART or InterPro but, across subtypes I-III, all were  
256 predicted to contain two to four transmembrane domains (**Supp. Table 1**, see olive green arrows  
257 following teal LXG genes in **Fig. 1**).

258

259 DUF4176 proteins are also commonly encoded in the vicinity of T7SSb LXG proteins but their roles in  
260 T7SSb are unclear. GBS T7SS subtype I, II, and III strains encode two to three loci associated DUF4176  
261 genes, and most encode an orphaned DUF4176 elsewhere in the genome (occasionally fragmented or  
262 annotated as pseudogenes) (**Supp. Fig. 4C**). Of the genes downstream of GBS *essC*, DUF4176  
263 exhibited the most overall homology across subtypes (**Fig. 1B**) as well as to proteins in other T7SSb+  
264 Gram-positive bacteria (30-60% identity across several species; **Supp. Fig. 1**). Further, all GBS  
265 DUF4176 proteins encode a central “FXG” motif (**Supp. Fig. 4D**). Interestingly, the orphaned DUF4176  
266 proteins encoded by subtype II, III, and IV strains were almost identical (94-100% identity; subtype I’s  
267 orphaned DUF4176 is a pseudogene). Of the locus-associated DUF4176 proteins in subtype I strain  
268 CJB111, that encoded by ID870\_4240 exhibited more homology to the orphan DUF4176 proteins (81-  
269 85% identity) compared to its locus-associated DUF4176 proteins (35-48% identity) (**Supp. Fig. 4E**).  
270 Using Alpha Fold, we sought to predict if these putative transmembrane proteins or DUF4176 proteins  
271 might interact with their cognate GBS LXG proteins. In CJB111, the transmembrane protein encoded for  
272 downstream of LXG (by ID870\_4220) yielded the highest confidence score (0.8) indicating there may be  
273 a stable interaction between this protein pair, but this would need to be confirmed experimentally.

274

275 Some T7SS loci encode additional similarly arranged partial LXG modules further downstream, but these  
276 additional modules typically encode a fragmented LXG protein (usually retaining only the central linker  
277 region or the C-terminal toxic domain (Zhang *et al.*, 2012, Chatterjee *et al.*, 2021)). We observed  
278 fragmented LXG proteins in all subtypes, including subtype I strain CJB111 (ID870\_4230, 4245, and  
279 4250) and in subtype III CNCTC 10/84 (W903\_RS05415, RS05395), the majority of which were similarly  
280 followed by genes encoding for hypothetical transmembrane and DUF4176 proteins (**Fig. 1A,C**).  
281 Following these partial LXG modules, the furthest downstream regions of T7SS loci are more variable  
282 but sometimes contain blocks of T7SS genes that can be found across subtypes (**Fig. 1**), indicating that  
283 this region of the putative T7SS locus may be prone to recombination, as recently shown in *S. aureus*

284 (Garrett *et al.*, 2022). This region includes genes encoding hypothetical and transmembrane proteins,  
285 CHAP domain containing proteins, FtsK domain containing proteins (C-terminal *essC* fragments),  
286 lipoproteins, and  $\alpha\beta$  hydrolases (**Supp. Table 1, Fig. 1A,D**).

287

### 288 Genomic location and genes flanking GBS T7SSb

289 In all GBS isolates examined, T7SS genes are located between carbamoyl phosphate synthase genes  
290 (*carB*; CJB111 ID870\_4155) and the *LtdRS* two component system (Deng *et al.*, 2018, Faralla *et al.*,  
291 2014) (**Fig. 1**). Two highly conserved genes downstream of *carB* encode for a 107 amino acid  
292 hypothetical protein with no predicted function or domains (CJB111 ID870\_4160) and a *WxcM* domain-  
293 containing protein predicted to contain  $\alpha\beta$  hydrolase and/or lipase-like domains (CJB111 ID870\_4165).  
294 While this *WxcM* protein is prematurely truncated in subtype IV strains, it maintains >97% identity to the  
295 subtype I - III homologs within the first 60% of the amino acid sequence. ID870\_4160 homologs were not  
296 found within other T7SSb containing Gram-positive species, and the GBS *WxcM* domain containing  
297 protein exhibited minimal identity to homologs in *E. faecalis*, *S. intermedius*, and *Streptococcus suis*,  
298 indicating that these genes are fairly specific to GBS.

299

300 To confirm the boundaries of the T7SS operon in subtype I strain CJB111, we assessed which genes are  
301 co-transcribed within this genomic region by performing RT-PCR using primers spanning each T7SS  
302 gene junction. Primers were confirmed using gDNA and non-specific amplification was assessed using  
303 no-RT controls. We observed bands for almost every cDNA RT-PCR reaction, indicating that most genes  
304 are capable of being co-transcribed; however, *esxA1* consistently appears to be transcribed separately  
305 from the rest of the T7SS operon, indicated by a lack of amplicon from cDNA using primers spanning the  
306 *esxA1* - *esxA2* junction (**Supp. Fig. 5**). Bands were observed between *esxA2* and machinery genes of  
307 the T7SS indicating they are transcribed together, as has been shown in *S. aureus* and *S. gallolyticus*  
308 (Kneuper *et al.*, 2014, Taylor *et al.*, 2021). Further, we observed bands for all gene junctions until the  
309 junction spanning the *LtdS* operon for reactions including cDNA as template (but no bands were detected

310 in the no-RT controls), indicating that the genes within the T7SS locus from *esxA2* through the gene  
311 upstream of *ItdS* are capable of being co-transcribed. Although, as RT-PCR can detect low levels of  
312 transcript, more sensitive methods should be used in the future to assess potential differential regulation  
313 within the T7SS locus.

314

## 315 **2.2 | Intra-subtype diversity of the GBS T7SS**

316 While the above bioinformatic analysis assessed diversity of the GBS T7SS between subtypes and  
317 between Gram-positive species, we also observed extensive intra-subtype diversity. Within subtype I  
318 strains, the region from *carB* through the 5 genes downstream of *essC* (CJB111 ID870\_4225) was highly  
319 conserved (~99% nucleotide identity). However, heterogeneity in the form of insertion or loss of gene  
320 blocks occurred further downstream (**Fig. 3A**). For example, 12 out of 46 subtype I strains (representative  
321 strain CJB111) encode 18 genes downstream of *essC*. In other subtype I strains (12/46, representative  
322 strain A909), the T7SS locus is truncated to just 11 genes downstream of *essC* (ID870\_4245 through  
323 ID870\_4285 have been lost). Additional subtype I T7SS loci (10/46 strains) are almost identical to A909  
324 but have a 73 bp deletion after the first DUF4176 encoding gene (which deletes a putative terminator)  
325 and a 69 bp deletion in the intergenic region before A909 gene SAK\_RS05570. Finally, compared to the  
326 A909 T7SS arrangement, a few strains (representative strain Sag153) have undergone further reductive  
327 evolution, losing additional genes in the T7SS locus.

328

329 While most GBS T7SS subtype II strains encode the same set of genes downstream of *essC*, diversity  
330 within subtype II is most commonly based on nonsense and frameshift mutations in *essC* and *esaA* (**Fig.**  
331 **3B**). Of the 15 closed GBS genomes that encode GBS T7SS subtype II, 13 encode a putatively  
332 truncated/multiple-CDS *EssC*. The majority of these are due to a slip-strand mutation within a  
333 homopolymeric G<sub>n</sub> tract in *essC*, resulting in the loss of either one or two nucleotides, and frameshift and  
334 truncation of *EssC* to 166 or 173 amino acids, respectively. In some subtype II strains, an additional  
335 nonsense mutation occurs before this homopolymeric tract resulting in an *EssC* truncation to 56 amino

336 acids (C166T Gln56\*). However, NCBI ORF Finder predicts a second EssC ORF may be possible in  
337 these subtype II strains, resulting in a 1291 amino acid protein (of the usual 1469 amino acid protein),  
338 which may still allow T7SSb activity. A second common area for mutation within subtype II strains is *esaA*  
339 (in 5 of 15 subtype II strains, including strain 515), also potentially due to slippage within a homopolymeric  
340  $A_n$  tract, resulting in deletion of one nucleotide, frameshift, and truncation of *EsaA* at 465 amino acids  
341 (full length *EsaA* is 1005 amino acids). Similar to *EssC* truncation, NCBI ORF Finder predicts a second  
342 *esaA* ORF may be encoded; therefore, this mutation may also not necessarily inactivate the T7SS.  
343 Despite the T7SS machinery being largely conserved across GBS subtypes (including these  
344 homopolymeric tracts), these mutations within T7SS machinery genes rarely occur in GBS subtype I, III,  
345 and IV loci.

346

347 Subtype III is the least prevalent subtype of GBS T7SS and encompasses immense diversity, with four  
348 unique versions of the downstream T7SS region existing in just five isolates (**Fig. 3C**). Similar to subtype  
349 I, diversity of subtype III is due to the presence/absence of downstream genes between *essC* and *ltdS*,  
350 with some subtype III strains encoding different LXG modules (e.g., strains CNCTC 10/84 and C001  
351 encode different full length LXG proteins). Because these strains are unique in their downstream T7SS  
352 gene arrangement, each strain's downstream repertoire has been independently analyzed in **Supp.**  
353 **Table 1**. Subtype IV strains are also rarer, which impairs intra-subtype comparisons; however, two of 14  
354 subtype IV strains encode similar slip-strand mutations in *essC* as seen in subtype II strains (**Fig. 3D**).

