Streptococcus suis encodes multiple allelic variants of a phase-variable Type III DNA methyltransferase, ModS, that control distinct phasevarions

Greg Tram[a], Freda E.-C. Jen[a], Zachary N. Phillips[a], Jamie Timms[a], Asma-Ul Husna[a], Michael P. Jennings[a], Patrick J. Blackall[b] & John M. Atack[a]*

[a]Institute for Glycomics, Griffith University, Gold Coast, Queensland 4222, Australia

[b]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St. Lucia, Queensland, 4072, Australia

Running title: Characterisation of *modS* phasevarions in *S. suis*

*Address for correspondence – John M. Atack, Institute for Glycomics, Griffith University, Gold Coast, Queensland 4215, Australia, Tel: (+61) 07 56780580; Fax: (+61) 07 55527050; E-mail: j.atack@griffith.edu.au

Abstract

Streptococcus suis is a significant cause of bacterial meningitis in humans, particularly in S.E. Asia, and is a leading cause of respiratory and invasive disease in pigs. Phase-variable DNA methyltransferases, associated with Restriction-Modification (R-M) systems, are a source of epigenetic gene regulation, controlling the expression of multiple genes. These systems are known as phasevarions (phase-variable regulons), and have been characterised in many host-adapted bacterial pathogens. We recently described the presence of a Type III DNA methyltransferase in *S. suis*, ModS, which contains a simple sequence repeat (SSR) tract within the open reading frame of the *modS* gene, and which varied in length between individual strains. We also observed multiple allelic variants of the *modS* gene were present in a population of *S. suis* isolates. Here, we demonstrate that a biphasic ON-OFF switching of expression occurs in the two most common ModS alleles, ModS1 and ModS2, and that switching is dependent on SSR tract length. Further, we show that ModS1 and ModS2 are active methyltransferases in *S. suis* using Single-Molecule, Real Time (SMRT) sequencing. ON-OFF switching of each ModS allele results in the regulation of distinct phasevarions, with the ModS2 phasevarion impacting growth patterns and antibiotic resistance. This is the first demonstration of a phase-variable Type III DNA methyltransferase in a Gram-positive organism that controls a phasevarion. Characterising the phenotypic effects of phasevarions in *S. suis* is key to understanding pathogenesis and the development of future vaccines.

Importance

Streptococcus suis is a causative agent of meningitis, polyarthritis and polyserositis in swine, and is a major cause of zoonotic meningitis in humans. Here we investigate epigenetic gene

regulation in *S. suis* by multiple phasevarions controlled by the phase-variable Type III DNA methyltransferase ModS. This is the first characterised example of a Type III R-M system regulating a phasevarion in a Gram-positive organism. We demonstrate that biphasic ON-OFF switching of ModS expression results in differences in bacterial growth and antibiotic resistance. Understanding the effects of ModS phase variation is required to determine the stably expressed antigenic repertoire of *S. suis*, which will direct and inform the development of antimicrobial treatments and vaccines against this important pathogen.

Introduction

Streptococcus suis is an important veterinary pathogen that contributes a substantial disease burden to the swine industry (1). *S. suis* is also a significant pathogen in humans, causing zoonotic meningitis, most commonly associated with occupational exposure (2) and consumption of contaminated pork products (3). *S. suis* frequently colonises the upper respiratory tract of pigs, where it is considered a commensal organism (4). Virulent strains of *S. suis* are able to adhere to and invade epithelial cells of the airway allowing access to the bloodstream. In both pigs and humans, *S. suis* is able to breach the blood brain barrier, resulting in the development of meningitis and septic shock (4).

Phase variation is the rapid and reversible switching of gene expression, and plays an important role in the pathogenesis of many organisms, where it is usually associated with expression of surface factors such as capsules (5), adhesins (6, 7) and lipooligosaccharide biosynthesis (8). Randomised gene expression as a result of phase variation can complicate vaccine development as it results in an unstable antigenic repertoire, and is potentially a major cause of vaccine escape. Many bacterial pathogens also encode cytoplasmically located phase-variable DNA methyltransferases, that are associated with restriction-modification (R-M) systems (9). Variable methyltransferase expression results in genome wide methylation differences, resulting in differential gene regulation by epigenetic mechanisms in systems called phasevarions (phase variable regulons) (9, 10). Many phasevarions are controlled by the ON-OFF switching of Type III DNA methyltransferases, encoded by *mod* genes. Phasevarions controlled by Type III *mod* genes have been described in *Haemophilus influenzae* (11, 12), *Moraxella catarrhalis* (13), *Neisseria* spp. (14), *Helicobacter pylori* (15) and *Kingella kingae* (16). The phasevarions in these human adapted

pathogens all control expression of genes involved in the pathogenesis of these organisms (17-19). All these phase variable *mod* genes contain simple DNA sequence repeat (SSR) tracts within their open reading frame. These SSRs tracts are highly unstable and prone to variation in length due to slipped-strand mispairing during DNA replication, resulting in the biphasic ON-OFF switching of gene expression; the *mod* gene is either in-frame and Mod is expressed (ON), or a variation in SSR tract length results in a frameshift and premature stop codon, and Mod is not expressed (OFF) (20).

The methyltransferase specificity of a Type III Mod protein is dictated by the central target recognition domain (TRD) of the encoding *mod* gene (9, 10). This TRD varies between the alleles of individual *mod* genes, with the 5′ and 3′ regions of individual *mod* genes being highly conserved between alleles (9, 10). For example, there are twenty-one *modA* alleles encoded by non-typeable *Haemophilus influenzae* (NTHi) and *Neisseria* species; six *modB* and seven *modD* alleles have been identified in *Neisseria gonorrhoeae* and *Neisseria meningitidis*; and nineteen *modH* alleles are present in *H. pylori* (10, 21). Our previous analysis of the restriction enzyme database REBASE demonstrated the presence of a *mod* gene in *S. suis*, which we have named *modS*, that contained a $GAGCA_{(n)}$ SSR tract (22). A follow up study analysing of a large collection of *S. suis* isolates determined the presence of three distinct *modS* alleles, each of which methylated an adenine in distinct DNA sequences. ModS1 methylated 5′-$GCG^{(m6)}$**A**DT-3′ (D is either A,G or T), ModS2 methylated 5′-$VTC^{(m6)}$**A**TC-3′ (V is either A,G or C), and ModS3 (present in a single strain identified in GenBank) methylated 5′-$GTTC^{(m6)}$**A**NNNB-3′ (B is either C, G or T; N is any nucleotide) (23). These specificities were determined through heterologous expression of the methyltransferase in *E. coli*. Whilst variable length $GAGCA_{(n)}$ SSR tracts were present in

different strains of *S. suis* encoding the same *modS* allele, it was not determined whether *modS* is phase variable, or whether the ModS protein is an active methyltransferase in *S. suis* when expressed.

In this study we investigated phase variation of the two most prevalent *modS* alleles, *modS1* and *modS2*, by enriching populations of *S. suis* strains for specific $GAGCA_{(n)}$ SSR tract lengths in each encoding *modS* gene. We then determined if differential protein expression occurred in enriched ON-OFF populations of *S. suis* using SWATH-MS. Clinically relevant phenotypes were assessed *in vitro* to determine if phase variation of the ModS methyltransferase could result in relevant phenotypic changes or advantages *in vivo*. Studying the impact of these systems provides an understanding of not only disease pathogenesis, but will also guide the development of vaccines by defining the stably expressed antigenic repertoire of *S. suis*.

Results

ModS is a phase variable DNA methyltransferase

In all previous examples of phase-variable Type III *mod* genes, SSR tract length variation results in a biphasic ON-OFF switching of expression. In order to determine if variation in length of the $GAGCA_{(n)}$ repeat tract (Figure 1A) located in the *modS* open reading frame led to phase variable expression of the gene, isogenic strains were generated containing defined lengths of this SSR tract. The isolation of three consecutive repeat tract lengths would theoretically result in one strain where the respective *modS* gene is ON, ($GAGCA_{(n)}$ repeat tract length will place the gene in frame and expressed), and two strains of the triplet

will be OFF ($GAGCA_{(n)}$ repeat tract length will place the gene out of frame and not expressed). These enriched populations were generated in *S. suis* strains LSS89 (*modS1*) or SS1056 (*modS2*), as our analysis of the TRDs of these two alleles showed they were highly variable, and therefore predicted to methylate different target sequences (Figure 1B) as we demonstrated with heterologous over-expression of ModS1 and ModS2 previously (23). Strains were enriched for SSR tract lengths containing either 19, 20 or 21 repeats in *modS1* and 17, 18 or 19 repeats in *modS2.* Fragment analysis of each strain confirms enrichment of each SSR tract length to above 80% (Figure 1C).

