Multi-continental detection of *Streptococcus pyogenes* $M1_{UK}$: Impact of ssrA SNP on SpeA expression in ancestral and $M1_{UK}$ isolates.

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Abstract

The *Streptococcus pyogenes* $M1_{UK}$ lineage, characterised by an intrinsic ability to express SpeA toxin and defined by 27 single nucleotide polymorphisms in the core genome, dominates the population of *emm*1 *S. pyogenes* isolates throughout Europe, Canada, South America, Japan and Oceania. While not the sole deterministic factor, enhanced SpeA expression is likely to have contributed to $M1_{UK}$ lineage expansion, but was not sufficient to support expansion of intermediate lineage $M1_{23SNP}$, that expresses SpeA at a similar level to $M1_{UK}$.

A single nucleotide polymorphism (SNP) in the *ssrA* leader sequence upstream of *speA* is one of a limited number of SNPs that distinguish intermediate sublineages that differ in SpeA production. It was recently shown that this SNP leads to increased *ssrA* terminator read-through, and consequent increased transcription of *speA*, which lies downstream of *ssrA*. In this work, introduction of the *ssrA* SNP into representative isolates of the widely disseminated $M1_{global}$ clone and the intermediate $M1_{13SNP}$ lineage, that cannot otherwise produce readily-detectable SpeA in culture, resulted in SpeA expression, confirming the importance of the *ssrA* SNP to SpeA phenotype. Consistent with this, correction of the *ssrA* SNP in $M1_{UK}$ abrogated SpeA expression. However, RNAseq analysis of 8 *emm*1 clinical pharyngitis strains showed that presence of the SNP was not invariably linked to read-through from the ssrA leader sequence or SpeA expression. Read-through was observed in isolates that did not possess the ssrA SNP. Critical review of existing data suggests that *speA* mRNA transcript length may be impacted by the two-component regulator CovRS, pointing to a complex regulatory network interaction between the bacterial chromosome and phage-encoded superantigens.

Introduction

A marked increase in serious *Streptococcus pyogenes* infections was reported in several countries following relaxation of public health interventions designed to limit the spread of COVID-19 [1]. In England, notifications of both scarlet fever and invasive *S. pyogenes* infections increased throughout 2022; infections peaked at the end of the calendar year but remained high thereafter [2]. In the 30 weeks from mid September 2022-mid April 2023 there were 54,394 notifications of scarlet fever and 2965 invasive *S. pyogenes* infections in England alone [2]. The high case fatality rate of 14.2% [2] underlines the major public health impact of the observed increase, and the importance of understanding the relative roles of strain pathogenicity, population immune susceptibility, co-infections, season, and access to healthcare.

*Emm*1 *S. pyogenes* strains are known to be inherently invasive and normally account for one quarter of invasive infections; however, in the 2022-2023 season, *emm*1 isolates accounted for over half of invasive infections, rising to over 60% in children in England [2]. The population of *emm*1 *S. pyogenes* isolates in England is dominated by a novel sublineage, $M1_{UK,}$ that has an intrinsic ability to express the phage-encoded superantigenic toxin, streptococcal pyrogenic exotoxin (Spe) A [3,4]. $M1_{UK}$ strains can be differentiated from strains belonging to the highly successful *emm*1 *S. pyogenes* globally-disseminated clone that emerged in the 1980's ($M1_{global}$) by just 27 single nucleotide polymorphisms in the core genome [3]. The $M1_{UK}$ lineage has been identified elsewhere in Europe [5], North America [6,7], Japan [8], China, Russia [9] and Australia, where isolates from Queensland and Victoria date from 2013 [10], just 3 years after its first detection in England [3]. While $M1_{UK}$ represented ~60% of Australian *emm*1 isolates by 2020 [10], in England, this proportion reached 91% by 2020 [4]. The detection of $M1_{UK}$ Queensland, underlines the ability of this lineage to spread in temperate and more tropical climates, although notably *emm*1 strains appear to be uncommon in sub-Saharan Africa [11]. Of added concern are reports of strains infected with a ΦHKU488.vir-like phage, that carries both *speC* and *ssa* superantigen genes in Australia [10]. To date, this phage has not been identified in *emm*1 strains from England, although ~10% of *emm*1 strains in England do possess a phage encoding *speC* and *spd*.

