Sensing and responding to diverse extracellular signals: An updated analysis of the sensor kinases and response regulators of *Streptomyces* spp.

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Abstract

Streptomyces venezuelae is a Gram-positive, filamentous actinomycete with a complex developmental life cycle. Genomic analysis revealed that *S. venezuelae* encodes a large number of two-component systems (TCS): comprised of a membrane-bound sensor kinase (SK) and a cognate response regulator (RR). These proteins act together to detect and respond to diverse extracellular signals. Some of these systems have been shown to regulate antimicrobial biosynthesis in *Streptomyces* species, making them very attractive to researchers. The ability of *S. venezuelae* to sporulate in both liquid and solid cultures has made it an increasingly popular model organism in which to study these industrially and

medically important bacteria. Bioinformatic analysis identified 58 TCS operons in *S. venezuelae* with an additional 27 orphan SK and 18 orphan RR genes. A broader approach identified 15 of the 58 encoded TCS to be highly-conserved in 93 *Streptomyces* species for which high quality and complete genome sequences are available. This review attempts to unify the current work on two-component systems in the streptomycetes, with an emphasis on *S. venezuelae.*

Introduction

A response to a stimulus or changes in the environment is one of the seven criteria of life (1). Bacteria sense their environment mainly via two-component systems (TCS) and the number of two-component systems varies greatly depending on the environmental niche the bacterium occupies. Intracellular pathogens living in a homeostatic environment encode very few TCSs whereas bacteria living in competitive environments like the soil contain many more (2). Soil dwelling *Streptomyces* bacteria have some of the largest bacterial genomes and the highest numbers of TCS found in bacteria, perhaps reflecting an adaptation to their highly variable environmental niche (3).

TCSs were first described in the late 1980s (4) and since then they have been found in all Domains of life (5). The absence of TCSs in humans or animals makes them an ideal target for antibiotics, particularly since some TCS are essential (6,7) and others regulate virulence and antibiotic resistance (8). There are three different classes of TCSs: the classical TCS has a transmembrane sensor kinase (SK) that senses the signal and transfers a phosphate group to its cognate response regulator (RR); the hybrid TCS has an SK and RR fused together thus the whole TCS complex is membrane bound; finally the phosphorelay TCS includes phosphotransferases that transfer the phosphoryl group from the SK to the ultimate target via multiple phosphotransfer steps (9). In general SKs receive a stimulus and autophosphorylate at a conserved histidine residue using ATP. The phosphate group is then transferred to a

50 conserved aspartate in the cognate RR. SKs contain two domains: a variable sensor region
51 and a highly-conserved kinase core (10). The kinase core contains a dimerization domain and
52 a catalytic domain which contains the ATP binding site (11). RRs also consist of two domains:
53 a conserved N-terminal receiver domain containing the conserved aspartate and a variable
54 output domain. The receiver domain contains the phosphorylation site and the output domain
55 is usually a helix-turn-helix (HTH) DNA binding domain, but can also be an RNA binding,
56 protein binding or an enzymatic domain (2). For a schematic diagram see Figure 1.

57 In this review we compare the conservation of TCSs in 93 *Streptomyces* complete genome
58 sequences and review all TCSs in the model organism *S. venezuelae* NRRL B-65442 to give
59 an update on the known function of TCS in the genus *Streptomyces*.

60 TCSs in *Streptomyces* species

61 Despite the vast number of *Streptomyces* strains isolated so far, much of our knowledge of
62 this genus has been obtained from two model organisms: *Streptomyces coelicolor* and *S.*
63 *venezuelae. S. coelicolor* was the first model organism to be adopted due to the pioneering
64 work of Sir David Hopwood and colleagues (12). *S. coelicolor* produces five antibiotics under
65 laboratory conditions: the blue actinorhodin [ACT] (13), the red undecylprodigiosin [RED] (14),
66 the colourless calcium-dependent antibiotic [CDA] (15), the yellow pigmented coelimycin P1
67 [yCPK] (16,17) and the unusual, plasmid encoded cyclopentanone antibiotic methylenomycin
68 (18). Most of the developmental regulatory genes were first identified and studied in *S.*
69 *coelicolor* and it was the first streptomycete to have its genome sequenced (3). In recent years,
70 *S. venezuelae* has been adopted as a model to study the regulation of development and
71 antibiotic production. *S. venezuelae* is unusual for the genus because it sporulates in liquid
72 culture and is known to make three antibiotics under laboratory conditions: chloramphenicol,
73 jadomycin and watasemycin (19–21).