Western blotting of whole cell lysates of these enriched strains with antisera generated against the conserved region of all ModS proteins demonstrate that variation in SSR tract length led to a biphasic ON-OFF switching of expression (Figure 1D; full blot in Supplementary Figure 1). The ModS1 protein was only produced in the LSS89 strain which contained 21 GAGCA repeats in the *modS1* gene. ModS2 was only produced in the SS1056 strain enriched for 18 GAGCA repeats. This matches the prediction from the annotated start codon of both genes (Supplementary Figure 2).

To demonstrate methyltransferase activity of the expressed ModS protein, Pacific Biosciences (PacBio) Single-Molecule, Real-Time (SMRT) sequencing was carried out on genomic DNA isolated from our triplet sets of enriched isogenic strains (19, 20, 21 repeats for *modS1* in strain LSS89; 17, 18, 19 repeats for *modS2* in strain SS1056). We detected a separate, distinct Type I methyltransferase motif in strain LSS89 compared to strain SS1056 (Table 1), with these motifs being fully methylated in all three strains of each enriched triplet set. When comparing the methylomes of enriched LSS89 strains, we detected a Type III methyltransferase motif, 5'-GCG$^{(m6)}$**A**T, only present in the strain where ModS1 was

expressed (21 repeats ON; Table 1). This methylation by ModS1 occurred at 2859 5'-GCG$^{(m6)}$**A**T sites throughout the genome, and was not detected in strains enriched for 19 or 20 GCACA repeats in the *modS1* gene. SS1056 enriched strains exhibited a different Type III motif, 5'-VTC$^{(m6)}$**A**TC, which was only present when ModS2 was expressed (18 repeats ON; Table 1). This motif was methylated 4810 times in the SS1056 genome, and again was not methylated in strains enriched for 17 or 19 repeats (OFF) in the *modS2* gene. These motifs are the same as detected previously when these ModS alleles were heterologously over-expressed in *E. coli* (5).

Biphasic switching of ModS1 and ModS2 results in the expression of distinct phasevarions

Quantitative proteomics was carried out to determine if distinct protein expression profiles occurred as a result of ModS expression in *S. suis*, i.e., if these phase-variable methyltransferases control phasevarions. The expression profiles of an ON-OFF ModS1 pair from strain LSS89 (21 repeats ON and 19 repeats OFF) and an ON-OFF ModS2 pair from strain SS1056 (18 repeats ON and 17 repeats OFF) were assessed using SWATH-MS. Both strain pairs exhibited unique changes in protein expression dependent on phase variation of their respective *modS* gene, with SWATH-MS covering ~23% of total proteins in LSS89 strains and ~22% of total in SS1056 strains. Significant differences in protein abundance between the ON and OFF populations are represented as volcano plots in Figure 2. In strain LSS89, ModS1 ON resulted in higher expression of several proteins involved in a range of cellular processes compared to strains where ModS1 was OFF. Multiple transcriptional regulators were upregulated by expression of ModS1 such as the replication initiator protein DnaA (24), and a YlbF/ YmcA type protein (25), as well as transcription factors and

161 repressors. Many ribosomal subunits were upregulated as well as those involved in general
162 metabolism such as a nitroreductase (26), glucokinase (27) and a protein involve in
163 alkylphosphonate utilisation (28) (Table 2). Conversely, in strains where ModS1 was not
164 expressed, proteins such as ribosomal proteins, several components of ABC transporters,
165 and the fatty acid binding protein DegV (29) were all increased in expression (Table 2).

166 In strain SS1056, ModS2 ON-OFF switching resulted in varied expression of a distinct set of
167 proteins (Table 3). ModS2 expression increased the levels of proteins involved in amino acid
168 metabolism such as cysteine synthase and aminopeptidase D (30). Proteins involved in
169 general metabolism also showed increased expression in ModS2 ON, such as
170 dihydroxyacetone kinase (31). The DNA biosynthesis enzymes ribonucleotide-diphosphate
171 reductase and ribonucleotide-triphosphate reductase (32) were increased in expression in
172 ModS2 ON, as was an acyl carrier protein involved in fatty acid biosynthesis (33). Three
173 proteins were upregulated in strains which did not express ModS2 (OFF); an ATP-dependent
174 Clp protease, the nucleotide exchange factor GrpE and a glyoxalase/bleomycin
175 resistance/extradiol dioxygenase family protein involved in resistance to antimicrobials.

176

177 ***ModS switching results in differences in growth***

178 Our proteomic analysis of ModS switching demonstrated several proteins which could
179 potentially impact growth rate exhibited differential regulation in both ModS alleles. These
180 included proteins involved in general metabolic enzymes, DNA transcription factors and
181 repressors as well as biosynthesis enzymes (Tables 2 & 3). In order to determine if these
182 altered protein expression levels could affect *S. suis* growth rates, we conducted standard
183 growth curves of our enriched ModS ON-OFF pairs in rich media. These growth curves

184 showed a small but repeatable (n = 3) difference in growth rate for both strain pairs (Figure
185 3): when ModS1 was ON, growth was to a lower final OD (P value <0.05), and conversely,
186 when ModS2 was ON, growth was to a higher final OD (P Value <0.05).

187

188 ***ModS2 phase variation results in differences in antibiotic resistance***

189 SWATH-MS also demonstrated differential expression of an antibiotic resistance protein in
190 ModS2 (WP_002935876.1), annotated as a glyoxalase/bleomycin resistance/extradiol
191 dioxygenase family protein, which exhibited higher expression in strains which did not
192 express ModS2 (OFF) compared to ModS2 ON. We therefore carried out a minimum
193 inhibitory concentration (MIC) analysis using several beta lactam antibiotics (ampicillin,
194 penicillin and amoxicillin) and a glycopeptide antibiotic (vancomycin) (Table 4). Although
195 there was no change in MIC to amoxicillin, vancomycin and penicillin, there was a two-fold
196 increase in resistance to ampicillin when ModS2 was OFF compared to ModS2 ON (0.32
197 µg/mL vs 0.16 µg/mL respectively).

198

199 **Discussion**

200 Over the last 15 years, phasevarions (phase-variable regulons) controlled by ON-OFF
201 switching of Type III *mod* genes have emerged as an important gene regulation strategy in a
202 diverse range of host-adapted pathogens (9, 12, 13, 15, 16, 21). In all these examples, phase
203 variable ON-OFF switching of *mod* expression occurs via changes in the length of locus
204 encoded SSR tracts. In this study, using a combination of strain enrichment, Western
205 blotting, and SMRT sequencing, we conclusively demonstrate that *S. suis* contains multiple

allelic variants of a new phase-variable Type III *mod* gene, *modS*, with this representing the first example of a phasevarion controlled by biphasic ON-OFF switching of a Type III DNA methyltransferase in a Gram-positive organism (Figure 1). The antisera used to detect ModS was generated against the conserved regions of ModS, and should recognise all ModS alleles with equal affinity, as sequence variation of Mod proteins only occurs in the central TRD region (9, 34). Both ModS alleles were only expressed in strains with specific SSR tract lengths; ModS1 was only present in strains with 21 GAGCA repeats and ModS2 was only expressed in strains with 18 GAGCA repeats. In both cases, the repeat length placed the *modS* gene in the correct reading frame and demonstrates that the expression of the ModS protein is dependent on the SSR tract length.

SMRT sequencing was used to determine methyltransferase activity and specificity of ModS1 and ModS2. LSS89 strains of *S. suis* enriched for ModS1 expression (21 GACAC repeats ON) exhibited methylation of the adenine in 5'-GCG^{m6}**A**T motifs which was not seen in strains in which ModS1 was OFF (19 or 20 GACAC repeats). ModS2 expressing strains (SS1056) of *S. suis*, exhibited methylation of a Type III Mod motif of 5'-VTC^{m6}**A**TC motifs (where V can be either A, G or C) when ModS2 was predicted to be ON (18 GACAC repeats). This confirmed our previous findings when we over-expressed ModS1 and ModS2 using a recombinant *E. coli* system (23). SMRT sequencing also demonstrates that both ModS1 and ModS2 are active methyltransferases in *S. suis*, methylating at thousands of sites in their respective genomes (in strain LSS89, 2861 5'-GCG^{m6}**A**T sites are present; in SS1056, 4812 5'-TC^{m6}**A**TC sites are present; Supplementary Data 1).