66

67 Previous genomic analysis of *emm*1 *S. pyogenes* strains in England identified two intermediate
68 sublineages in addition to $M1_{global}$ and $M1_{UK,}$ with just 13 and 23 of the 27 SNPs that define $M1_{UK}$ [3],
69 namely $M1_{13SNP}$ and $M1_{27SNP}$ (Figure 1). We recently reported that strains from the $M1_{13SNP}$
70 intermediate sublineage produce negligible amounts of SpeA, similar to $M1_{global}$ [12]. Strains from
71 the $M1_{23SNP}$ sublineage, in contrast, produce SpeA at an increased level that is indistinguishable from
72 $M1_{UK}$ isolates [12]. Despite increased SpeA production, $M1_{23SNP}$ isolates could not be detected when
73 screening all *emm*1 isolates submitted to the reference laboratory in England in 2020 [4], suggesting
74 an added fitness advantage in $M1_{UK}$ strains beyond production of SpeA alone. We postulate this
75 further adaptation may be conferred by the additional 4 SNPs that distinguish $M1_{UK}$ from $M1_{23SNP}$
76 isolates (3 non-synonymous SNPs and an intergenic SNP 39bp upstream of *glpF2*, aquaporin,
77 expression of which is significantly reduced in $M1_{UK}$) [10,12]. In this report we seek to further
78 understand the mechanisms that differentiate SpeA-producers from non-producers among *emm*1 *S.*
79 *pyogenes* strains that carry the phage that encodes *speA*.

80

81 **Results and Discussion**

82 The $M1_{UK}$ SNP at position 983438 of the MGAS5005 reference genome, within the leader sequence
83 of *ssrA* and upstream of the *speA* start site (referred to hereafter as the 'ssrA SNP'), is present in
84 both $M1_{23SNP}$ and $M1_{UK}$ sublineages, but is absent in $M1_{global}$ and $M1_{13SNP}$ sublineages. Screening of
85 natural mutants arising during outbreaks identified the ssrA SNP to be associated with increased
86 SpeA production [12]. Davies et al [10] showed that introduction of the ssrA SNP resulted in
87 increased expression of SpeA by $M1_{global}$. We introduced the ssrA SNP into an $M1_{global}$ strain, and
88 into an intermediate $M1_{13SNP}$ strain (Figure 2A). Consistent with the findings of Davies et al [10], SpeA
89 production in culture supernatants increased in both transformants (Figure 2B). Enhancement of
90 SpeA was similar in both the $M1_{global}$ and $M1_{13SNP}$ backgrounds, suggesting that the accumulated
91 SNPs in the $M1_{13SNP}$ lineage, which include almost half of the SNPs that characterize $M1_{UK}$, do not
92 appreciably contribute to the SpeA phenotype. We considered if our mutagenesis strategy itself
93 might lead to increased SpeA expression but this was ruled out by use of a control construct in each