74

75 ## Identification and classification of TCSs

76 We searched the genome of *Streptomyces venezuelae* NRRL B-65442 (22) and *S. coelicolor*
77 A3(2) (3) for TCS, orphan RR, unpaired SK and other regulatory genes with the online tool
78 P2RP (23). Within this software regulatory proteins are identified by using RPSBLAST
79 (reverse PSI-BLAST) which searches a query sequence against the SMART and Pfam
80 databases (23). The program uses an E-value cut-off of 0.01 (24) and the secondary structure
81 of regulatory proteins is computed using the PSIPRED method with the presence or absence
82 of transmembrane segments predicted using the HMMTOP predictor. This method identifies
83 more regulatory proteins than previous studies (3,25) due to the improved protein domain
84 identification and classification (Table 1).

85 Analysis of the *S. venezuelae* NRRL B-65442 genome showed that there are more than twice
86 the number of one-component systems as there are TCSs. One-component systems are
87 transcription factors containing known or predicted input and output domains but lack the
88 histidine kinase and receiver domains. This is consistent with the work of Ulrich *et al.* (2005)
89 (26) which argued that TCSs are evolutionary derivatives of one-component systems. In total
90 58 TCS were identified in *S. venezuelae.* The SK / RR pair *vnz_32020* / *vnz_32020* is judged
91 to be a "false positive" TCS because the genes are convergent and not in an operon. We
92 therefore classified the SK and RR as orphans and identified a further 26 unpaired SKs and
93 17 orphan RRs (Tables 3 and 4). Whilst many of these SKs were listed as potentially
94 incomplete in the analysis due to lack of an identifiable histidine residue for phosphorylation,
95 some of them may still be able to pair with some of the orphan RRs or RRs of paired TCSs as
96 exemplified by Spo0F (27).

97 ## Evolution and conservation of TCSs

98 In addition to the P2RP analysis we investigated the pan genome of 93 fully-sequenced
99 streptomycetes using the BPGA tool (28) identifying the conservation of TCS throughout
100 *Streptomyces* species. Fifteen TCSs are highly conserved in these 93 genomes and 12 are of

101 known function or predicted by homology to TCS in other genera. Three highly conserved TCS
102 have no known function. The TCS *vnz_24545/50* consists of a classic SK and an OmpR RR
103 and is not only conserved throughout *Streptomyces* spp. but also in closely related genera
104 and the suborders Corynebacterineae, Micrococcineae and Bifidobacteriales (29). The TCSs
105 *vnz_07060/65* and *vnz_08930/35* both consist of a classical SK and a NarL RR and seem to
106 be conserved only within *Streptomyces* and closely related genera. OmpR and NarL RRs both
107 contain a DNA binding HTH domain at the output domain and bind to DNA, controlling gene
108 expression (5).

109 Analysis of total TCSs in genomes have shown that typically the number of TCSs roughly
110 correlates with the square of the genome size (30,31). The gain of a new TCS has been
111 attributed to two avenues: (1) lineage specific expansion (LSE), where there is a duplication
112 event or reshuffling and (2) horizontal gene transfer (HGT), where genes are transferred from
113 one species to another (32). The gain of new TCSs may be evenly spread between LSE and
114 HGT as is seen in *Bradyrhizobium japonicum* or may be biased towards one of these methods
115 such as for *Pseudomonas aeruginosa* which has gained the majority of its new TCSs from
116 HGT. This is in contrast to *S. coelicolor* which has evolved nearly all of its new TCSs through
117 LSE (32). This is not surprising because *Streptomyces* spp. are known for their tendency for
118 gene duplication (3). Thus, it is likely that acquiring TCSs through LSE may not be a common
119 theme in *Streptomyces* spp. as TCS expansion in *S. coelicolor* has largely occurred though
120 lineage specific means. This may explain why streptomycete RR effector domains appear to
121 be largely restricted to transcriptional regulation (2). The gain of new TCSs through HGT is
122 thought to be more likely to preserve the positioning of the genes in their adjacent position or
123 closely within a regulon; however, those gained through duplication events are more likely to
124 cause separations (32). Most investigated TCS in Streptomyces are involved in secondary
125 metabolite production (Table 2) either directly binding to cluster situated regulators (e.g.
126 AfsQ1) or as global regulators (e.g. PhoP). However, biosynthetic gene clusters (BGC) do not

consistently contain a TCS which autoregulates the biosynthesis of the respective secondary metabolite.