355

### 356 **2.3 | GBS T7SS orphaned WXG100 modules**

357 In addition to locus associated T7SS genes, many GBS strains encode for “orphaned” WXG100 proteins  
358 elsewhere in the genome. Comparative genomic analysis of example strains from each T7SS subtype  
359 revealed two orphaned WXG100 groups (here termed Orphaned Modules 1 and 2) based on genomic  
360 location/neighboring genes and WXG100 protein sequence alignment (**Fig. 4A** and **Supp. Fig. 6**).  
361 Module 1 WXG100 orphan proteins are encoded in 64 of the 80 T7SS<sup>+</sup> GenBank isolate cohort (**Supp.**

362 **Table 3**), often upstream of a MutR family transcriptional regulator (**Fig. 4A**) and are well conserved,  
363 exhibiting 97-100% identity across strains (**Supp. Fig. 6A-B**). Module I WXG100 genes are often  
364 encoded either in proximity to a RelE/ParE family toxin protein and plasmid recombinase genes in some  
365 strains (as in NEM316) or downstream of a DEAD/DEAH box helicase gene in others (CJB111 and 515).  
366 Furthermore, Module 1 WXG100 orphan genes are commonly followed by a cluster of conserved  
367 hypothetical genes. In a subset of subtype IV strains, the orphan WXG100 gene is encoded downstream  
368 of *rpsI* (sometimes with restriction-modification and abortive infection system genes immediately  
369 preceding the WXG100 gene) and this “Module 1b” contains a ~15kb insertion (encoding for tyrosine  
370 recombinases, sigma factors, and *tetM*) following the second hypothetical gene of the cluster downstream  
371 of the WXG100 gene.

372

373 Module 2 WXG100 orphans are less common (encoded in just 45 of the 80 T7SS<sup>+</sup> GenBank isolates)  
374 and are only observed in strains encoding the Module 1 WXG100 orphan (**Fig 4B, Supp. Table 3**). While  
375 the genomic region surrounding Module 2 orphans are more variable, this WXG100 gene (ID870\_10565  
376 in subtype I strain CJB111) is consistently encoded upstream of at least one DUF1310 gene and a few  
377 genes upstream of a Type II toxin-antitoxin system RelB/DinJ family antitoxin fragment (**Fig. 4A**). Module  
378 2 WXG100 orphans are well-conserved at the protein sequence level (**Supp. Fig. 6C**) and orphaned  
379 WXG100 proteins are more similar to each other compared to locus associated *EsxA* in subtype I strain  
380 CJB111 (**Fig. 4C**). However, because orphaned WXG100 proteins are distinct from WXG100 proteins  
381 found in other species, we have continued the *esxA* nomenclature and have annotated these genes as  
382 *esxA3* (Module 1 orphan) and *esxA4* (Module 2 orphan). Integrase/recombinase genes and toxin-  
383 antitoxin system genes are often associated with phage (Harms *et al.*, 2018), which are known to mediate  
384 horizontal gene transfer and to modulate bacterial fitness and virulence (Borodovich *et al.*, 2022, Basler  
385 *et al.*, 2012, Hobbs & Mattick, 1993); therefore, we investigated whether GBS T7SS genes may be  
386 encoded within prophage. Interestingly, neither T7SS loci nor orphaned WXG100 proteins appear within  
387 prophage regions (**Fig. 4D**).

388

#### 389 **2.4 | EsxA secretion across T7SS subtypes**

390 We next sought to determine GBS T7SS activity across subtypes by measuring EsxA secretion *in vitro*  
391 as previously described (Spencer *et al.*, 2021b). We observed EssC-dependent secretion in subtype I  
392 strain CJB111 and subtype III strain CNCTC 10/84 but could not detect EsxA in the supernatant or cell  
393 pellet of subtype II strain 2603V/R (**Fig. 5**). This lack of detection was not due to inability of the anti-EsxA  
394 antibody (raised against CJB111 EsxA1) to bind the 95% identical subtype II EsxA2, since it was cross-  
395 reactive against subtype II strain NEM316 EsxA in both the supernatant and cell pellet. These results  
396 indicate that T7SS may be repressed or that EsxA may be degraded or unstable in the 2603V/R  
397 background. Alternatively, T7SS subtype II strains may require different inducing conditions to express  
398 and secrete EsxA *in vitro*. Subtype IV strain COH1 served as a negative control in these studies as it  
399 does not encode any EsxA homolog.

400

#### 401 **2.5 | Impact of GBS T7SS subtype on vaginal colonization**

402 Secretion systems are often induced by stress, competing bacteria, and/or host pressures. We have  
403 previously shown that GBS T7SS promotes systemic infection but hypothesized that T7SS may also play  
404 a role during vaginal colonization and ascending infection, niches in which GBS must encounter host  
405 immune responses and compete with the native vaginal microbiota. As each GBS T7SS subtype  
406 expresses unique putative effectors, we further hypothesized that subtypes may have varying impacts  
407 on GBS interaction with the host. As subtypes I and III T7SS are active *in vitro* (as seen by EsxA secretion  
408 in **Fig. 5**), we utilized our *essC* deletion mutants in subtype I (CJB111 and A909) and III (CNCTC 10/84)  
409 to assess the role of T7SS in vaginal persistence and ascending female genital tract infection in a murine  
410 model of colonization. CD1 mice were vaginally inoculated with parental or  $\Delta$ *essC* mutant strains and  
411 GBS persistence over time was assessed by vaginal lavage or swab and tissue burdens were evaluated  
412 at the experimental endpoint. Loss of *essC* did not impact subtype I GBS persistence in the vaginal lumen  
413 as indicated by percentages of mice vaginally colonized between parental and  $\Delta$ *essC* colonized groups



414 in CJB111 (**Fig. 6A**) and A909 (**Supp. Fig. 7A**) over time. However, we recovered a marked increase of  
415 parental CJB111 from vaginal, cervical, and uterine tissues at the experimental endpoint compared to  
416 the CJB111 $\Delta$ essC mutant (**Fig. 6B-D**), indicating that the subtype I T7SS promotes tissue invasion and  
417 ascending infection in the female genital tract. Similar trends were observed in the A909 background  
418 (**Supp. Fig. 7B-D**). Interestingly, for GBS T7SS subtype III, the CNCTC 10/84 $\Delta$ essC mutant persisted  
419 better than the parental CNCTC 10/84 strain in the murine vaginal lumen (**Fig. 6E**). At the experimental  
420 endpoint, the CNCTC 10/84 $\Delta$ essC mutant exhibited higher tissue burdens in the vagina, cervix, and  
421 uterus compared to parental CNCTC 10/84-colonized mice (**Fig. 6F-H**). Together these data indicate that  
422 GBS T7SS subtype I may provide an advantage to GBS in colonization of the female genital tract while  
423 T7SS subtype III may be disadvantageous in this environment.

424

## 425 **2.6 | Multiplex PCR to T7SS type GBS clinical isolates**

426 Given this striking subtype-specific phenotype, we sought to validate whether the T7SS subtype  
427 distribution observed in our GenBank GBS isolates is representative of recent clinical GBS isolates using  
428 collections of vaginal isolates from pregnant women and from diabetic foot ulcers that our laboratory has  
429 characterized previously (Burcham *et al.*, 2019, Keogh *et al.*, 2022). To determine GBS T7SS subtypes,  
430 we developed a multiplex PCR assay using primers against subtype specific transmembrane encoding  
431 genes, the amplicons of which could be distinguished by size (**Fig. 7A; Supp. Table 4**). Within the clinical  
432 isolate cohorts, most encode T7SS subtype I and subtype II (24/64, 37.5% and 33/64, 51.6%,  
433 respectively) (**Fig. 7B-C**). A small percentage of isolates encode subtype IV (7/64, 10.9%); however, we  
434 did not identify any subtype III in our clinical isolate cohorts, corroborating its low prevalence in whole  
435 genome sequences available on GenBank. We compiled relevant information on these strains as well as  
436 those from GenBank including sequence type, serotype, T7SS locus subtype, prophage cluster, and  
437 orphan modules encoded to determine if any associations exist between these metrics using Fisher's  
438 exact tests (**Supp. Tables 3 and 5**). Similar to a previous report on GBS T7SS genomic arrangement  
439 (Zhou *et al.*, 2022) we found that GBS T7SS subtype is associated with sequence-type ( $p < 2.2e-16$ ) and

440 is also associated with GBS serotype ( $p < 2.158E-13$ ). Within our clinical isolate cohorts, trends were  
441 particularly observed between T7SS subtype I and serotype Ib, T7SS subtype II and serotypes Ia and II,  
442 and T7SS subtype IV with serotype III sequence type 17 (ST-17) (**Supp. Tables 3 and 5**). While we also  
443 observed significant associations between prophage cluster, orphaned modules, and T7SS subtype in  
444 our vaginal isolate cohort, it is possible these associations may be artifacts of T7SS association with  
445 sequence type.

446

447 To achieve a larger dataset and to ensure that our isolate banks are not biased due to collection, we  
448 performed *in silico* T7SS subtyping of WGS GBS sequences available in contigs. Of 1342 sequences,  
449 1130 were able to be typed using BLAST of the 225 C-terminal amino acids of EssC and the subtype  
450 specific gene used for physical typing of isolates in **Supp. Table 4** and **Fig. 7**. Strains were only  
451 considered typable if positive for both a specific subtype's EssC and downstream subtype-specific gene.  
452 Upon screening of these WGS sequence contigs of GBS isolates with typable T7SS, 424 were subtype  
453 I (37.5%), 496 were subtype II (43.9%), 43 were subtype III (3.8%), and 167 were subtype IV (14.8%)  
454 (**Fig. 7D**). As these sequences are in contigs, T7SS typing was not possible for all isolates (sequences  
455 may have gaps within the genomic region containing the T7SS locus or, occasionally, some strains do  
456 not encode a T7SS). Sequence analysis of these additional strains further confirmed that subtypes I and  
457 II are the most prevalent and that extensive diversity exists within GBS T7SS.