94 strain (Figure 2B). In contrast, the enhanced SpeA expression in an $M1_{UK}$ background could be
95 reduced considerably upon correction of the ssrA SNP (Figure 2B).
96
97 To understand the basis for difference in *speA* expression between the lineages, we compared
98 RNAseq reads from four randomly selected $M1_{global}$ strains, which do not have the ssrA SNP, and
99 four randomly selected $M1_{UK}$ strains, which all have this SNP. None of the strains possessed
100 regulatory gene mutations other than those that characterize $M1_{UK}$. Similar to Davies et al [10], we
101 found that there was indeed evidence of read-through in the intergenic regions between the end of
102 *ssrA* and the start of *speA*. (Figure 2C). However, we did not see clear differentiation between the
103 four $M1_{global}$ strains and the four $M1_{UK}$ strains within the intergenic region, where read abundance in
104 two of the $M1_{global}$ strains was almost identical to $M1_{UK}$ strains (Figure 2C). Regardless, transcription
105 of *speA* was significantly reduced in all four $M1_{global}$ strains compared with $M1_{UK}$. Interestingly, *speA*
106 read abundance was somewhat higher in the two $M1_{global}$ strains that had more intergenic reads, but
107 not as high as the $M1_{UK}$ strains. These data provide support for intergenic read-through affecting
108 *speA* transcription. However, based on short read RNA sequencing data, we cannot definitively
109 ascribe read abundance in the intergenic region to the ssrA SNP in all cases, noting that all four of
110 the $M1_{global}$ strains studied lack any SNP in this region. Comparative WGS analysis between the four
111 $M1_{global}$ strains failed to detect any other genetic features associated with higher levels of read-
112 through. Taken together, transcriptional read-through was observed in this region, but the association
113 with the ssrA SNP and SpeA over-expression was not absolute. Despite not possessing the ssrA
114 SNP, two of four $M1_{global}$ strains tested showed similar read-through to $M1_{UK}$ strains, yet made less
115 SpeA, pointing to additional regulatory factors that influence SpeA expression. The ssrA SNP
116 however was important, at least in the strains examined.
117
118 A polycistronic transcript for speA was originally reported ~25 years ago by Cleary *et al*, who
119 identified both ~2kB and ~900bp *speA* mRNA transcripts in a single *emm*1 *S. pyogenes* strain [13].
120 In that case, the longer 2kB transcript was associated with colonies that failed to produce SpeA,
121 while the shorter 900bp transcript, corresponding to the approximate expected size for the *speA*

122 transcript, was associated with mucoid *emm*1 colonies that produced abundant SpeA. Based on the
123 date of publication, we infer the *emm*1 strain in that study [13] to be from an $M1_{global}$ background.
124 The within-strain variation in *speA* transcript length coupled with mucoid phenotype points to
125 involvement of the two-component regulator covRS (csrRS) which is now known to repress capsule
126 and *speA* transcription in *S. pyogenes* [14–16]. Although $M1_{global}$ strains produce little detectable
127 SpeA protein, SpeA production can be markedly upregulated following mutation of *covS* [14,15] or
128 *covR* [15,16] (Figure 3A), likely accounting for previous reports of upregulation of SpeA following *in*
129 *vivo* passage of *emm*1 *S. pyogenes* isolates [17].
130
131 Consistent with the above experimental observations, contemporary $M1_{global}$ invasive strains with
132 *covRS* mutations arising in patients produce SpeA at amounts equivalent to $M1_{UK}$ strains [12]. A
133 historic *emm*1 isolate NCTC8198 (SF130; H250; H305) [18,19] produces 5-10 fold more SpeA than
134 $M1_{UK}$ strains (Figure 3B) yet does not have the $M1_{UK}$ ssrA SNP. It does however possess a stop
135 mutation in *covS* at residue 318. Northern blotting has previously identified only a single dominant
136 ~750bp *speA* mRNA transcript for this strain [20], potentially consistent with abundant SpeA
137 production. We postulate that release from indirect or direct covRS repression may allow *speA*
138 transcription to start at a native promoter site proximal to *speA*, triggered by growth-phase related
139 factors. Alternatively, this may result in more rapid processing of any longer transcript. Whether
140 there is an interaction between the ssrA SNP and covRS, other signals, or RNAse Y is unknown.
141
142 $M1_{UK}$ is associated with the recent upsurge in invasive infections in Europe [21,22]. The two
143 intermediate sublineages, $M1_{13SNP}$ and $M1_{23SNP,}$ appear to be no longer present in England [4].
144 Despite the $M1_{23SNP}$ sublineage being able to produce SpeA at a level that is indistinguishable from
145 $M1_{UK}$ isolates [12], it has been fully outcompeted by $M1_{UK}$ in England [4]. Although increased SpeA
146 production is predicted to perpetuate community transmission, the fitness advantage(s) of $M1_{UK}$ may
147 yet require additional features that promote *emm*1 survival in the human population, underlining a
148 need for further research and surveillance.
149