SWATH-MS proteomic analysis confirmed that the altered methylation resulting from ModS phase-variation results in a change in the expression of numerous proteins in distinct

229 phasevarions. These changes in protein expression were seen across many families of
230 protein, including those involved in central metabolism, gene regulation, and transporters.
231 Phase-variable switching of ModS resulted in differences in growth rate, but it is unclear
232 whether this is a result of an alteration of regulatory genes due to ModS switching, or
233 another, as yet uncharacterised effect of ModS phase variation. Although the final
234 differences in final OD between enriched ON and OFF strain pairs were small, they are
235 statistically significant (P value <0.05), and could have an effect on both long-term carriage
236 and disease, particularly if one population has an advantage resulting from differential
237 protein expression over time. ModS2 phase variation also resulted in a difference in
238 antibiotic resistance. A protein repressed by ModS2 expression, annotated as a glyoxalase,
239 bleomycin resistance and extradiol dioxygenase family protein, described as involved in
240 resistance to beta-lactam and glycopeptide antibiotics (35), which are commonly used to
241 treat bacterial infections in humans as well as commercial pig farms (36). The MIC of *S. suis*
242 was assessed against three commonly used beta lactams (ampicillin, amoxicillin and
243 penicillin) and a glycopeptide from the same antibiotic class as bleomycin (vancomycin). A
244 strain in which ModS2 was OFF, where the expression of this resistance protein was
245 increased, showed a small (2-fold) increase in MIC towards ampicillin, compared to the
246 isogenic strain in which ModS2 was ON. Although small, there may be a cumulative effect,
247 particularly as many beta lactam antibiotics are extensively used prophylactically in
248 commercial pig farms (37-40). This has the potential to result in a long-term increase in
249 ampicillin resistance in *S. suis* populations, although this would need to be studied in a
250 model system and is beyond the scope of this work.

251 Although many of the changes in protein expression determined by our SWATH-MS analysis
252 of enriched ON-OFF population in each ModS allele were small (1.5-fold to 2-fold), SWATH-

MS is a technique which quantitates protein expression based on abundance (41). Differential expression of proteins will therefore only identify differences in highly expressed proteins, and expression differences of proteins beyond the limit of detection will be missed. We recognise this is a limitation of our approach, but a SWATH-MS proteomics approach has been previously used to characterise phasevarion mediated changes to protein expression (13, 42), with similar small differences in expression also reported in these phasevarions. Therefore, whilst we have determined that both ModS1 and ModS2 affect expression of small distinct sets of proteins, it is likely that further changes to gene and protein expression remain undetected. In order to thoroughly characterise each phasevarion, further analysis will be needed. It will also be important to characterisation the phasevarions controlled by other *modS* alleles; in previous work we showed that a third *modS* allele – *modS3* – was present in *S. suis*. The ModS3 allele methylated a distinct sequence to ModS1 and ModS2 (23), and is therefore highly likely to control a different phasevarion. The presence of additional *modS* alleles should also be determined, and studied, in order to detail all gene and protein expression changes mediated by phasevarions in *S. suis*.

This characterisation of ModS is the first described instance of a phasevarion in a Gram-positive organism controlled by a Type III methyltransferase. Different alleles of ModS methylate distinct motifs and result in distinct phasevarions. These distinct ModS phasevarions result in gross differences in growth rate, and in the case of ModS2, differential resistance to antibiotics. Both these phenotypes could affect disease and pathology, and this remains to be studied using both *in vitro* models and ideally, an *in vivo* challenge using the natural host (pigs). A thorough understanding of phase variation of gene expression, and in particular phasevarions, is required in order to determine the stably

277 expressed antigenic repertoire of a bacterial species. The prevalence of phase variable
278 methyltransferases across the bacterial domain demonstrates that phasevarions are a
279 widespread contingency strategy (22, 43, 44), and that characterisation of these systems is
280 imperative in order to rationally design effective vaccines that only target stably expressed
281 antigens in the organisms where they are present.

282

283 **Materials and methods**

284 ***Bacterial strains and growth***

285 *S. suis* strains LSS89 and SS1056 (45) used in this study were grown in THB broth (Oxoid)
286 supplemented with 2% yeast extract (THB-Y) or on THB-Y plates (THB-Y broth supplemented
287 with 1.5% w/v agar). Cultures were incubated overnight at 37°C.

288 ***Strain enrichment***

289 Fragment length analysis of the SSR tract in each *modS* allele was conducted to determine
290 the length of the $GAGCA_{(n)}$ SSR tract. A PCR was performed using a fluorescently labelled
291 forward primer SsuT3-F-FAM (5'-FAM-CAT CAA AAA CGG CTT GAC AGC C) and the reverse
292 primer SsuT3-R (5'-GCA ATG TTG TCT GAT AAA ACA TCT TTT G) as described previously (23).
293 DNA fragment length analysis was carried out at the Australian Genome Research Facility
294 (AGRF; Brisbane, Australia). This technique was used to enrich populations of *S. suis* strains
295 for defined SSR lengths through a combination of fragment length analysis and subculturing.
296 *S. suis* populations of strain LSS89 (encoding *modS1*) were enriched for 19, 20 and 21
297 GAGCA repeats, and strain SS1056 (encoding modS2) were enriched for 17, 18, 19 GAGCA

298 repeats. Populations containing >80% of each single tract length were considered to be
299 enriched, and were used in subsequent studies.

300 ***SDS-PAGE and Western blot***

301 Cell lysates of each enriched strain were prepared from overnight liquid cultures of *S. suis*
302 grown in THB-Y broth. Samples were normalised to $O.D._{600}$ 30.0, pelleted at 12,000xg for 1
303 minute and resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% SDS, 50 mM
304 Tris HCl, pH 8). Samples were sonicated twice for 30 seconds at a probe intensity of 15
305 amplitude microns, on ice. Lysates were clarified by centrifugation at 14,000xg for 20
306 minutes to remove cellular debris.

307 Supernatants were prepared for SDS-PAGE using BOLT 4x loading dye containing 100 mM
308 dithiothreitol (Sigma) and boiled for 30 minutes, then loaded on precast 4-12% BOLT Bis-Tris
309 gel (Thermo Fisher) and run for 45 minutes at 165V in MOPs 1x running buffer according to
310 manufacturer's instructions (Thermo Fisher). Proteins were visualised by Coomassie brilliant
311 blue staining. Twice the amount of lysate was loaded onto gels for use in Western blotting
312 and proteins were transferred to pre-equilibrated polyvinylidene difluoride (PVDF)
313 membranes (BioRad) at 20 volts for 60 minutes using the BOLT transfer system (Thermo
314 Fisher). Following transfer, membranes were blocked with 10% skim milk in TBS-T for 60
315 minutes. Membranes were incubated with a 1:1,000 dilution of anti-ModS antisera (raised
316 as detailed below) overnight at 4°C, followed by a 1:10,000 anti-mouse alkaline-
317 phosphatase secondary antibody (Sigma). Blots were developed using NBT/BCIP (Roche)
318 according to manufacturer's instructions.

319 ***Construction of a TRD-less ModS protein***

The *modS1* gene was amplified from *S. suis* strain LSS89 excluding the $CAGCA_{(n)}$ SSR tract using primers SsuT3-oE-F (5'-AGTCAG CATATG AGC AGA GCA AAG CAA AAG CTT GGA GAA TAC ACT CAA G) and SsuT3-oE-R (5'-AGTCAG GGATCC CTA CAC CAC CTT CAC TTT GGT ACC) with KOD Hot Start DNA polymerase (Merck Millipore) according to manufacturer's instructions. The resulting product was then cloned into the NdeI-BamHI site of the expression vector pET15b (Novagen) with the gene in-frame with the N-terminal His-tag to generate vector pET15b:SsuT3-His_tag. The TRD coding region of *modS* was then removed and the conserved 5' and 3' coding regions were fused. This was generated by using the pET15b::SsuT3-His_tag vector as a template via inverse PCR with primers SsuT3-TRD-remove-F (5'-CCCA AAT ATC TCA TCA CAG ATA GCT TTG AGG TTG G) and SsuT3-TRD-remove-R (5'-GCT GTT ATG CAG CTG AAT GCA GAA GAT GG) binding either side of the TRD, using KOD Hot Start DNA polymerase (Merck Millipore) according to manufacturer's instructions. His-tagged TRD-less ModS was over-expressed in *E. coli* BL21 using isopropyl β-d-1-thiogalactopyranoside (IPTG) induction (0.5 mM) overnight at 37°C with 200 rpm shaking, and protein purified using TALON resin (Takara Bio) using standard protocols in 50 mM phosphate buffer pH7.4 containing 300mM NaCl.