458

### 459 **3 | DISCUSSION**

460 This study highlights the heterogeneity of GBS T7SS subtypes, which could affect GBS interactions in  
461 the host. One purpose of this work was to compare GBS T7SS to T7SS loci encoded by other Gram-  
462 positive species. Despite having a similar arrangement of T7SS genes, GBS core proteins and putative  
463 effectors were largely unique compared to T7SS proteins described in eight other Gram-positive  
464 organisms. The secreted substrate/putative core protein EsxA and ATPase EssC were the most  
465 conserved T7SS components, consistent with previous observations that these two proteins are general

466 features of T7SS (Pallen, 2002). Despite low sequence homology, GBS T7SS components are commonly  
467 found within T7SSb in other species, including WXG-like DUF3130 containing proteins, putative LXG  
468 toxins, putative immunity factors and chaperones, CHAP domain containing putative lysins,  
469 transmembrane proteins, lipoproteins, fragments of *essC*, and DUF4176 proteins.

470  
471 Within GBS, T7SS loci reside in a common genomic location, contain homologous machinery, subtype-  
472 specific putative effectors/immunity factors/chaperones downstream of *essC*, and, with the exception of  
473 subtype IV, encode at least one copy of *esxA* upstream of T7SS machinery. However, we also observed  
474 extensive intra-subtype GBS T7SS heterogeneity. Within subtype I specifically, strains encoding the  
475 A909 type locus lack approximately eight genes compared to the subtype I CJB111 locus; yet A909  
476 maintains functional *EsxA* secretion (**Fig. 3A, Fig. 5**). In vaginal colonization experiments, parental  
477 CJB111 and A909 exhibited higher tissue invasion compared to  $\Delta$ *essC* mutants (**Fig. 6B-D, Supp. Fig.**  
478 **7B-D**). It is unclear whether this eight gene cluster contributes to T7SS activity and future work will  
479 investigate its possible contributions to GBS-host interactions. GBS T7SS subtype II loci differ from each  
480 other based on truncations of T7SS machinery, particularly within *essC* due to either nonsense or slip  
481 strand mutations. However, these putative truncated *EssC* proteins may retain function as subtype II  
482 strain NEM316 is capable of *EsxA* secretion (**Fig. 5**). It is possible that a second *EssC* ORF is transcribed  
483 and translated, and whether this second CDS works with *EssC* CDS1 or is functional on its own would  
484 be interesting to investigate.

485  
486 Across species and strains, T7SSb proteins also commonly contain T7-associated motifs, including the  
487 [W/F]XG motif (found within *EsxA* and other WXG100-like proteins), the LXG motif (found within toxins  
488 that promote interbacterial competition), and the YxxxD/E motif. The YxxxD/E motif is thought to form  
489 one part of a bipartite signal required for homing of a substrate to the machinery for export in  
490 mycobacterial T7SSa and Firmicute T7SSb (Champion *et al.*, 2006, Daleke *et al.*, 2012, Anderson *et al.*,  
491 2013, Sysoeva *et al.*, 2014). Permutations of these motifs also exist, as a C-terminal FxxxD/E motif was

492 found within GBS Lap1 and “FXG” motifs were identified in GBS Lap1 and DUF4176 proteins. While the  
493 presence of a given motif does not necessitate a role in T7SS, we observed that many machinery and  
494 putative effector proteins encode YxxxD/E motifs, which may be important for substrate recognition.  
495 Interestingly, unlike mycobacterial and staphylococcal EsxA homologs, GBS EsxA1 and EsxA2 do not  
496 encode a C-terminal YxxxD/E motif. Previously, Poulsen *et al.* showed that some WXG100 proteins may  
497 encode a less specific C-terminal motif, which might direct these substrates to the T7SS machinery:  
498 HxxxD/ExhxH (in which the H and h, stand for high and low conservation of hydrophobic residues,  
499 respectively (Poulsen *et al.*, 2014)). Both GBS EsxA1 and EsxA2 C-terminal sequences include an  
500 HxxxD/ExhxH motif, as does the *S. gallolyticus* EsxA (Taylor *et al.*, 2021); however, further studies  
501 are needed to determine whether this C-terminal motif is required for GBS EsxA secretion.

502

503 LXG modules (an LXG gene preceded by two small alpha helical protein genes and followed by one or  
504 two hypothetical proteins(s) and a DUF4176 gene) are widespread in T7SS<sup>b+</sup> Gram-positive species. In  
505 *S. aureus*, two WXG-like proteins named EsxC and EsxD can heterodimerize with EsxA and EsxB,  
506 respectively, and substrate secretion is interdependent as deletion of these WXG-like genes impacts  
507 EsxA/B export (Anderson *et al.*, 2013). In *S. intermedius*, WXG-like genes adjacent to *essC* facilitate  
508 secretion of a downstream LXG toxin (Klein *et al.*, 2022). We have observed that a double mutant in both  
509 these WXG-like genes in GBS subtype I did not affect EsxA secretion (data not shown); therefore, we  
510 hypothesize that GBS WXG-like proteins may function in LXG protein secretion, similar to *S. intermedius*.  
511 Transmembrane protein and DUF4176 protein encoding genes, commonly observed in the vicinity of  
512 T7SS loci and toxins, appeared downstream of the LXG genes in GBS subtypes I-III. In *S. aureus*,  
513 cognate immunity factor *esaG* (DUF600) is encoded downstream of nuclease *EsaD* (Cao *et al.*, 2016)  
514 and in *S. intermedius*, Tel immunity proteins are encoded either adjacent to (in the case of TelB, TelC,  
515 TelD) or one gene separated (in the case of TelA) from the LXG toxin (Whitney *et al.*, 2017, Klein *et al.*,  
516 2022). We therefore expect that one of these downstream GBS genes encodes a subtype specific  
517 immunity factor and determining these LXG toxin-immunity factor pairs is the subject of our future work.

518

519 In GBS T7SS subtypes I-III, LXG proteins are typically encoded by the third gene downstream of *essC*  
520 and are associated with unique upstream genes (putative chaperones) and unique downstream genes  
521 (putative immunity factors). These LXG proteins have highly conserved  $\alpha$ -helical N-termini within and  
522 across GBS subtypes, which are structurally similar to the WXG100 proteins (as originally described by  
523 Aravind *et al.*, 2011), but have unique, globular C-terminal (and putatively toxic) domains (**Supp. Fig.**  
524 **4A**). Our bioinformatic analysis failed to identify domains/predicted functions for these C-terminal  
525 putatively toxic regions; thus, our future work will determine the putatively toxic activities of these proteins  
526 experimentally. Because the toxic activity and chaperone/accessory functions for these proteins have not  
527 yet been demonstrated experimentally, we have named these GBS gene products conservatively as  
528 LXG-domain containing proteins and the WXG100-like proteins as putative LXG-associated proteins.  
529 Many T7SS<sup>+</sup> Gram positive species also encode orphaned LXG proteins elsewhere in the genome. For  
530 example, LXG toxin TspA is secreted by the T7SSb in *S. aureus* but is not encoded in the T7SS locus  
531 (Ulhuq *et al.*, 2020). Further, *L. monocytogenes*, *B. subtilis*, *S. gallolyticus*, *S. intermedius* and *S. suis*  
532 encode multiple full-length LXG toxins, not all of which are associated with the T7SS locus (Bowran &  
533 Palmer, 2021, Whitney *et al.*, 2017, Kobayashi, 2021, Teh *et al.*, 2022, Liang *et al.*, 2022). While we did  
534 not identify any orphaned full-length GBS LXG proteins based on presence of an LXG motif with the first  
535 100 amino acids of the protein, N-terminal homology to other proteins, or by searching specifically in  
536 genomic regions that encode orphaned DUF4176 or WXG100 proteins, it is possible that orphaned C-  
537 terminal toxin fragments may exist in GBS strains.

538

539 GBS subtype I and II were the most commonly identified T7SS subtypes based on multiplex PCR typing  
540 of cohorts of clinical isolates or by *in silico* typing of whole genome sequences and contigs. Subtype IV  
541 strains, the next most common subtype, do not encode many common T7SS components, such as locus  
542 associated WXG100, DUF3130/SACOL2603, or full-length LXG or DUF4176 proteins. While the genes  
543 encoded between the COH1 subtype IV *essC* and *ltdS* are all annotated as hypothetical, interestingly,

544 some of these COH1 genes (the *essC*-lipoprotein-hydrolase module depicted in **Fig. 1D**) also appear in  
545 subtype I, II, and III loci; see **Fig 1** and **Fig. 3**), indicating that homologous recombination may occur in  
546 T7SS loci as has been reported in other species. While the biological purpose of the GBS T7SS subtype  
547 IV is currently unclear, we have observed in subtype IV strain COH1 background that T7SS genes are  
548 modulated in certain conditions, such as in a *cas9* deletion mutant (Spencer *et al.*, 2019), or during  
549 incubation with mucins (Burcham *et al.*, 2022b)], thus indicating that the COH1 T7SS may play a role  
550 during stress. As most subtype IV strains identified are from the hypervirulent ST-17, serotype III lineage,  
551 which is associated with neonatal meningitis, they may have lost some T7SS components due to  
552 acquisition of additional virulence factors. Lastly, while subtype I T7SS promoted GBS colonization of  
553 genital tract tissues, the T7SS encoded by subtype III strain CNCTC 10/84 appeared to be detrimental  
554 for vaginal colonization. We propose that these subtype specific phenotypes are likely due to subtype  
555 specific effectors encoded by each strain. Our future work will investigate whether this detrimental role  
556 for the CNCTC 10/84 T7SS is due to modulation of the vaginal microbiota or due to modulation of immune  
557 responses, resulting in parental CNCTC 10/84 clearance. This disadvantage of the CNCTC 10/84 T7SS  
558 in the vaginal tract may account for subtype III T7SS's rarity across GBS isolates. Indeed, subtype III  
559 constitutes just ~4% of T7SSb<sup>+</sup> GBS contig sequences ( $n=1130$ ).