150 **Methods and Materials**

151 **Bacterial strains and growth conditions**

152 All bacterial strains used in this study are described in Table 1. *S. pyogenes* isolates were routinely
153 cultured in Todd Hewitt Broth (THB; Oxoid), on THY (THB supplemented with 1% (w/v) yeast extract)
154 agar or Columbia blood agar at 37ºC in 5% CO_2. *Escherichia coli* One Shot™ TOP10 was grown in
155 lysogeny broth (LB) or LB agar at 37ºC. For maintaining and selecting pUCMUT-containing *E. coli*
156 and *S. pyogenes*, 50 µg/mL and 400 µg/mL of kanamycin were added to the growth medium,
157 respectively.

158

159 **Introduction of the ssrA G-to-C SNP into the $M1_{global}$ and $M1_{13SNP}$ strains**

160 The ssrA G-to-C SNP was introduced separately into *S. pyogenes* $M1_{global}$ and *S. pyogenes* $M1_{13SNP}$
161 by allelic exchange (Figure 2A) using the suicide vector pUCMUT [19], which harbours the *aph3*
162 gene (coding for kanamycin resistance cassette (Kan^R)). *aph3* is flanked by two combinations of
163 restriction sites on either side: PstI + SalI and KpnI + EcoRI.

164

165 The 5' region upstream of ssrA was PCR-amplified from the genomic DNA of *S. pyogenes* $M1_{UK}$
166 H1490 using primers 993P1 (5'-AACTGCAGCTATCATCCTTGCATTTGC-3') and 993P2 (5'-
167 ACGCGTCGACGGTTTGGTGTTAATTTGATAAAC-3'), and the resulting 554-bp PCR product was
168 cloned into the PstI and SalI sites of pUCMUT, resulting in pUCMUT5s. Next, the 3' region containing
169 the ssrA SNP was PCR-amplified using primers 994P3 (5'-
170 GGGGTACCTTAATGCTGTAAGAACCAAGATATAA-3') and 994P4 (5'-
171 AGCGAATTCATAATGCCTGTCGAATCC-3'), and the 735-bp PCR product was cloned into the KpnI
172 and EcoRI sites of pUCMUT5s to yield the suicide plasmid pUCMUTssrA_M1UK. All constructed
173 plasmids were verified by sequencing at Full Circle Labs.

174

175 pUCMUTssrA_M1UK was electroporated into the recipient strains, *S. pyogenes* $M1_{global}$ H1499 and
176 *S. pyogenes* $M1_{13SNP}$ H1750, and was allowed to cross into the chromosome by homologous