Generation of ModS Antisera

A cohort of five 6-8 week old female BALB/c mice (Animal Resources Centre, WA, Australia) were immunized subcutaneously with 25 µg of recombinant TRD-less ModS protein in 25uL PBS, mixed with 25 µL Freund's Adjuvant (Merck, Darmstadt, Germany; Freund's Complete Adjuvant (FCA) on day 0 and Freund's Incomplete Adjuvant (FIA) subsequently on days 14, 21, 28 and 42. Terminal bleeds were collected on day 58 and serum separated via centrifugation. Pre-immune (naïve) serum was collected from cohorts prior to immunization

via tail bleed. Serum was stored in 50% glycerol at -20°C. This antisera recognises all ModS alleles with equal affinity as it was raised against the conserved regions of ModS shared by all alleles. All animal work was carried out according to the Australian Code for the Care and Use of Animals for Scientific Purposes, with approval from the Griffith University Animal Ethics Committee (GLY/16/19/AEC).

Single-Molecule, Real-Time (SMRT) sequencing and methylome analysis

Genomic DNA from our enriched triplet sets of *S. suis* strains LSS89 (*modS1* 19, 20, 21 repeats) and SS1056 (*modS2* 17, 18, 19 repeats) was prepared from an overnight culture in THB-Y broth and high-molecular-weight genomic DNA was isolated using the Sigma Genelute kit (Sigma Aldrich) according to the manufacturer's instructions. SMRT sequencing and methylome analysis was carried out as previously described (46, 47). Briefly, DNA was sheared to an average length of approximately 5-10 kb (genomic DNA) using g-TUBEs (Covaris, Woburn, MA, USA) and SMRTbell template sequencing libraries were prepared using sheared DNA. DNA was end repaired, then ligated to hairpin adapters. Incompletely formed SMRTbell templates were degraded with a combination of Exonuclease III (New England Biolabs; Ipswich, MA, USA) and Exonuclease VII (USB; Cleveland, OH, USA). Primer was annealed and samples were sequenced on the PacBio Sequel system (Menlo Park, CA, USA) using standard protocols for long insert libraries. SMRT sequencing and methylome analysis was carried out at SNPSaurus (University of Oregon, USA).

SWATH-MS proteomics

Overnight cultures of each *S. suis* strain (10^7 CFU/ml) were harvested, lysed in guanidium buffer (6 M guanidium chloride, 50 mM Tris-HCl pH8, 10 mM dithiothreitol) and incubated at 30°C for 30 minutes with shaking (500 rpm). Cysteines of the total protein were alkylated

by addition of acrylamide to a final concentration of 25 mM and incubated at 30°C for 60 minutes with shaking (500 rpm). Concentration of samples was assessed using a Nanodrop 2000 (Thermo Fisher). A 100 µg aliquot of the protein was then precipitated by addition of 1:1 methanol: acetone at -20°C overnight. The protein was pelleted at 18,000xg for 10 minutes and supernatant was removed before the pellet was resuspended in 50 µL trypsin reaction buffer and 1 µg trypsin (New England Biolabs) added and the suspension incubated overnight at 37°C. Tryptic digested peptides were then desalted and purified using a Ziptip (Millipore) as per manufacturer instructions. SWATH-MS was performed as previously described (48). Briefly, tryptic peptides were analyzed by LC-ESI-MS/MS using a Prominence nanoLC system (Shimadzu) and Triple TOF 5600 mass spectrometer with a Nanospray III interface (SCIEX). Peptides were separated on a Vydac EVEREST reversed-phase C18 HPLC column at a flow rate of 1 µL/min. A gradient of 10−60% buffer B over 45 min, with buffer A (1% acetonitrile and 0.1% formic acid) and buffer B (80% acetonitrile and 0.1% formic acid) was used. An MS-TOF scan was performed from an m/z range of 350− 1800 for 0.5 s followed by information dependent acquisition of MS/MS of the top 20 peptides from m/z 40−1800 for 0.05 s per spectrum, with automated CE selection. Identical LC conditions were used for SWATH-MS. SWATH-MS of triplicate biological replicates was performed with the same MS-TOF scan, followed by high sensitivity information-independent acquisition with m/z isolation windows with 1 m/z window overlap each for 0.1 s across an m/z range of 400−1250. Collision energy was automatically assigned by Analyst software (AB SCIEX) based on m/z window ranges. Proteins were identified by searching against *S. suis* Lss89 and SS1056 genomes (NCBI Accession GCA_900059105.1 and GCA_900051945.1 respectively) and common contaminants with standard settings using ProteinPilot 5.0.1 (AB SCIEX). False discovery rate analysis was performed on all searches. ProteinPilot search results were used

as ion libraries for SWATH analyses. The abundance of proteins was measured automatically using PeakView (AB SCIEX) with standard settings. Comparison of protein relative abundance was performed based on protein intensities or ion intensities using a linear mixed-effects model with the MSstats package in R. Proteins with greater than X changes in abundance and with adjusted P-values. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (49) partner repository with the dataset identifier PXD023726.

Minimum Inhibitory Concentration (MIC) assay

The MIC was measured by broth microdilution in triplicate experiments based on CLSI guidelines as described previously (50). Briefly, an overnight culture of *S. suis* was diluted to OD=0.1 (A_{600}) and sub-cultured grown to mid log for 3 hours at 37°C. Mid log cultures were diluted to OD=0.2 (A_{600}) and 50 µl of each culture was added to 96-well plates containing serially diluted antibiotic concentrations (5 µg/mL - 0.08µg/mL), and plates grown at 37°C with 5% CO_2 for 24 hours. The MIC (mg/L) was determined as the last dilution at which turbidity was observed following overnight growth with all assays being performed in triplicate.

Acknowledgements

We thank Amanda Nouwens and Peter Josh in SCMB MS facility, University of Queensland for conducting SWATH-MS proteomic analysis. We thank Allison Banse and Eric Johnson at SNPsaurus (University of Oregon, USA) for conducting SMRT sequencing and methylome analysis. This work was funded by an Australian Research Council Discovery Project

412 DP180100976 to JMA and PJB, Australian National Health and Medical Research Council

413 (NHMRC) Principal Research Fellowship 1138466 to MPJ.

References

1. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. 2014. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect 3:e45.

2. Breton J, Mitchell WR, Rosendal S. 1986. Streptococcus suis in slaughter pigs and abattoir workers. Can J Vet Res 50:338-41.

3. Susilawathi NM, Tarini NMA, Fatmawati NND, Mayura PIB, Suryapraba AAA, Subrata M, Sudewi AAR, Mahardika GN. 2019. *Streptococcus suis*-Associated Meningitis, Bali, Indonesia, 2014-2017. Emerg Infect Dis 25:2235-2242.

4. Dutkiewicz J, Zajac V, Sroka J, Wasinski B, Cisak E, Sawczyn A, Kloc A, Wojcik-Fatla A. 2018. Streptococcus suis: a re-emerging pathogen associated with occupational exposure to pigs or pork products. Part II - Pathogenesis. Ann Agric Environ Med 25:186-203.

5. Tzeng YL, Thomas J, Stephens DS. 2016. Regulation of capsule in *Neisseria meningitidis*. Crit Rev Microbiol 42:759-72.

6. Atack JM, Winter LE, Jurcisek JA, Bakaletz LO, Barenkamp SJ, Jennings MP. 2015. Selection and Counterselection of Hia Expression Reveals a Key Role for Phase-Variable Expression of Hia in Infection Caused by Nontypeable Haemophilus influenzae. J Infect Dis 212:645-53.

7. Elango D, Schulz BL. 2020. Phase-Variable Glycosylation in Nontypeable *Haemophilus influenzae*. J Proteome Res 19:464-476.

8. Phillips ZN, Brizuela C, Jennison AV, Staples M, Grimwood K, Seib KL, Jennings MP, Atack JM. 2019. Analysis of Invasive Nontypeable *Haemophilus influenzae* Isolates Reveals Selection for the Expression State of Particular Phase-Variable Lipooligosaccharide Biosynthetic Genes. Infect Immun 87.

9. Seib KL, Srikhanta YN, Atack JM, Jennings MP. 2020. Epigenetic regulation of virulence and immunoevasion by phase-variable restriction-modification systems in bacterial pathogens. Annu Rev Microbiol 74:655-671.

10. Phillips ZN, Husna AU, Jennings MP, Seib KL, Atack JM. 2019. Phasevarions of bacterial pathogens - phase-variable epigenetic regulators evolving from restriction-modification systems. Microbiology 165:917-928.

11. Srikhanta YN, Maguire TL, Stacey KJ, Grimmond SM, Jennings MP. 2005. The phasevarion: a genetic system controlling coordinated, random switching of expression of multiple genes. Proc Natl Acad Sci U S A 102:5547-51.