560

561 As is common for many virulence factors, secretion systems in bacteria often are heavily regulated and  
562 may be minimally expressed *in vitro*. We have similarly observed that the GBS T7SS is lowly expressed  
563 *in vitro*, requiring concentration of supernatant to detect secreted EsxA (**Fig 5**). Yet, we observe striking  
564 GBS T7SS-dependent phenotypes in cell infections or in animal models of infection. Notably, it has been  
565 shown that staphylococcal T7SS genes were upregulated in the female genital tract (Deng *et al.*, 2019)  
566 and TN-seq studies in *E. faecalis* OG1RF revealed that T7SS Tn-mutants were underrepresented in the  
567 female genital tract (Alhajjar *et al.*, 2020, Burcham *et al.*, 2022a), further indicating that this system may  
568 be induced in host environments. A common regulatory mechanism for bacterial virulence factors such  
569 as adhesins, pili, and capsule is phase variation, which can involve transcriptional slippage resulting in

570 expression of a given factor in some environments and under certain conditions (Phillips *et al.*, 2019).  
571 For example, transcriptional slippage can occur within genes encoding pneumococcal capsule modifying  
572 enzymes, facilitating pneumococcal evasion of vaccine elicited antibodies (van Selm *et al.*, 2003, Rajam  
573 *et al.*, 2007, Spencer *et al.*, 2017). We observed many homopolymeric tracts within the GBS T7SS, in  
574 line with studies of potential slip-strand regulation in GBS (Janulczyk *et al.*, 2010). The most common  
575 T7SS slip-strand mutation occurred within *essC* in subtypes II and IV, due to a homopolymeric G<sub>n</sub> tract.  
576 This tract occurs within all subtypes; therefore, it is unclear why these *essC* mutations are not observed  
577 in subtype I strains. Because these mutations are primarily found in subtype II, it is possible that subtype  
578 specific effectors may induce host pressure, therefore necessitating stringent regulation of this the  
579 subtype II locus. Although more work is needed to determine if *EssC* is encoded as two coding sequences  
580 or if the true *EssC* start site is downstream of the homopolymeric tract, we observed numerous  
581 homopolymeric tracts within GBS T7SS loci and hypothesize that these may regulate T7SS gene  
582 expression. Finally, numerous single and two-component regulators control T7SS loci across Gram  
583 positive species, and this has been most extensively studied in *S. aureus* (Bowman & Palmer, 2021).  
584 Interestingly, GBS T7SS gene expression is induced upon deletion of *cas9* (Spencer *et al.*, 2019), in the  
585 presence of mucins (Burcham *et al.*, 2022b), in amniotic fluid (Sitkiewicz *et al.*, 2009), and other stress  
586 conditions including removal of nutrients, exposure to serum, and oxygen deprivation (Avican *et al.*,  
587 2021). Therefore, our investigation of a GBS T7SS regulator is ongoing.

588

589 Secretion systems are commonly associated with phage, with a well-established connection between  
590 T6SS and bacteriophage machinery in particular. A link between T7SS and bacteriophage infection may  
591 also exist as T7SS WXG100 and putative toxins have been identified on *Mycobacterium abscessus*  
592 prophage (Dedrick *et al.*, 2021) and phage infection induces *E. faecalis* T7SS (Chatterjee *et al.*, 2021).  
593 Although we do not observe T7SS proteins encoded within GBS prophage, we did observe that some  
594 GBS T7SS proteins exhibit homology to phage proteins and that many phage proteins contain T7SS-  
595 associated motifs. It is known that encoding of prophage can modulate bacterial resistance to

596 bacteriophage infection and antibiotics (Wendling *et al.*, 2021). Recently, integration of a temperate  
597 phage into *S. aureus* was shown to increase virulence, not by encoding virulence factors itself, but instead  
598 due to upregulation of various bacterial virulence factors including EsxA (Yang *et al.*, 2022). This was  
599 also recently shown in GBS, in which loss of prophage from CNCTC 10/84 modulated gene expression  
600 (Wiafe-Kwakye and Neely, *unpublished observation*). Therefore, future investigation of a link between  
601 GBS T7SS and prophage is warranted.

602

603 In summary, this study bioinformatically characterizes the diversity encoded within the GBS T7SS and  
604 indicates that GBS T7SS contains similar, but unique, individual effectors compared to T7SSb in other  
605 Gram-positive species. GBS can be classified into four subtypes, which encode unique effectors and  
606 appear to modulate GBS T7SS-dependent interactions within the host, such as during vaginal  
607 colonization. This study also identifies orphaned modules containing potential T7SS-associated WXG100  
608 proteins. Taken together, this study suggests a “one size does not fit all” approach to studying T7SS as  
609 phenotypes and implications for host and interbacterial phenotypes likely depend on the specific effectors  
610 encoded across and even within species. Future studies are warranted to further characterize GBS T7SS  
611 effectors and their impact on colonization and disease.

612

## 613 **4 | MATERIALS AND METHODS**

### 614 **4.1 | Bacterial strains**

615 Example strains from each of the GBS T7SS subtypes were used in this study (subtype I-CJB111;  
616 GenBank accession CP063198.2 (01-APR-2021 version) (Spencer *et al.*, 2021a), subtype II- 2603V/R;  
617 GenBank accession NC\_004116.1 (Tettelin *et al.*, 2002), subtype III- CNCTC 10/84; GenBank accession  
618 NZ\_CP006910.1 (Nizet *et al.*, 1996, Hooven *et al.*, 2014, Wilkinson, 1977), subtype IV- COH1; GenBank  
619 accession NZ\_HG939456.1 (Nizet *et al.*, 1996, Da Cunha *et al.*, 2014)). Further, previously described  
620 cohorts of clinical GBS isolates were utilized for molecular T7SS subtyping and analysis of T7SS activity  
621 *in vitro*. Vaginal isolates were obtained from Melody Neely from the Detroit Medical Center as described



622 previously (Burcham *et al.*, 2019). Diabetic wound isolates were obtained from Elizabeth Grice (University  
623 of Pennsylvania) as well as the CU-Anschutz Medical center (Keogh *et al.*, 2022). GBS was grown  
624 statically in Todd Hewitt Broth (THB; Research Products International, RPI) at 37°C. All strains used in  
625 this study can be found in **Supp. Table 4**.

626

## 627 **4.2 | Bioinformatic analysis of GBS T7SS**

628 All comparative genomics were performed in Geneious Prime 2022.0.2 using genomes of *Streptococcus*  
629 *agalactiae* downloaded from NIH GenBank (as described in Supplementary Table 1 of (Spencer *et al.*,  
630 2021b)) as well as using 1342 WGS contigs of *Streptococcus agalactiae* downloaded from NIH GenBank  
631 (as of December 2020). Protein BLAST was utilized in Geneious to determine presence/absence or  
632 conservation of T7SS-associated proteins across GBS subtypes and across other Gram-positive species.  
633 Protein sequences yielding a grade of 30% or less were considered not homologous. Protein alignments  
634 were performed using the EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics  
635 Institute) Clustal Omega (v.1.2.4). CDART (Geer *et al.*, 2002), and InterPro (Blum *et al.*, 2021) were used  
636 to identify domains within T7SS locus-associated proteins. Protter was used to characterize protein  
637 topology (Omasits *et al.*, 2014) and amino acid sequences were scanned manually for T7SS-associated  
638 motifs.

639

## 640 **4.3 | Modelling of GBS T7SS proteins**

641 The modelling of LXG proteins and Lap chaperones was performed using original or multimer AlphaFold2  
642 weights (Jumper *et al.*, 2021, Evans *et al.*, 2022) as implemented in ColabFold (Mirdita *et al.*, 2022) or  
643 using Robetta (Baek *et al.*, 2021). Structural illustrations were generated using PyMol (The PyMOL  
644 Molecular Graphics System, Version 2.3.5 Schrödinger, LLC. (Schrodinger, 2015)).

645

## 646 **4.4 | Cloning**

647 A clean *essC* deletion mutant in the CJB111 background was described in (Spencer *et al.*, 2021b).  
648 Additional deletion mutants were created via allelic exchange in this study in the A909, 2603V/R, CNCTC  
649 10/84, and COH1 backgrounds using the temperature sensitive plasmid pHY304 (Spencer *et al.*, 2019)  
650 and using a gene encoding spectinomycin resistance (*aad9*) in the knockout construct. Second crossover  
651 mutants were screened for erythromycin sensitivity and spectinomycin resistance. Strains created in this  
652 study, *essC* locus tags for each strain, and primers used in this study can be found in **Supp. Table 4**.