177 recombination. The presence of the ssrA G-to-C SNP in the selected strains resulting from a double
178 allelic exchange was verified by PCR and Sanger sequencing (Genewiz).
179
180 **Detection of SpeA via immunoblotting**
181 SpeA production in various *S. pyogenes* isolates was assessed using immunoblotting with anti-
182 rSpeA rabbit serum. An overnight culture of *S. pyogenes* (50 mL) was harvested by centrifugation
183 (2,500 rpm, 10 min, 4ºC, swing bucket rotor). The supernatant was passed through an 0.2-µm filter
184 (Sartorius) to obtain a cell-free supernatant. A 500-µL aliquot of this cell-free supernatant was
185 concentrated 5x using a 10 kDa MWCO ultra centrifugal filter (Amicon). The concentrated
186 supernatant was denatured in 1x Bolt™ LDS Sample Buffer (ThermoFisher) containing 300 mM DTT,
187 prior to being fractionated on an 10% Bolt™ Bis-Tris Plus Gel (ThermoFisher). Fractionated proteins
188 were electroblotted onto a nitrocellulose membrane. After blocking the membrane with 5% (w/v) skim
189 milk buffer and 0.05% Tween-20, it was then probed with anti-rSpeA rabbit serum (1:1,000 dilution)
190 [12], and followed by an HRP-conjugated anti-rabbit secondary antibody (1:80,000 dilution). Probed
191 SpeA was detected using chemiluminescence reagents (Cytiva Amersham™ ECL™ Prime Western
192 Blotting Detection Reagents), and the blot was visualised on a Li-COR Odyssey Fc imaging system.
193
194 **Quantification of the RNA reads in $M1_{global}$ and $M1_{UK}$ strains**
195 The average read coverage at genomic regions covering *ssrA*, *paratox*, and *speA* was determined
196 according to Davies et al [10].
197
198 **Author contributions**
199 AV, VWCS, HKL, and SS conceived the work described. AV, VS, HKL, XZ, EJ, LR, KKH, KYM, OC,
200 JC (UKHSA) undertook data collection, analysis, and data presentation. AV, VWCS, and SS wrote
201 the first draft of the report and all authors contributed to its revision.
202
203 **The authors declare no competing interests**
204

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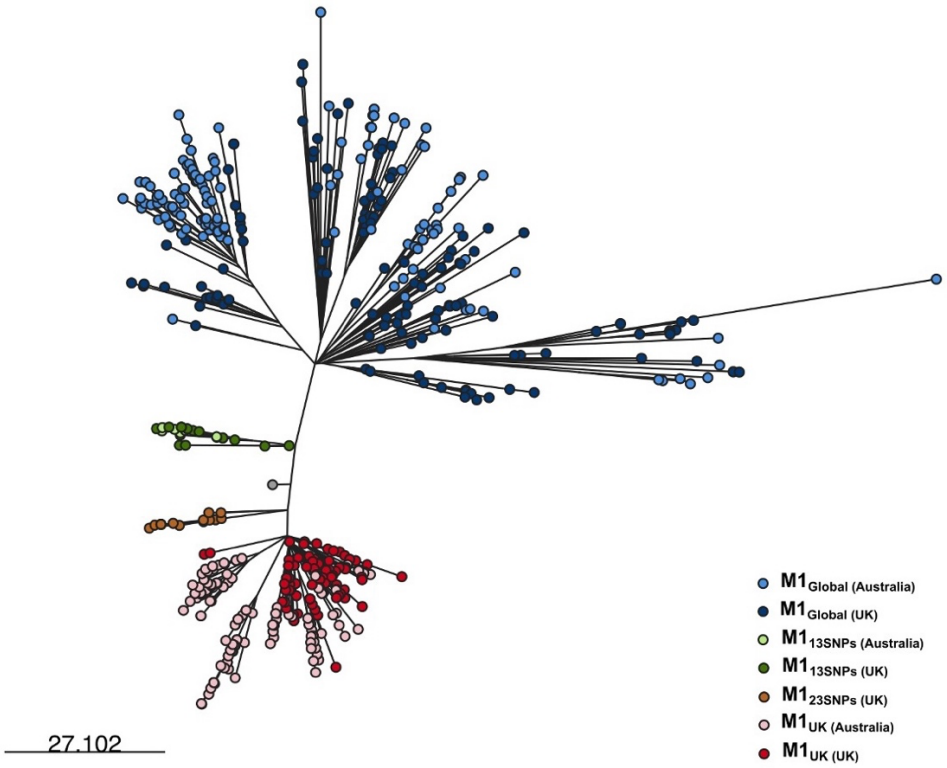


Figure 1. M1$_{UK}$ and sublineages

Phylogenetic tree constructed with 413 core SNPs (without recombination regions) obtained by mapping 269 *emm*1 *S. pyogenes* isolates selected from UK to demonstrate sublineages [12] with 319 isolates from Australia [10] to the reference genome MGAS5005. Coloured tips indicate the various sublineages, with Australian strains indicated by lighter colours and a UK strain with 19SNPs in grey.