12. Atack JM, Srikhanta YN, Fox KL, Jurcisek JA, Brockman KL, Clark TA, Boitano M, Power PM, Jen FE, McEwan AG, Grimmond SM, Smith AL, Barenkamp SJ, Korlach J, Bakaletz LO, Jennings MP. 2015. A biphasic epigenetic switch controls immunoevasion, virulence and niche adaptation in non-typeable Haemophilus influenzae. Nat Commun 6:7828.

13. Blakeway LV, Power PM, Jen FE, Worboys SR, Boitano M, Clark TA, Korlach J, Bakaletz LO, Jennings MP, Peak IR, Seib KL. 2014. ModM DNA methyltransferase methylome analysis reveals a potential role for Moraxella catarrhalis phasevarions in otitis media. FASEB J 28:5197-207.

14. Seib KL, Jen FE, Scott AL, Tan A, Jennings MP. 2017. Phase variation of DNA methyltransferases and the regulation of virulence and immune evasion in the pathogenic *Neisseria*. Pathog Dis 75.

15. Srikhanta YN, Gorrell RJ, Steen JA, Gawthorne JA, Kwok T, Grimmond SM, Robins-Browne RM, Jennings MP. 2011. Phasevarion mediated epigenetic gene regulation in *Helicobacter pylori*. PLoS One 6:e27569.

16. Srikhanta YN, Fung KY, Pollock GL, Bennett-Wood V, Howden BP, Hartland EL. 2017. Phasevarion-Regulated Virulence in the Emerging Pediatric Pathogen *Kingella kingae*. Infect Immun 85.

17. Brockman KL, Branstool MT, Atack JM, Robledo-Avila F, Partida-Sanchez S, Jennings MP, Bakaletz LO. 2017. The ModA2 Phasevarion of nontypeable Haemophilus influenzae Regulates Resistance to Oxidative Stress and Killing by Human Neutrophils. Sci Rep 7:3161.

18. VanWagoner TM, Atack JM, Nelson KL, Smith HK, Fox KL, Jennings MP, Stull TL, Smith AL. 2016. The modA10 phasevarion of nontypeable *Haemophilus influenzae* R2866 regulates multiple virulence-associated traits. Microb Pathog 92:60-67.

19. Gauntlett JC, Nilsson HO, Fulurija A, Marshall BJ, Benghezal M. 2014. Phase-variable restriction/modification systems are required for *Helicobacter pylori* colonization. Gut Pathog 6:35.

20. Phillips ZN, Tram G, Seib KL, Atack JM. 2019. Phase-variable bacterial loci: how bacteria gamble to maximise fitness in changing environments. Biochem Soc Trans 47:1131-1141.

21. Srikhanta YN, Dowideit SJ, Edwards JL, Falsetta ML, Wu HJ, Harrison OB, Fox KL, Seib KL, Maguire TL, Wang AH, Maiden MC, Grimmond SM, Apicella MA, Jennings MP.

2009. Phasevarions mediate random switching of gene expression in pathogenic *Neisseria*. PLoS Pathog 5:e1000400.

22. Atack JM, Yang Y, Seib KL, Zhou Y, Jennings MP. 2018. A survey of Type III restriction-modification systems reveals numerous, novel epigenetic regulators controlling phase-variable regulons; phasevarions. Nucleic Acids Res 46:3532-3542.

23. Atack JM, Weinert LA, Tucker AW, Husna AU, Wileman TM, F Hadjirin N, Hoa NT, Parkhill J, Maskell DJ, Blackall PJ, Jennings MP. 2018. *Streptococcus suis* contains multiple phase-variable methyltransferases that show a discrete lineage distribution. Nucleic Acids Res 46:11466-11476.

24. Katayama T, Kasho K, Kawakami H. 2017. The DnaA Cycle in *Escherichia coli:* Activation, Function and Inactivation of the Initiator Protein. Front Microbiol 8:2496.

25. Carabetta VJ, Tanner AW, Greco TM, Defrancesco M, Cristea IM, Dubnau D. 2013. A complex of YlbF, YmcA and YaaT regulates sporulation, competence and biofilm formation by accelerating the phosphorylation of Spo0A. Mol Microbiol 88:283-300.

26. Boddu RS, Perumal O, K D. 2020. Microbial Nitroreductases: A versatile tool for biomedical and environmental applications. Biotechnol Appl Biochem doi:10.1002/bab.2073.

27. Romero-Rodriguez A, Ruiz-Villafan B, Rocha-Mendoza D, Manzo-Ruiz M, Sanchez S. 2015. Biochemistry and regulatory functions of bacterial glucose kinases. Arch Biochem Biophys 577-578:1-10.

28. Schowanek D, Verstraete W. 1990. Phosphonate utilization by bacterial cultures and enrichments from environmental samples. Appl Environ Microbiol 56:895-903.

505 29. Schulze-Gahmen U, Pelaschier J, Yokota H, Kim R, Kim SH. 2003. Crystal structure of a
506 hypothetical protein, TM841 of *Thermotoga maritima*, reveals its function as a fatty
507 acid-binding protein. Proteins 50:526-30.
508 30. Asano Y, Lübbehüsen TL. 2000. Enzymes acting on peptides containing d-amino acid.
509 Journal of Bioscience and Bioengineering 89:295-306.
510 31. Wei D, Wang M, Jiang B, Shi J, Hao J. 2014. Role of dihydroxyacetone kinases I and II
511 in the dha regulon of *Klebsiella pneumoniae*. J Biotechnol 177:13-9.
512 32. Torrents E. 2014. Ribonucleotide reductases: essential enzymes for bacterial life.
513 Front Cell Infect Microbiol 4:52.
514 33. Chan DI, Vogel HJ. 2010. Current understanding of fatty acid biosynthesis and the
515 acyl carrier protein. Biochem J 430:1-19.
516 34. Gawthorne JA, Beatson SA, Srikhanta YN, Fox KL, Jennings MP. 2012. Origin of the
517 diversity in DNA recognition domains in phasevarion associated modA genes of
518 pathogenic *Neisseria* and *Haemophilus influenzae*. PLoS One 7:e32337.
519 35. dos Santos DF, Istvan P, Noronha EF, Quirino BF, Kruger RH. 2015. New dioxygenase
520 from metagenomic library from Brazilian soil: insights into antibiotic resistance and
521 bioremediation. Biotechnol Lett 37:1809-17.
522 36. Yongkiettrakul S, Maneerat K, Arechanajan B, Malila Y, Srimanote P, Gottschalk M,
523 Visessanguan W. 2019. Antimicrobial susceptibility of *Streptococcus suis* isolated
524 from diseased pigs, asymptomatic pigs, and human patients in Thailand. BMC Vet
525 Res 15:5.
526 37. Lugsomya K, Chatsuwan T, Niyomtham W, Tummaruk P, Hampson DJ, Prapasarakul
527 N. 2018. Routine Prophylactic Antimicrobial Use Is Associated with Increased
528 Phenotypic and Genotypic Resistance in Commensal *Escherichia coli* Isolates

529 Recovered from Healthy Fattening Pigs on Farms in Thailand. Microb Drug Resist
530 24:213-223.
531 38. Kouadio IK, Guessennd N, Dadie A, Koffi E, Dosso M. 2018. Comparative study of the
532 impact of the administration of Amoxicillin and Algo-Bio((R)) alternative substance to
533 antibiotics, on the level of selection of resistant *Enterobacteriaceae* in the digestive
534 flora of piglets. J Glob Antimicrob Resist 13:161-164.
535 39. Callens B, Persoons D, Maes D, Laanen M, Postma M, Boyen F, Haesebrouck F,
536 Butaye P, Catry B, Dewulf J. 2012. Prophylactic and metaphylactic antimicrobial use
537 in Belgian fattening pig herds. Prev Vet Med 106:53-62.
538 40. Lekagul A, Tangcharoensathien V, Yeung S. 2019. Patterns of antibiotic use in global
539 pig production: A systematic review. Vet Anim Sci 7:100058.
540 41. Krasny L, Bland P, Kogata N, Wai P, Howard BA, Natrajan RC, Huang PH. 2018.
541 SWATH mass spectrometry as a tool for quantitative profiling of the matrisome. J
542 Proteomics 189:11-22.
543 42. Brockman KL, Azzari PN, Branstool MT, Atack JM, Schulz BL, Jen FE, Jennings MP,
544 Bakaletz LO. 2018. Epigenetic Regulation Alters Biofilm Architecture and
545 Composition in Multiple Clinical Isolates of Nontypeable Haemophilus influenzae.
546 mBio 9.
547 43. Atack JM, Guo C, Litfin T, Yang L, Blackall PJ, Zhou Y, Jennings MP. 2020. Systematic
548 Analysis of REBASE Identifies Numerous Type I Restriction-Modification Systems with
549 Duplicated, Distinct hsdS Specificity Genes That Can Switch System Specificity by
550 Recombination. mSystems 5:e00497-20.