653

#### 654 **4.5 | T7SS operon mapping**

655 Mapping of the T7SS operon in the CJB111 strain background was performed similar to that described  
656 previously (Kneuper *et al.*, 2014, Taylor *et al.*, 2021). Briefly, primers were designed to span gene  
657 junctions within the putative T7SS locus and surrounding genomic area. These primers sets were used  
658 in PCR reactions with cDNA template (50 ng/ reaction) to determine which adjacent genes were co-  
659 transcribed. Genomic DNA (50 ng/ reaction) was used as a positive control for the primer sets and no-  
660 reverse transcriptase cDNA was generated and diluted equivalently to control for possible genomic DNA  
661 contamination of the cDNA. cDNA and no-RT cDNA were generated as previously described (Spencer  
662 *et al.*, 2021b). Briefly, GBS strains were grown to mid-log ( $OD_{600} = 0.4-0.6$ ) and RNA was purified using  
663 the MACHEREY-NAGEL NucleoSpin kit (catalog# 740955.250) according to manufacturer instructions  
664 with the addition of three bead beating steps (30 sec x 3, with one minute rest on ice between each)  
665 following the resuspension of bacterial pellets in RA1 buffer +  $\beta$ -mercaptoethanol. Purified RNA was  
666 treated with the Turbo DNase kit (Invitrogen, catalog# AM1907) according to manufacturer instructions.  
667 cDNA was synthesized using the SuperScript cDNA synthesis kit (QuantaBio, catalog# 95047-500), per  
668 manufacturer instructions. All RT-PCR reactions were performed using Q5 polymerase (New England  
669 Biolabs) under the following cycling conditions on a Bio-Rad T100 thermal cycler: 98°C, 2-min hot start;  
670 34 cycles (98°C, 10 seconds; 60 °C, 20 seconds; 72°C, 30-second extension/kb); and 72°C, 10-min  
671 extension. Primers used for these experiments can be found in **Supp. Table 4**. PCR amplicons were

672 visualized via gel electrophoresis using 1% agarose gels and GeneRuler 1 kb Plus DNA ladder (Thermo  
673 Scientific, SM1331). Three independent replicates were performed.

674

#### 675 **4.6 | EsxA secretion assay**

676 Secretion of EsxA during growth in THB was assessed for GBS clinical isolates as described previously  
677 (Spencer *et al.*, 2021b). Briefly, overnight cultures of GBS isolates were sub-cultured into 5 mL of THB  
678 and grown statically for 24 hours at 37°C. Bacteria were pelleted at 3214 x *g* for 10 minutes at 4°C.  
679 Supernatants were removed from pellets, filtered (Millex Low Protein Binding Durapore PVDF Membrane  
680 0.22µm filters, catalog #SLGVR33RS), and supplemented with an EDTA-free protease inhibitor cocktail  
681 (Millipore-Sigma set III, catalog # 539134; 1:250 dilution). Supernatants were then precipitated overnight  
682 with trichloroacetic acid (TCA) at 4°C. Precipitated proteins were centrifuged for 15 minutes at 13K x *g*  
683 and resulting pellets were gently washed with acetone. Pellets were then centrifuged again at the same  
684 settings. Acetone was removed and pellets were allowed to dry before being resuspended in Tris buffer  
685 (50 mM Tris HCl, 10% glycerol, 500 mM NaCl, pH 7). Bacterial pellets were washed once with PBS,  
686 frozen overnight, and resuspended in Tris buffer + protease inhibitor. Pellets were then bead beaten (2  
687 x one minute) using 0.1mm zirconia/silica beads (BioSpec). Triton-X-100 was then added to lysates at a  
688 final concentration of 1% to solubilize membrane proteins and vortexed to mix.

689

690 Supernatant and pellet samples were mixed 1:1 with Laemmli buffer + beta-mercaptoethanol, boiled 10  
691 minutes, and run on SDS-PAGE for Western blotting. Proteins were transferred to membranes via the  
692 BioRad Trans-Blot Turbo Transfer System (high molecular weight settings). Membranes were washed  
693 three times in TBST and blocked in LI-COR's Intercept Blocking Buffer (catalog# 927-60001) for one  
694 hour at room temperature. Membranes were probed with an anti-EsxA1 rabbit polyclonal antibody (0.5  
695 µg/ml; GenScript) in the above LI-COR blocking buffer overnight at 4°C. Following washes in TBST,  
696 membranes were incubated with IRDye 680RD goat anti-rabbit IgG (H + L) secondary antibodies from

697 LI-COR (1:10,000 dilution; 1 hour, room temperature; catalog# 926–68071). Following washes in TBST  
698 and water, western blots were imaged using the LI-COR Odyssey.

699

#### 700 **4.7 | Murine Model of GBS Vaginal Colonization**

701 GBS vaginal colonization was assessed using a previously described murine model of vaginal  
702 persistence and ascending infection (Patras & Doran, 2016). Briefly, 8–10-week-old female CD1 (Charles  
703 River) mice were synced with beta-estradiol at day -1 and inoculated with  $1 \times 10^7$  GBS in PBS on day 0.  
704 Post-inoculation, mice were lavaged with PBS or swabbed daily, and the samples were serially diluted  
705 and plated for CFU counts to determine bacterial persistence on differential and selective GBS  
706 CHROMagar [catalog# SB282(B)]. At experimental end points, mice were euthanized, and female genital  
707 tract tissues (vagina, cervix, and uterus) were collected. Tissues were homogenized and samples were  
708 serially diluted and plated on CHROMagar for CFU enumeration. Bacterial counts were normalized to  
709 the tissue weight. These experiments were approved by the committee on the use and care of animals  
710 at the University of Colorado-Anschutz Medical Campus (protocol #00316) and at Baylor College of  
711 Medicine (protocol AN-8233).

712

#### 713 **4.8 | Molecular T7SS typing of GBS clinical isolates**

714 Molecular typing was performed by multiplex PCR amplification using primers within subtype-specific  
715 genes: subtype I, CJB111 ID870\_4220; subtype II, 2603V/R SAG\_RS07870; subtype III, CNCTC 10/84  
716 W903\_RS05410; and subtype IV, COH1 GBSCOH1\_RS05060. Primers used for these experiments can  
717 be found in **Supp. Table 4**. Multiplex PCR reactions were performed using Q5 polymerase (New England  
718 Biolabs) under the following cycling conditions on a Bio-Rad T100 thermal cycler: 98°C, 30 second hot  
719 start; 35 cycles (98°C, 10 seconds; 59 °C, 30 seconds; 72°C, 30 second extension/kb); and 72°C, 2-  
720 minute extension. PCR amplicons were visualized via gel electrophoresis using 1.4% agarose gels and  
721 Gene Ruler 100bp DNA ladder (Thermo Fisher Scientific, SM0243).

722

## 723 **4.9 | Data analysis and statistics**

724 Fisher's exact test were performed to assess associations between T7SS subtype, T7SS orphaned  
725 modules, sequence type, serotype, and prophage cluster. Tables and R script used for Fisher's exact  
726 tests can be found in **Supp. Table 5**. For vaginal colonization experiments, statistical analysis was  
727 performed using Prism version 9.4.1 (458) for macOS (GraphPad Software, La Jolla, CA, United States).  
728 Significance was defined as  $p < \alpha$ , with  $\alpha = 0.05$ .

729

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732 Manzer for contributing the Circos plot in **Fig. 4D** and Uday Tak for contributing to the Robetta models in  
733 **Supp. Fig. 4A**.

734

## 735 **Figure legends**

736 **Fig. 1. Intra-species diversity of the GBS T7SS. A)** Example GBS T7SS loci by subtype (roughly to  
737 scale). GBS T7SS loci are flanked by carbamoyl phosphate synthesis genes and two-component system  
738 genes *ltdS/ltdR* (light red shading). GBS T7SS encode conserved machinery genes (light blue shading)  
739 but subtypes vary in copies of *esxA* (purple) and in putative downstream effectors, including WXG100-  
740 like proteins (purple), putative LXG toxins (teal), transmembrane proteins (olive green), hypothetical  
741 proteins (gray), DUF4176 proteins (orange), CHAP domain-containing proteins (navy blue),  $\alpha\beta$   
742 hydrolases (light blue), and lipoproteins (light brown). Arrows with patterns indicate fragmented genes.  
743 Incompletely filled arrows indicate genes encoding prematurely truncated products (e.g., CNCTC 10/84  
744 *esxA2*). **B)** Heatmaps indicating homology of T7SS-associated genes downstream of *essC* across T7SS  
745 subtype. Color intensity based on Geneious alignment grade, which considers query coverage and  
746 percent identity. Many downstream GBS T7SS effectors have little homology across subtypes or are  
747 entirely T7SS subtype specific. **C)** Commonly occurring "LXG modules" are encoded for downstream of  
748 *essC* in subtypes I-III, which include putative chaperones (WXG100-like proteins, purple), a putative LXG

749 toxin (teal), a transmembrane protein (olive green), and a DUF4176 protein (orange). Partial LXG  
750 modules were also identified, which contain only a fragmented LXG gene (patterned teal) and the  
751 subsequent transmembrane and DUF4176 protein encoding genes. **D)** Another commonly found module  
752 within T7SS loci consists of a gene fragment encoding the C-terminal end of the COH1 *essC* (red/white  
753 stripe), followed by  $\alpha\beta$  hydrolase (light blue) and lipoprotein (light brown) encoding genes. This module  
754 is often preceded by an amidase encoding gene (navy blue).