After duplication events, the build-up of mutations and reshuffling events can change the function and recognition of TCSs to generate new TCSs. In addition to reshuffling and duplication, hybridisation can also occur, resulting in hybrid TCSs. This can occur at stop codons or independently of this but reshuffling and hybridisation are both very rare, especially in *Streptomyces* (31). In the process of mutation build up, the two components may gain or lose functions and pseudogenes may be formed or it may result in multiple RRs being activated by a single SK or multiple SKs regulating a single RR (branched pathways). The regulatory circuit provided by a TCS enables the coupling of the target regulon genes to each system via the RR. The eco-evolutionary processes that determine how these regulons differ in closely related species depends upon the presence of orthologous genes and may reflect horizontal gene transfer events or the occupation of different environmental niches. This is an area of research that will develop given the availability of genetic tools to dissect gene function in these organisms (33).

Function and Phenotypes of *Streptomyces* TCSs

The conserved TCSs

MacRS: vnz_08725/vnz_08730

The function of the novel TCS MacRS (SCO2120/21) was discovered by transposon mutagenesis in *S. coelicolor* (34). Further studies showed that deletion of *macRS* blocked production of ACT and enhanced formation of aerial mycelium. The NarL-type RR MacR regulates the expression of eight target genes encoding the lipoproteins SCO0607 and SCO7460 and the integral membrane proteins SCO1700, SCO4011 (MmpC), SCO4225,

SCO4924 (MmpB), SCO6728 (MmpA) and SCO7613. The effect of MacRS on ACT production is most likely indirect because MacR did not bind to the ACT biosynthetic gene cluster (BGC) in the reported chromatin immunoprecipitation and sequencing (ChIP-seq) experiment. Interestingly, the deletion of *macRS* caused rapid aerial mycelium formation and the authors speculated that this phenotype could be due to the involvement of the MacRS target genes in membrane integrity and / or other membrane associated activities (35). MacRS homologs from *S. venezuelae* and *S. avermitilis* complemented the deletion mutant which indicates that MacRS function is conserved throughout *Streptomyces*. The signal sensed by MacS is unknown.

MtrAB: vnz_13525/vnz_13520

MtrAB is highly conserved throughout the phylum Actinobacteria and has been characterised in *Mycobacterium* species, *Corynebacterium glutamicum*, *S. coelicolor* and *S. venezuelae.* It is the only essential TCS in *Mycobacterium tuberculosis* but is not essential in other tested mycobacteria or in *C. glutamicum* (36–38). MtrA (*M. tuberculosis* regulator A) is the RR in this system and MtrB the SK. MtrA$_{MT}$ (from *M. tuberculosis*) regulates expression of *dnaA* and *dnaN*, encoding the DNA replication initiator and DNA polymerase III stabilising protein, respectively, and binds to the *oriC* region between these two genes, sequestering the chromosome origin to prevent further DNA replication. Direct interaction of MtrA with the DnaA protein was also demonstrated and may facilitate the binding of MtrA-P (phosphorylated MtrA) to *ori*C. This occurs in the post replication period of the cell (39). MtrA is thought to stop further DNA replication and move the cycle towards cell division. MtrB$_{MT}$ localises to the cell membrane in a FtsZ (cell division initiator) dependent but phosphorylation independent manner and has also been shown to interact with Wag31 (DivIVA), FtsI and PknA/PknB (Protein kinase A and B; Ser/Thr kinases), which are all involved in controlling cell division and shape (40–42). There is a third component, the genetically linked lipoprotein, LpqB, which has been shown to interact with the sensor domain of *M. smegmatis* MtrB to modulate signalling (43). An *M. smegmatis* Δ*lpqB* mutant demonstrated multidrug sensitivity and *Streptomyces*-

179 like filamentous growth with polyploidy. A similar phenotype was also exhibited by an *mtrA*
180 transposon insertion inactive mutant in *M. smegmatis* (43). The lipoprotein *lpqB* is highly
181 conserved as the third gene in the *mtrAB-lpqB* operon and described as a signature protein
182 for the phylum Actinobacteria (44). This may indicate its importance for the effective
183 functioning of MtrAB TCS signalling.