551 44. Atack JM, Guo C, Yang L, Zhou Y, Jennings MP. 2020. DNA sequence repeats identify
552 numerous Type I restriction-modification systems that are potential epigenetic
553 regulators controlling phase-variable regulons; phasevarions. FASEB J 34:1038-1051.
554 45. Weinert LA, Chaudhuri RR, Wang J, Peters SE, Corander J, Jombart T, Baig A, Howell
555 KJ, Vehkala M, Valimaki N, Harris D, Chieu TT, Van Vinh Chau N, Campbell J, Schultsz
556 C, Parkhill J, Bentley SD, Langford PR, Rycroft AN, Wren BW, Farrar J, Baker S, Hoa
557 NT, Holden MT, Tucker AW, Maskell DJ, Consortium BRT. 2015. Genomic signatures
558 of human and animal disease in the zoonotic pathogen *Streptococcus suis*. Nat
559 Commun 6:6740.
560 46. Clark TA, Murray IA, Morgan RD, Kislyuk AO, Spittle KE, Boitano M, Fomenkov A,
561 Roberts RJ, Korlach J. 2012. Characterization of DNA methyltransferase specificities
562 using single-molecule, real-time DNA sequencing. Nucleic Acids Res 40:e29.
563 47. Murray IA, Clark TA, Morgan RD, Boitano M, Anton BP, Luong K, Fomenkov A, Turner
564 SW, Korlach J, Roberts RJ. 2012. The methylomes of six bacteria. Nucleic Acids Res
565 40:11450-11462.
566 48. Peak IR, Chen A, Jen FE, Jennings C, Schulz BL, Saunders NJ, Khan A, Seifert HS,
567 Jennings MP. 2016. *Neisseria meningitidis* Lacking the Major Porins PorA and PorB Is
568 Viable and Modulates Apoptosis and the Oxidative Burst of Neutrophils. J Proteome
569 Res 15:2356-65.
570 49. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ,
571 Inuganti A, Griss J, Mayer G, Eisenacher M, Perez E, Uszkoreit J, Pfeuffer J,
572 Sachsenberg T, Yilmaz S, Tiwary S, Cox J, Audain E, Walzer M, Jarnuczak AF, Ternent
573 T, Brazma A, Vizcaino JA. 2019. The PRIDE database and related tools and resources
574 in 2019: improving support for quantification data. Nucleic Acids Res 47:D442-D450.

575 50. Peak IR, Jennings CD, Jen FE, Jennings MP. 2014. Role of *Neisseria meningitidis* PorA
576 and PorB expression in antimicrobial susceptibility. Antimicrob Agents Chemother
577 58:614-616.

578

Table 1. Summary of methylomes for *S. suis* strains LSS89 (ModS1) and SS1056 (ModS2). Strains were enriched for $GAGCA_{(n)}$ SSR tracts of 19, 20, or 21 GAGCA repeats in the *modS1* gene in strain LSS89, or 17, 18, or 19 GAGCA repeats in the *modS2* gene of strain SS1056. Type III methyltransferase motifs are only detected in strains enriched for an ON number of repeats (ModS1 in red in strain LSS89 21 repeats; ModS2 in blue in strain SS1056 18 repeats) and matches the protein expression detected in Figure 1. In ModS2, V represents A, G or C. % values represent motifs detected/motifs present. Full methylome data is presented in Supplementary Data 1.

	LSS89 *modS1*			**SS1056 *modS2***		
motif	19 reps	20 reps	21 reps	17 reps	18 reps	19 reps
5'-G$^{(m6)}$**A**AGNNNNNTTC/ 5'-G$^{(m6)}$**A**AHNNNNCTTC	100%/ 100%	100%/ 100%	100%/ 100%			
5'-GCG$^{(m6)}$**A**T (ModS1)	ND	ND	99.9%			
5'-GYT$^{(m6)}$**A**NNNNNNNNTTC/ 5'-GA$^{(m6)}$**A**NNNNNNNNTARC				100%/ 100%	100%/ 99.6%	100%/ 100%
5'-VTC$^{(m6)}$**A**TC (ModS2)				ND	99.9%	ND

587 **Table 2. Differentially regulated proteins (>1.5 fold) in the ModS1 phasevarion.** Fold

588 change presented as *modS1* ON vs *modS1* OFF

Down regulated in *modS1* ON			
Accession	**Protein**	**Fold change (ON vs OFF)**	***p*-value**
WP_002940030.1	30S ribosomal protein S12	2.87	1.43E-8
WP_002938143.1	ABC transporter permease	2.48	6.54E-7
WP_009909754.1	50S ribosomal protein L27	1.92	9.04E-7
WP_014638653.1	ABC transporter substrate-binding protein	1.65	4.81E-7
WP_002936253.1	DegV family protein	1.62	2.53E-4
WP_023369127.1	Sugar ABC transporter substrate-binding protein	1.55	7.86E-14
WP_004195491.1	50S ribosomal protein L23	1.54	9.68E-8
Up regulated in *modS1* ON			
WP_002936659.1	50S ribosomal protein L29	9.14	2.76E-8
WP_002940438.1	Amino acid ABC transporter ATP-binding protein	2.1	8.50E-4
WP_012027640.1	Ribosome biogenesis GTPase Der	2.08	3.12E-6
WP_002942806.1	CsbD family protein	2.03	6.94E-8
WP_014636557.1	Transcriptional repressor	1.99	1.78E-15
WP_002936247.1	HU family DNA-binding protein	1.97	1.81E-6
WP_002936483.1	50S ribosomal protein L7/L12	1.89	1.82E-14
WP_002938891.1	Acyl carrier protein	1.88	≤1.00E-17
WP_002936048.1	DUF1846 domain-containing protein	1.86	≤1.00E-17
WP_014736429.1	Nitroreductase family protein	1.77	2.17E-5
WP_014735259.1	30S ribosomal protein S10	1.76	≤1.00E-17
WP_002940682.1	30S ribosomal protein S16	1.73	6.91E-8
WP_002934959.1	DUF1149 family protein	1.67	4.09E-14
WP_023368958.1	Chromosomal replication initiator protein DnaA	1.66	≤1.00E-17
WP_023369777.1	dTDP-4-dehydrorhamnose reductase	1.64	1.96E-10
WP_002936660.1	50S ribosomal protein L16	1.62	5.40E-10

WP_004194840.1	DUF2829 domain-containing protein	1.6	1.60E-4
WP_012027178.1	PTS sugar transporter subunit IIA	1.6	3.27E-3
WP_002936656.1	50S ribosomal protein L24	1.59	3.52E-6
WP_002937025.1	Response regulator transcription factor	1.59	2.89E-3
WP_011922043.1	DivIVA domain-containing protein	1.59	8.40E-4
WP_002938966.1	YlbF/YmcA family competence regulator	1.58	≤1.00E-17
WP_012775074.1	Alkylphosphonate utilization protein	1.54	8.72E-4
WP_023370402.1	ROK family glucokinase	1.53	≤1.00E-17
WP_002936622.1	30S ribosomal protein S13	1.51	1.00E-4
WP_023369315.1	Glycine cleavage system protein H	1.5	1.94E-12

589

590 **Table 3. Differentially regulated proteins (>1.5 fold) in the ModS2 phasevarion.** Fold

591 change presented as *modS2* ON vs *modS2* OFF

Down regulated in *modS2* ON			
Accession	**Protein**	**Fold change (ON vs OFF)**	***p*-value**
WP_002935876.1	Glyoxalase/bleomycin resistance/extradiol dioxygenase family protein	1.79	≤1.00E-17
WP_044673510.1	ATP-dependent Clp protease ATP-binding subunit	1.62	4.35E-4
WP_024387015.1	Nucleotide exchange factor GrpE	1.55	2.81E-12
Up regulated in *modS2* ON			
WP_012028296.1	50S ribosomal protein L20	2.13	4.82E-9
WP_012027287.1	Peptidylprolyl isomerase	1.77	4.40E-2
WP_002938891.1	Acyl carrier protein	1.68	≤1.00E-17
WP_044754372.1	Cysteine synthase A	1.66	≤1.00E-17
WP_002936486.1	50S ribosomal protein L10	1.62	2.35E-4
WP_044680965.1	Class 1b ribonucleoside-diphosphate reductase subunit alpha	1.61	1.54E-4
WP_044771061.1	Dihydroxyacetone kinase subunit L	1.55	8.68E-3
WP_024384331.1	Aminopeptidase P family protein	1.54	3.24E-4
WP_044681922.1	Ribonucleoside-triphosphate reductase	1.54	2.97E-9
WP_079394259.1	Amino acid ABC transporter substrate-binding protein	1.52	1.07E-8
WP_044674536.1	Winged helix-turn-helix transcriptional regulator	1.5	1.70E-4