755

756 **Fig. 2. Subtype-specific LXG toxins are encoded by GBS T7SS.** **A)** Predicted models of complexes  
757 between specific Lap1 (green) and Lap2 (blue/purple) pairs with the LXG domain (pink) of their cognate  
758 LXG protein. The putative effector domains of LXG proteins and the predicted disordered regions of Lap  
759 proteins are omitted for clarity. The conserved residues of LxG and FxxxD motifs are shown as spheres.  
760 The modelling confidence metrics are presented in **Supp. Fig. 3. B-D)** Percent identity matrix of full-  
761 length LXG protein sequences and Clustal Omega alignment of the LXG domain from CJB111 (subtype  
762 I), 2603V/R (subtype II), CNCTC 10/84 (subtype III), and C001 (subtype III). In the above matrix, the  
763 purple shading corresponds to the level of identity between two strains (on a spectrum of 0 to 100%  
764 identity), with darker shading indicative of higher percent identity. The longer motif found in all GBS LXG  
765 proteins is denoted by a red box in **D**.

766

767 **Fig. 3. Intra-subtype diversity of the GBS T7SS.** GBS T7SS loci representing genomic diversity within  
768 **A)** subtype I, **B)** subtype II, **C)** subtype III, and **D)** subtype IV. GBS T7SS encode putative downstream  
769 effectors including WXG100-like proteins (purple), putative LXG toxins (teal), transmembrane proteins  
770 (olive green), hypothetical proteins (gray), DUF4176 proteins (orange), CHAP domain-containing  
771 proteins (navy blue),  $\alpha\beta$  hydrolases (light blue), and lipoproteins (light brown). Arrows with patterns  
772 indicate fragmented genes. Incompletely filled arrows indicate genes encoding prematurely truncated  
773 products (e.g., CNCTC 10/84 *esxA2*). Subtype I and III strains differ in presence/absence of downstream

774 genes (**A, C**). Subtype II and IV strains differ in mutations within *essC* (**B, D**) and the length of the  
775 homopolymeric tract within *essC* is annotated as  $G_n$ .

776

777 **Fig. 4. GBS T7SS orphaned modules.** **A)** Representative GBS T7SS orphaned modules 1 and 2. The  
778 subtype I strain CJB111 Module 1 region includes genes encoding a DEAH/DEAD box helicase (green),  
779 WXG100 protein (purple), hypothetical proteins (gray), and a MutR family transcriptional regulator (black).  
780 The Module 2 region includes genes encoding hypothetical proteins (gray), a WXG100 protein (purple),  
781 DUF1310 protein(s) (orange), and a type II RelB/DinJ family antitoxin (pink). **B)** Heatmap indicating  
782 presence (purple) or absence (white) of locus associated *EsxA1* and *EsxA2* and of GBS T7SS orphaned  
783 modules 1 and 2 (containing *EsxA3* and *EsxA4*, respectively) in various GBS clinical isolates. **C)** Percent  
784 identity matrix of *EsxA1-4* protein sequences from CJB111 (subtype I). In the above matrix, the purple  
785 shading corresponds to the level of identity between two strains (on a spectrum of 0 to 100% identity),  
786 with darker shading indicative of higher percent identity. **D)** Circos plot showing distinct genomic locations  
787 of the T7SS locus, orphaned modules, and prophage in subtype I strain CJB111.

788

789 **Fig. 5. *EsxA* secretion across GBS T7SS subtypes.** Western blot showing *EssC*-dependent secretion  
790 of *EsxA* from subtype I strain CJB111 and subtype III strain CNCTC 10/84, but not from subtype II strain  
791 2603V/R, *in vitro*. *EsxA* was also shown to be secreted in subtype I strain A909 and subtype II strain  
792 NEM316. Subtype IV strain COH1 does not encode *EsxA* and served as a negative control. Blot pictured  
793 is representative of 3 independent experiments.

794

795 **Fig. 6. Varying impact of *EssC* deficiency on GBS vaginal colonization by T7SS subtype I and III**  
796 **strains.** **A, E)** Percent colonization curves of 8-week-old CD1 female mice vaginally inoculated with **A)**  
797 subtype I strain CJB111 or CJB111 $\Delta$ *essC* or **E)** subtype III strain CNCTC 10/84 or CNCTC 10/84 $\Delta$ *essC*.  
798 Graphs are representative of three or two independent experiments, respectively (n = 10/group).  
799 Statistics reflect the Log rank (Mantel-Cox) test. Recovered CFU counts from the **B, F)** vaginal **C, G)**

800 cervical, and **D, H**) uterine tissue of colonized mice. In panels **B-D** and **F-H**, each dot represents one  
801 mouse and all independent experiments' data are combined in these figures (n = 30/group for CJB111  
802 experiments and n = 20/group for CNCTC 10/84 experiments). Plots show the median and statistics  
803 represent the Mann Whitney U test.

804

805 **Fig. 7. Multiplex PCR to identify T7SS subtype amongst GBS isolates.** A multiplex PCR was  
806 developed for GBS T7SS typing using primers that amplify subtype specific genes, designed to yield  
807 distinct amplicon sizes across T7SS subtypes (see **Supp. Table 4**). **A**) Agarose gel showing subtype  
808 specific amplicon products from single-plex and multiplex PCR (of GBS genomic DNA), which can be  
809 differentiated by size using a 100 bp DNA ladder. This multiplexed PCR system was utilized in the  
810 laboratory or *in silico* using Geneious to T7SS type GBS **B**) vaginal (n =37) and **C**) diabetic wound isolates  
811 (n = 27) or **D**) GBS contig sequences available from GenBank (n =1130), respectively.

812

### 813 **Supplemental Figure legends**

814 **Supp. Fig. 1. Comparison of GBS T7SS effectors to other Gram-positive organisms.** Heat map  
815 comparing CJB111 T7SS protein homology to a panel of eight Gram-positive organisms in which the  
816 T7SSb has been studied previously: *S. aureus* (variants 1-4), *S. lugdunensis* (variants 1, 2), *Listeria*  
817 *monocytogenes* (variants 1-7), *E. faecalis* (strain OG1RF), *Bacillus subtilis* (PY79), *S. gallolyticus* (strain  
818 TX20005), *S. intermedius* (strains B196 and GC1825), and *S. suis* (strains GZ5065 and WUSS351). See  
819 **Supp. Table 2** for individual strain information. Heat map color intensity is based on Geneious alignment  
820 grade, which considers query coverage and percent identity. Shading of the locus tag numbers on the x-  
821 axis corresponds to the key for gene color in **Fig 1**. Most downstream CJB111 T7SS effectors have little  
822 to no homology across species.

823

824 **Supp. Fig. 2. Subtype-specific LXG-associated proteins are encoded by GBS T7SS.**



825 Percent identity matrices and Clustal Omega alignments of **A-C**) LXG-associated protein 1 (Lap1) and  
826 **D-F**) LXG-associated protein 2 (Lap2) sequences from CJB111 (subtype I), 2603V/R (subtype II),  
827 CNCTC 10/84 (subtype III), and C001 (subtype III). In the above matrices, the purple shading  
828 corresponds to the level of identity between two strains (on a spectrum of 0 to 100% identity), with darker  
829 shading indicative of higher percent identity. Conserved putative T7SS-associated FXG and FxxxD/E  
830 motifs are highlighted by red boxes in **C**.

831

832 **Supp. Fig. 3. Confidence scores and predicted aligned error for LXG-Lap Alpha Fold models in**  
833 **Fig. 2A. A)** Predicted LXG-Lap complex models shown in **Fig. 2A** but with color corresponding to per-  
834 residue confidence level (predicted local distance difference test [pLDDT] score 1-100; pLDDT > 90 are  
835 expected to be modelled to high accuracy). **B)** Predicted aligned error for each LXG-Lap complex, with  
836 colors indicating the confidence of domain positions (higher predicted error in red, lower predicted error  
837 in blue).

838

839 **Supp. Fig. 4. Modeling of full-length LXG proteins and homology of downstream putative**  
840 **immunity factor and DUF4176 genes**

841 **A)** Robetta predicted structures for full-length putative GBS LXG toxins across subtypes I, II, and III. **B)**  
842 Percent identity matrix of putative immunity factors (transmembrane domain containing proteins)  
843 encoded for downstream of LXG genes in CJB111 (subtype I), 2603V/R (subtype II), CNCTC 10/84  
844 (subtype III), and C001 (subtype III). **C-E)** Percent identity matrices and Clustal Omega alignments of  
845 DUF4176 protein sequences from CJB111 (subtype I), 2603V/R (subtype II), CNCTC 10/84 (subtype III),  
846 and COH1 (subtype IV). Green highlighting in **C-E** indicate orphaned DUF4176 proteins. A conserved  
847 central FXG motif within GBS DUF4176 proteins is highlighted by a red box in **D**. In all of the above  
848 percent identity matrices, the purple shading corresponds to the level of identity between two strains (on  
849 a spectrum of 0 to 100% identity), with darker shading indicative of higher percent identity.

850

851 **Supp. Fig. 5. Transcriptional landscape of the CJB111 T7SS locus**

852 RT-PCR was performed to evaluate transcriptional organization of the CJB111 T7SS locus. Using cDNA  
853 as template (and no RT-cDNA and genomic DNA as negative and positive controls, respectively), PCRs  
854 were performed using primer pairs spanning every gene junction in the putative T7SS locus. Primers  
855 used for these experiments can be found in **Supp. Table 4**. Agarose gels shown are representative of  
856 three independent experiments.