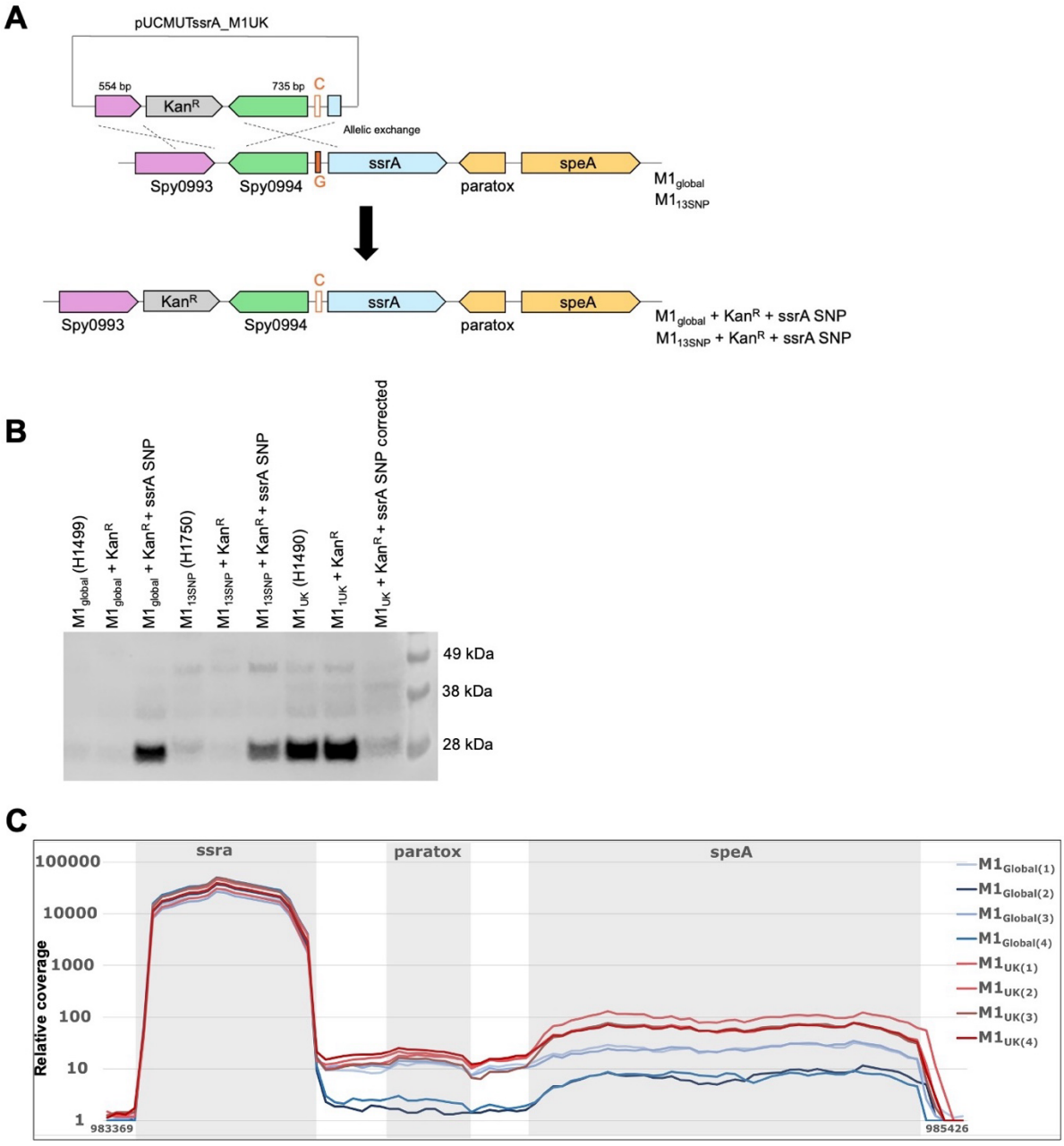
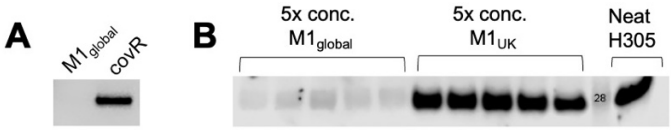