592

593 **Table 4. MICs of ModS2 ON vs ModS2 OFF enriched *S. suis* strains.**

	ModS2 ON	ModS2 OFF
Ampicillin (µg/mL)	**0.16**	**0.31**
Vancomycin (µg/mL)	1.25	1.25
Amoxicillin (µg/mL)	0.16	0.16
Penicillin (µg/mL)	0.63	0.63

594

595 **Figure Legends**

596

597 **Figure 1. Expression of ModS alleles. A)** The *modS* gene contains a variable length $GAGCA_{(n)}$
598 SSR tract (grey box) near the start of the gene, and a variable central target recognition
599 domain (TRD) represented by the hatched box. The 5' and 3' regions of the modS gene are
600 highly (>95% nucleotide identity) conserved (white). PCR over the SSR tract was determined
601 by FAM labelled PCR using primers SsuT3-F-FAM and SsuT3-R, and analysed using fragment
602 length analysis; **B)** Alignment of the TRD regions of ModS1 and ModS2 showing <25% amino
603 acid identity. * represents identical amino acid residues, · represents similar amino acid
604 residues (basic, acidic, neutral), TRD region underlined. Alignments carried out in ClustalW;
605 **C)** Fragment length analysis traces of the enriched *modS1* and *modS2* populations of strains
606 LSS89 and SS1056, respectively, containing three consecutive $GAGCA_{(n)}$ SSR tract lengths; **D)**
607 Western blot analysis using ModS antisera demonstrates that the ModS protein is only
608 present in *S. suis* populations enriched for 21 repeats in ModS1 (LSS89) and 18 repeats in
609 ModS2 (SS1056), demonstrating phase-variable expression of this protein.

610

611 **Figure 2. Volcano plot demonstrating changes to protein expression as a result of ModS.**
612 SWATH-MS proteomics demonstrated a coverage of 450 of 1964 identified proteins (~23%)
613 in LSS89 ModS1 ON-OFF strain pair, and 411 of 1905 (~22%) identified proteins in SS1056
614 ModS2 ON-OFF strain pair. The x axis indicates relative fold difference in protein abundance
615 in ON compared to OFF; the y axis indicates statistical significance.

616

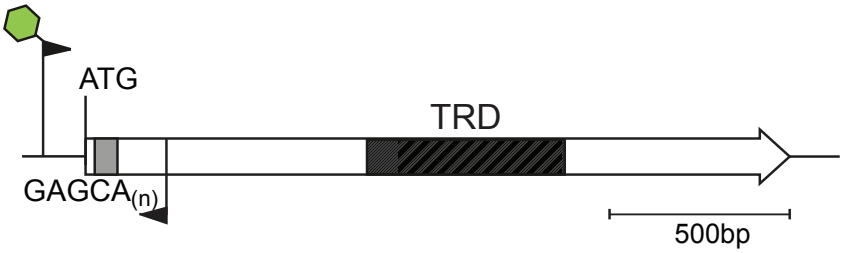
Figure 3. Growth Curves of *S. suis* populations enriched for *modS1* and *modS2*. ON-OFF strain pairs for ModS1 (strain LSS89) and ModS2 (SS1056) were grown in rich media (THB-Y broth) for 18 hours with shaking. Statistically significant differences (*P* value <0.05) in absorbance at each time point are indicated by asterisks, assessed using Student's t-test.

Supplementary figure legends

Supplementary Figure 1. Expression of ModS is dependent on SSR tract length. (A) Coomassie staining of whole cell lysates from enriched strains of S. suis. (B) Western blot using anti-ModS antisera demonstrates that ModS1 is only produced in strains enriched for 21 GAGCA repeats and that ModS2 is only produced in strains enriched for 18 repeats. Dotted line represents region of blot presented in Figure 1D.

Supplementary Figure 2. Three open reading frames occur in the *modS* gene due to variation in length of the $GAGCA_{(n)}$ simple sequence repeat tract. Due to a frameshift down-stream of the $GAGCA_{(n)}$ SSR tract, 19 and 20 repeats result in a premature stop codon (in bold, highlighted with *), and consequently no expression of the ModS protein; 21 $GAGCA_{(n)}$ repeats in the SSR tract results in the gene being in-frame, and therefore ModS protein is produced.