857

858 **Supp. Fig. 6. Homology of orphaned GBS WXG100 proteins.**

859 **A)** Locus tag tables and percent identity matrices of **B)** Orphaned Module 1 WXG100 protein EsxA3 and  
860 **C)** Orphaned Module 2 WXG100 protein EsxA4 sequences across a panel of GBS isolates representing  
861 T7SS subtypes I-IV. Green highlighting in **A** indicates orphaned WXG100 proteins. In the above matrices  
862 (**B-C**), the purple shading corresponds to the level of identity between two strains (on a spectrum of 0 to  
863 100% identity), with darker shading indicative of higher percent identity.

864

865 **Supp. Fig 7. Impact of EssC deficiency on GBS vaginal colonization by T7SS subtype I strain**

866 **A909. A)** Percent colonization curve of 8-week-old CD1 female mice vaginally inoculated with subtype I  
867 strain A909 or A909 $\Delta$ essC. Statistics reflect the Log rank (Mantel-Cox) test. Recovered CFU counts from  
868 the **B)** vaginal **C)** cervical, and **D)** uterine tissue of colonized mice. In panels **B-D**, each dot represents  
869 one mouse and two independent experiments' data are combined in these figures (n = 16/group total).  
870 Plots show the median and statistics represent the Mann Whitney U test.

871

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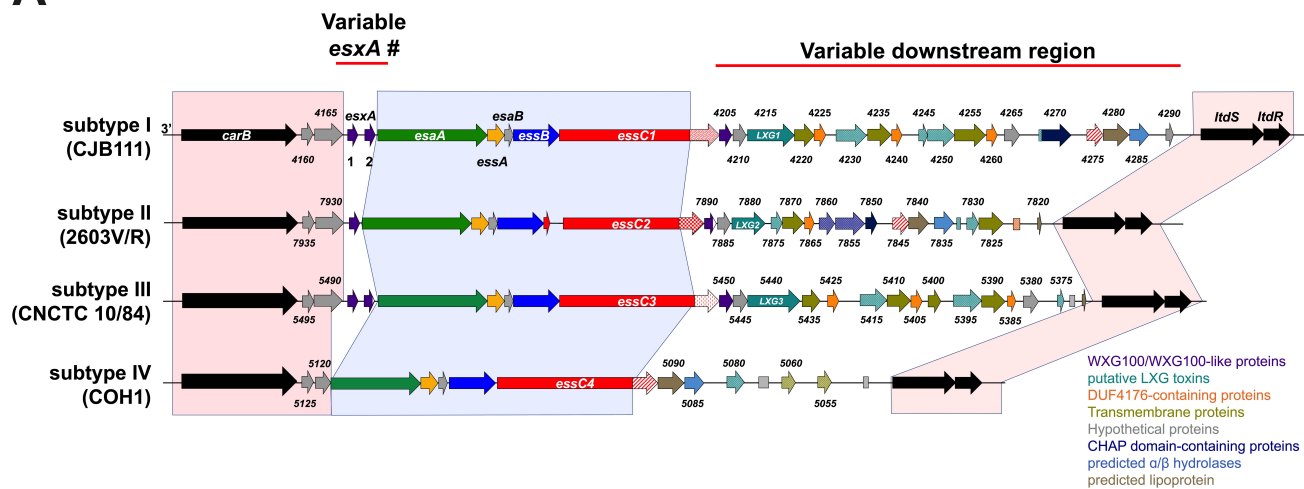
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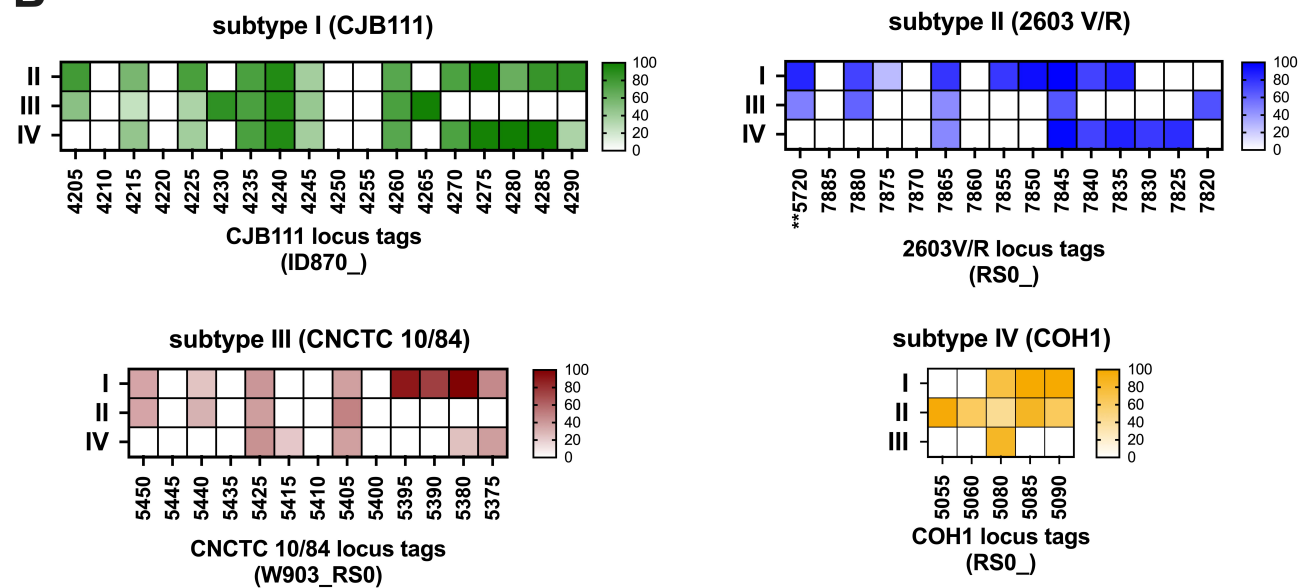


# Figure 1

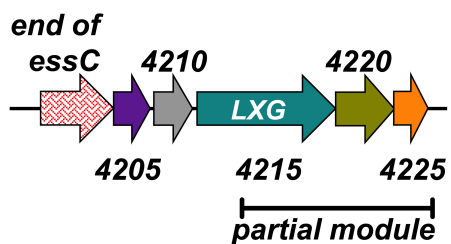
## A



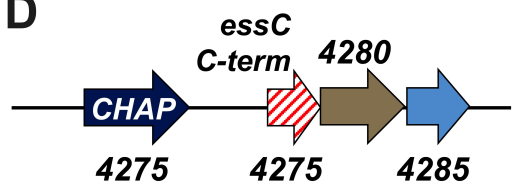
## B



## C



## D

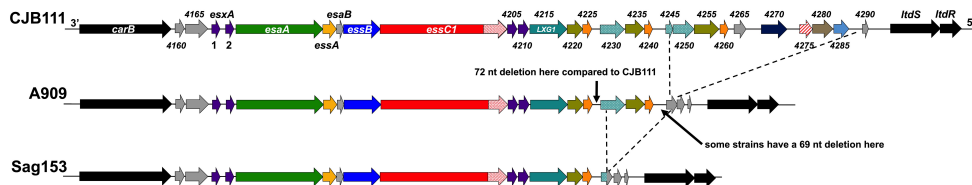




# Figure 3

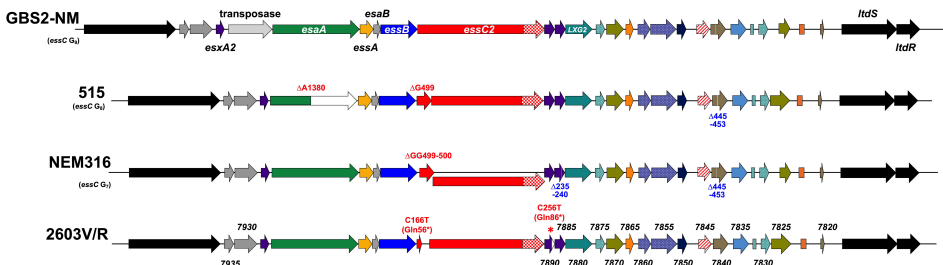
## A

### Subtype I diversity



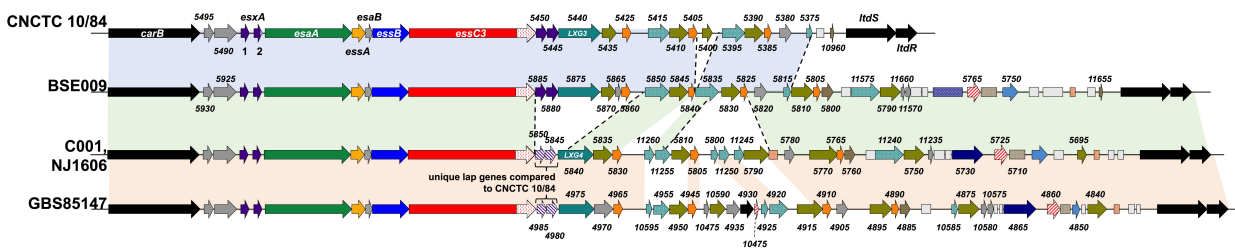
## B

### Subtype II diversity



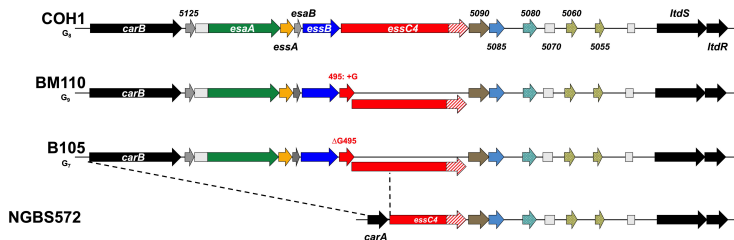
## C

### Subtype III Diversity



## D

### Subtype IV Diversity

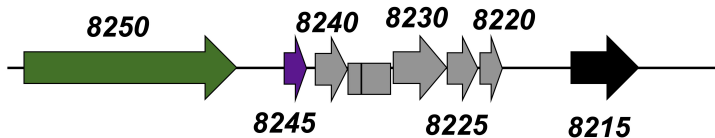


WXG100/WXG100-like proteins  
 putative LXG toxins  
 DUF4176-containing proteins  
 Transmembrane proteins  
 Hypothetical proteins  
 CHiAP domain-containing proteins  
 predicted α/β hydrolases  
 predicted lipoprotein