Figure 2. Complex regulation of SpeA production

A. The overall schematic for introducing the ssrA SNP into a *S. pyogenes* $M1_{global}$ and $M1_{13SNP}$ lineages. **B.** Introduction of the ssrA SNP into $M1_{global}$ strain H1499 and $M1_{13SNP}$ strain H1750 results in detectable SpeA in supernatants when tested using SpeA immunoblot. AphA3 (KanR) was introduced upstream of the ssrA leader sequence as a selectable marker in all transformants. Only transformants with the ssrA SNP introduced expressed detectable SpeA. In contrast, M1UK strain H1490 expressed considerably lower SpeA upon correction of its ssrA SNP. **C.** RNA sequencing read abundance across *ssrA*; an intergenic region which includes *ptx (paratox)*; and *speA* comparing four $M1_{global}$ strains (blue lines) and four $M1_{UK}$ strains (red lines). All were non-invasive throat isolates. $M1_{global}$ isolates were BHS0674 (H1499); BHS0162 (H1489); BHS0130 (H1504); and BHS0151 (H1488). $M1_{UK}$ isolates were BHS0170 (H1490); BHS0128 (H1503); BHS0258 (H1491); and BHS0581 (H1496).

313



314

315 **Figure 3. Regulation of SpeA production beyond ssrA SNP**

316 **A**. SpeA immunoblot of supernatant from $M1_{global}$ strain H584 and an isogenic covR T65P mutant (H1565).

317 **B.** SpeA immunoblot of concentrated *emm*1 supernatants from $M1_{global}$ strains (H1488; H1489; H1492;

318 H1495; H1499) and $M1_{UK}$ strains (H1490; H1491; H1493; H1539; H1496) (all non-invasive throat strains)

319 compared with non-concentrated supernatant from *emm*1 strain H305 (NCTC8198).

320 **Table 1. Bacterial strains used in this study.**

Strain	Description	Reference/Source
S. pyogenes $M1_{global}$ H1499	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H1499 + Kan^R	Isogenic strain of H1499 with only the Kan^R introduced upstream of ssrA	This study
S. pyogenes $M1_{global}$ H1499 + Kan^R + ssrA SNP	Isogenic strain of H1499 with both the Kan^R and ssrA SNP (G-to-C) introduced	This study
S. pyogenes $M1_{13SNP}$ H1750	PHE iGAS collection	[12]
S. pyogenes $M1_{13SNP}$ H1750 + Kan^R	Isogenic strain of H1750 with only the Kan^R introduced upstream of ssrA	This study
S. pyogenes $M1_{13SNP}$ H1750 + Kan^R + ssrA SNP	Isogenic strain of H1750 with both the Kan^R and ssrA SNP (G-to-C) introduced	This study
S. pyogenes $M1_{UK}$ H1490	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H584	Invasive puerperal sepsis blood isolate	[23]
S. pyogenes $M1_{global}$ covR H1565	Isogenic CovR T65P mutant of H584	[24]
S. pyogenes $M1_{global}$ H1488	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H1489	Imperial ENT isolate collection	[3]

S. pyogenes $M1_{global}$ H1492	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H1495	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H1504	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H1488	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{UK}$ H1491	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{UK}$ H1493	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{UK}$ H1539	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{UK}$ H1496	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{UK}$ H1503	Imperial ENT isolate collection	[3]
S. pyogenes $M1_{global}$ H305	NCTC8198	[19]
E. coli One Shot™ TOP10	Cloning host	Invitrogen

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