184 More recently, MtrA has been studied in *Streptomyces* species and has been found to be a
185 regulator of antibiotic production. Deletion of *mtrB* induced a >30-fold increase in
186 chloramphenicol production in *S. venezuelae* and MtrA binds directly within the
187 chloramphenicol cluster (45). Further analysis of MtrA gene targets in *S. coelicolor* using ChIP-
188 seq shows that it binds to sites in BCGs including upstream of *actII-orf4* and *redZ* (45)
189 suggesting that MtrA may also modulate ACT and RED production. Consistent with this, an
190 *S. coelicolor* Δ*mtrB* mutant over-produced both antibiotics. The fact that MtrA is essential in
191 the pathogen *M. tuberculosis* and regulates antibiotic production in *Streptomyces* makes it a
192 very attractive TCS for further study, whether as a target for antibiotic therapy or to activate
193 antibiotic production. It is particularly interesting that $MtrA_{TB}$ was able to switch on
194 chloramphenicol production in *S. venezuelae* (45).

195 In addition to the regulation of antibiotics in *Streptomyces* species*,* MtrAB has been found to
196 play a critical role in the regulation of development. Deletion of *mtrA* resulted in a conditional
197 bald phenotype in *S. coelicolor*, *S. lividans* and *S. venezuelae* that was dependent on the
198 growth medium, in a similar manner to deletion mutants of *sapB*, or chaplins or *bld* genes (46).
199 Moreover in *S. coelicolor*, MtrA is involved in nutrient uptake, regulating the Mce sterol uptake
200 system which is important for the colonisation of plant roots (47).

201 DraRK: vnz_13760/vnz_13755

202 The function of this highly conserved TCS was investigated in *S. coelicolor* (48). Deletion of
203 *draRK* led to the conditional repression of ACT biosynthesis but increased RED production on
204 minimal medium supplemented with a high concentration of a nitrogen source. The same
205 mutant overproduced the yellow-pigmented type-I polyketide Cpk on minimal medium

206 supplemented with glutamate. The phosphorylated RR DraR binds to the promoter of *actII-*
207 *ORF4* and *kasO* and likely directly regulates the production of these antibiotics. However,
208 DraR does not bind to *redZ* and therefore the effect of DraRK on increased production of RED
209 is likely to be indirect. Additionally, DraRK represses the growth of *S. coelicolor* under high
210 glutamine and glutamate conditions, most likely by regulating the transcription of genes
211 involved in primary metabolism (*SCO6748* a putative enoyl-CoA hydratase (ECH), *SCO2014*,
212 encoding a pyruvate kinase and *gltB*, encoding a glutamate synthase (48).

213 Consistent with our analysis, Yu *et al.*, 2012 (48) stated that DraRK is highly conserved
214 throughout the genus *Streptomyces.* Furthermore, the DNA binding domain is nearly identical
215 in *S. coelicolor*, *S. griseus*, *S. venezuelae*, *S. clavuligerus*, *S. scabies* and *S. avermitilis.* The
216 functional conservation was demonstrated by deleting *draKR* in *S. avermitilis*. This strain
217 showed increased production of avermectin B1a and slightly reduced oligomycin A production.
218 EMSAs using the regulatory gene promoters of the avermectin and oligomycin cluster
219 indicated that DraR directly regulates production of oligomycin A but the repression of
220 avermectin biosynthesis is indirect (48).

221 TunRS: vnz_15960/vnz_15965

222 The role of this highly-conserved TCS remains elusive but it is always genetically linked to a
223 *tmrB*-like gene (*vnz_15955*). The membrane-associated ATP-binding protein TmrB is
224 responsible for resistance to the antibiotic tunicamycin in *Bacillus subtilis* either by blocking
225 passive diffusion or by acting as a tunicamycin efflux pump (49). Tunicamycins are fatty acyl
226 nucleoside antibiotics originally isolated in 1971 from *Streptomyces lysosuperificus* and inhibit
227 bacterial cell wall biosynthesis by preventing the formation of undecaprenyl-pyrophosphoryl-
228 *N*-acetylmuramoyl pentapeptide, a key peptidoglycan precursor, via MraY. Not all species of
229 *Streptomyces* are capable of producing tunicamycin and the *tmrB* homologue is not located
230 within the tunicamycin biosynthetic gene cluster. Thus, it is possible that it has another role
231 beyond protection from self-made antibiotics (50). The *tmrB* homologue *SCO3388* shares
232 38.9% identity to TmrB and is likely to be under the control of the TunRS TCS with a possible

233 operon promoter region detected by differential RNA-seq (51). A *SCO3388::tsr* mutant was
234 defective in growth and sporulation on R4 agar and is suggested to be a spore cell wall-
235 remodelling factor with TmrB-like proteins playing a role in streptomycete cell wall metabolism
236 (52). Deletion of *tunRS* in *S, venezuelae* increases tunicamycin sensitivity relative to the wild-
237 type but this may be due to polar effects on the downstream *tmrB* gene (unpublished data).