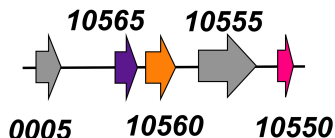
# Figure 4

## A

### Orphaned Module 1



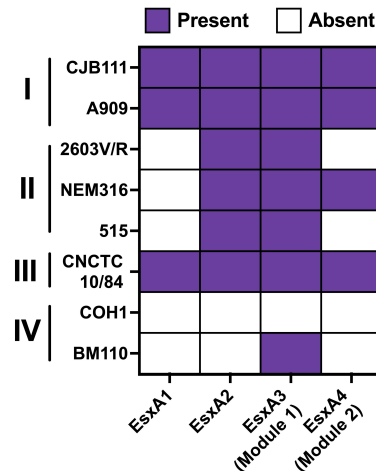
### Orphaned Module 2



■ DEAD/DEAH box helicase family protein  
■ WXG100 protein  
■ Hypothetical protein  
■ MutR family transcriptional regulator  
■ DUF1310 family protein  
■ type II, RelB/DinJ family antitoxin

## B

### GBS WXG100 Proteins

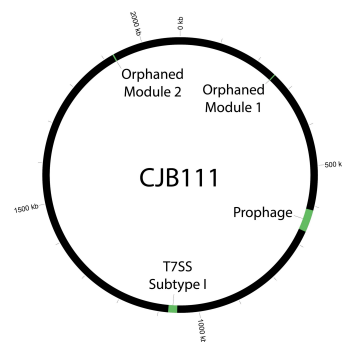


## C

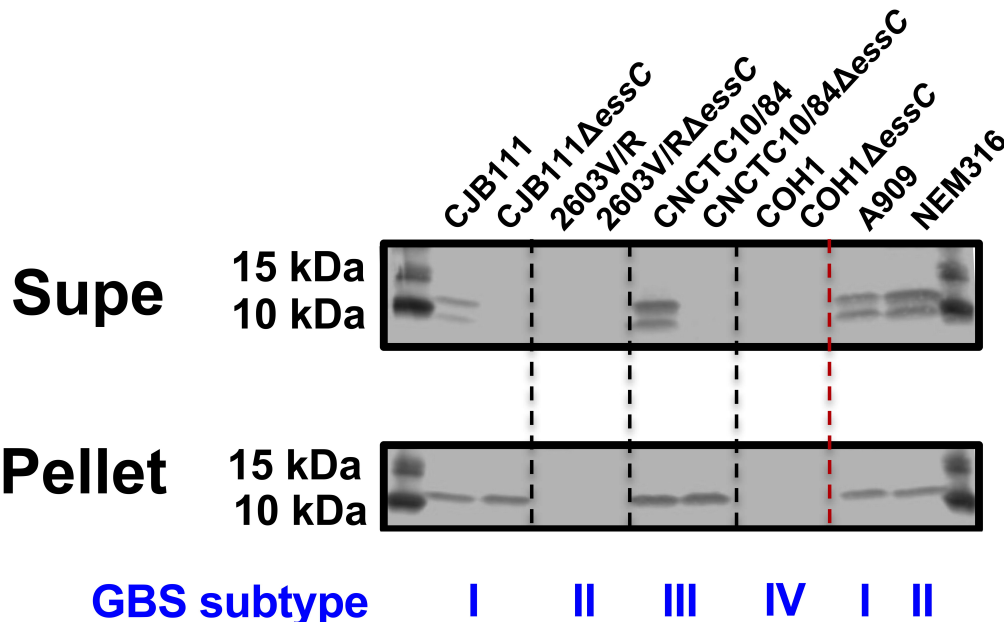
### CJB111 WXG100 protein alignment

	EsxA1	EsxA2	EsxA3	EsxA4
EsxA1		95	87	85
EsxA2	95		85	82
EsxA3	87	85		93
EsxA4	85	82	93	

## D

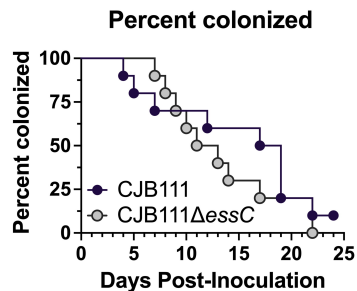


# Figure 5

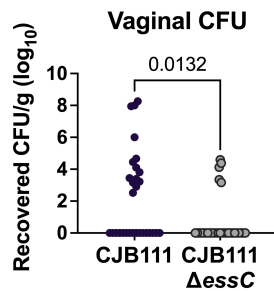


# Figure 6

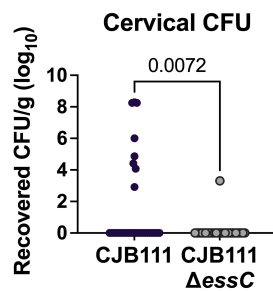
## A



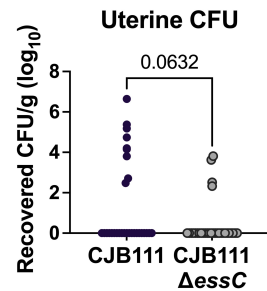
## B



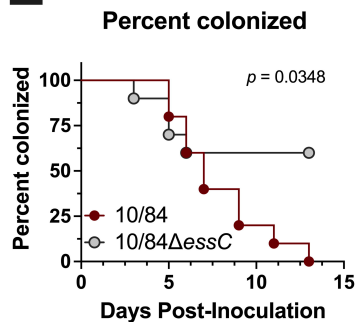
## C



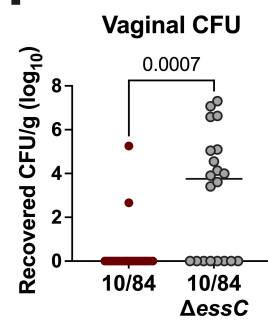
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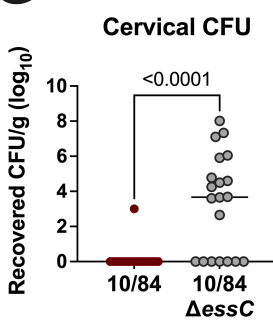
## E



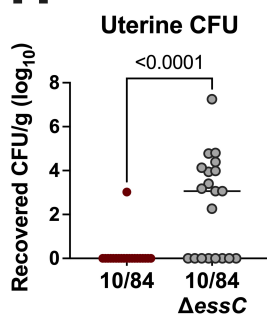
## F



## G

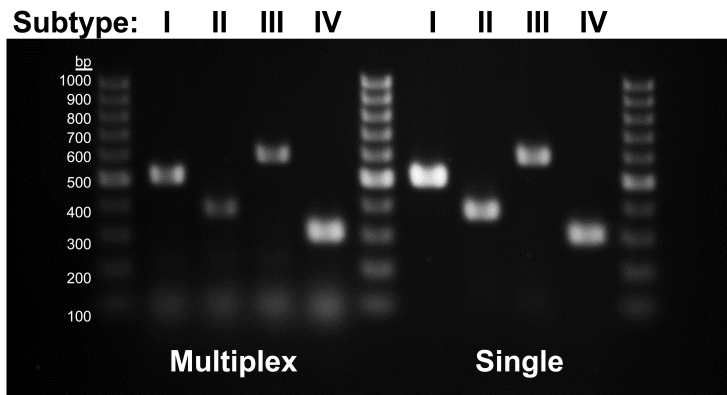


## H



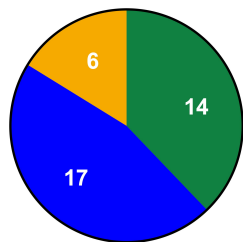
# Figure 7

## A



## B

Vaginal Isolates

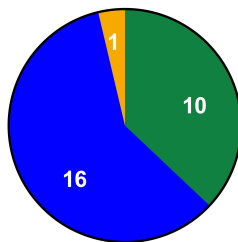


Total=37



## C

Diabetic Wound Isolates

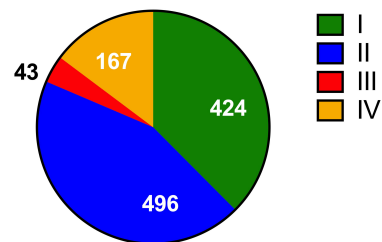


Total=27



## D

GBS GenBank Contigs



Total=1130