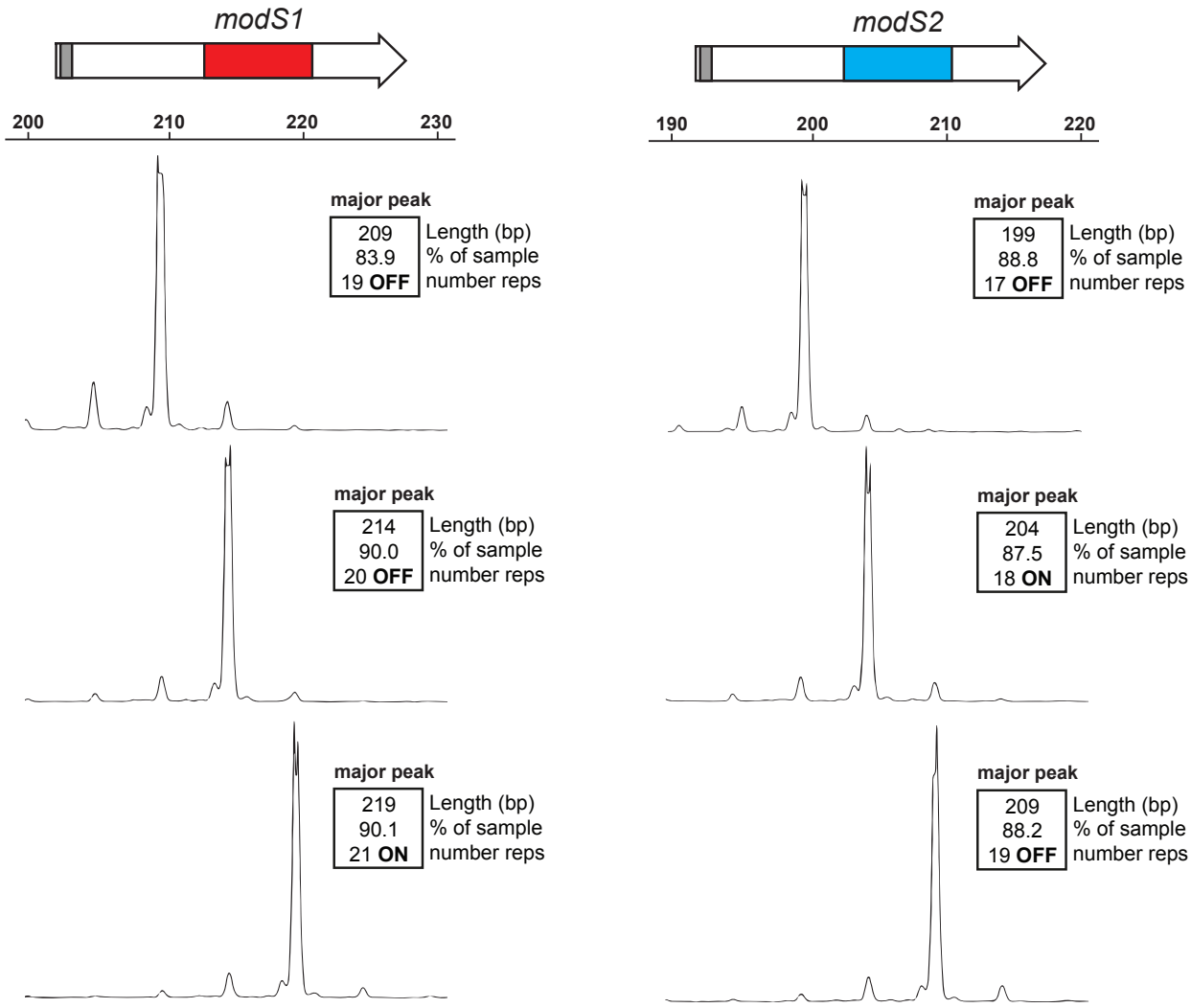
A



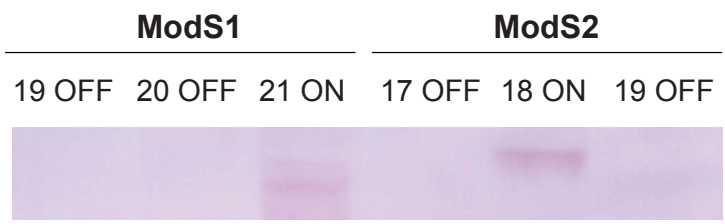
B

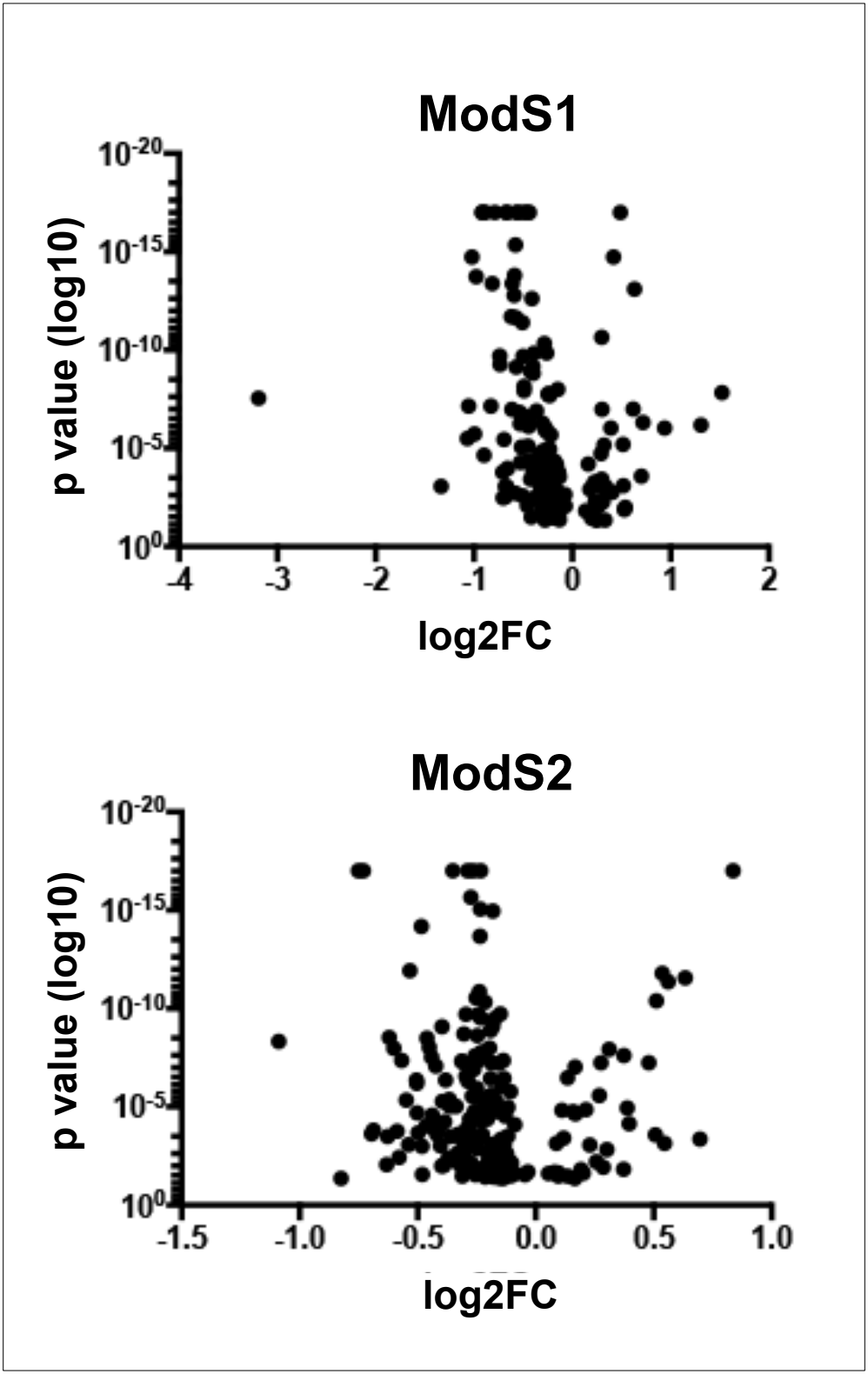
```
ModS1  247 QANLKAICDEIFGEENFVADVIWERAFAPVNLK------KHFSESHDYILVYAKNVEFAVNHGIPRSEDSNSRYQNPDNDVR 322
ModS2  243 QANLKAICDEIFGEENFRNTLLVRRRTKSLNLQFADNGLKSFNVGTEHIFVYAKS-NLALFNPIEKVKDKTSE--------K 315
           ****************   ..  *    .**.      * *    . * ****    *. . * .  * .*          .

ModS1  323 GPWTSGDLSVGPAVPSNIYEIISPSGRSIFPPSGRSWLLSKERFEEFIADNRIWFGANGDNV---------PRIKRFLSEVK 395
ModS2  316 GSWNV--FWSNADRPSMRYDVLG------FTPSTGQWRWSKEKADEAIENYIEFIANYSDKMTLEEYSSLNPQ-KKFIRRIE 378
           * *.      .   **  *...       * **   *  ***. .* *           * .         *. *.*.  .

ModS1  396 NTVTPMTIWKY----ADVGHSQSASQDLKKLFDGKAY---FTYPKPVPLMKQIVQLYSEKDGLILDFFAGSATTADAVM 467
ModS2  379 NGIGKNGGVQYYVAPSNTSLRTSNWTDLEVSQIAKEYDLPFDNPKNKSLIREILTSKTDSDSLILDFFAGSATTADAVM 457
           *.      .*    .       *   **      * *   *  **   *...*.   .. * ****************
```

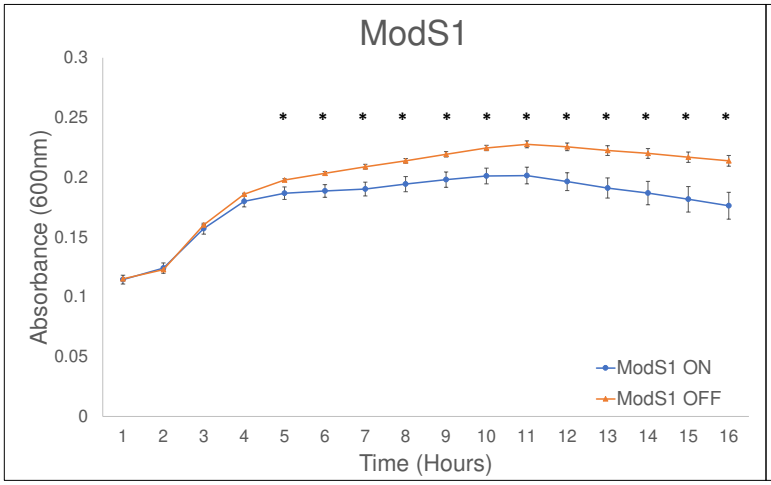
C

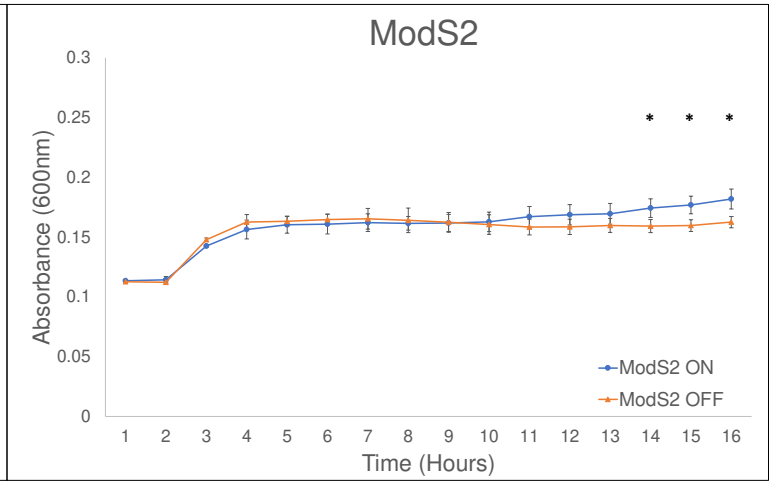


D




ModS1
p value (log10)
10-20
10-15
10-10
10-5
100
-4
-3
-2
-1
0
1
2
log2FC
ModS2
p value (log10)
10-20
10-15
10-10
10-5
100
-1.5
-1.0
-0.5
0.0
0.5
1.0
log2FC


ModS1
Absorbance (600nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (Hours)
ModS1 ON
ModS1 OFF
* * * * * * * * * * * *


ModS2
Absorbance (600nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Time (Hours)
ModS2 ON
ModS2 OFF
* * *