238 ### CssRS: vnz_19335/vnz_19330

239 *Bacillus subtilis* copes with misfolded protein stress by activating the expression of HtrA-like
240 proteases via the TCS CssRS (53). This TCS was identified by searching the genome
241 sequence of *S. coelicolor* (3) for the *B. subtilis* CssRS homolog. The homology was
242 experimentally confirmed via deletion mutants. Removal of CssRS in *S. lividans* leads to the
243 accumulation of misfolded proteins outside the cell and it was shown that the expression of
244 the HtrA-like proteases is downregulated in the *cssR* and *cssS* mutants (54). The conservation
245 of function in the very distant related bacteria *B. subtilis* and *S. lividans* indicates an overall
246 conservation of this TSC.

247 ### PhoPR: vnz_19585/19585

248 Perhaps the best studied *Streptomyces* TCS, and the only one with a well-defined activating
249 signal, It is unfeasible to cover the entirety of PhoPR research in a few short paragraphs thus
250 what follows is a directed summary.

251 Inorganic phosphate is a vitally important macronutrient for biological life, necessary for
252 genetic material (e.g. DNA, RNA), energy transfer (e.g. ATP, GTP) and regulatory signalling,
253 to name just a few of its roles. PhoP is a master regulator which governs both primary and
254 secondary metabolism in streptomycetes (55). It forms an operon with genes encoding a
255 sensor kinase, PhoR, and another regulatory protein PhoU (25,56). PhoP binds to defined
256 promoter sequences called PHO boxes which help define the PhoP regulon. These are eleven
257 nucleotide direct repeat units (DRUs) with a consensus sequence of GTTCACC each
258 recognised by a single PhoP monomer with a minimum of two DRUs required for PhoP-DNA

259 binding. In *Escherichia coli* the DNA-binding domain (DBD) is permanently exposed allowing
260 efficient interaction with PHO boxes. This is in contrast to PhoP of *S. coelicolor* which requires
261 phosphorylation by PhoR to expose the DBD, suggesting a more tightly controlled response
262 to phosphate (55,56). Under phosphate limiting conditions the PhoP regulon works to
263 scavenge phosphate from both the extracellular and intracellular environments whilst also
264 delaying or completely repressing morphological differentiation (55). In *S. coelicolor* this
265 includes expression of the alkaline phosphatase PhoA and a phospholipase PhoD to
266 scavenge inorganic phosphate from phosphorylated proteins alongside a phytase
267 (*vnz_00450*) to hydrolase phytate. Transport systems such as the high-affinity phosphate-
268 specific transporter *pstSCAB* are also highly upregulated by PhoP when phosphate levels are
269 low (57–59). Glycerophosphodiesters from membrane phophlipids can be hydrolysed by
270 glycerophosphodiester phosphodiesterases (GCPDs) such as *glpQ1* and *glpQ2* to form an
271 alcohol and sn-glycerol-3-phosphate, a cellular phosphate source. PhoP directly activates
272 both *glpQ1* and *2* (60,61). It also becomes imperative to store cellular phosphate and this can
273 be done via a reversible polymerization catalysed by the polyphosphate kinase *ppK*, also
274 activated by PhoP (62). Finally, there are morphological differentiation genes controlled by
275 PhoP including five *bld* genes (*bldA/C/D/K/M*) and two ECF sigma-factors *sigU* and *chpC*, all
276 of which are repressed to delay development whilst overcoming the phosphate limitation (63).
277 Little is known about the regulator PhoU except that expression of *phoU* is dependent on PhoP
278 activation and it acts to negatively modulate *pho* regulon expression forming a self-regulation
279 feedback mechanism (64).

280 In general, when phosphate levels are high secondary metabolism is repressed. Deletion of
281 *phoP* in *S. coelicolor* leads to varied metabolite biosynthesis dependant on the media with
282 hastened ACT production in R5 but repressed ACT and undecylprodigiosin in defined starch
283 media. PhoP acts at a higher level, repressing genes such as *afsS:* a positive regulator of
284 *actII-ORF4* and *redZ* (65,66). PhoP also represses the expression of *scbR*, in turn repressing
285 gamma-butyrolactones leading to downstream repression of coelimycin P1 biosynthesis as

286 well as *cdaR* thus repressing CDA biosynthesis (55). It has also been shown that *phoP*
287 disruption increases production of the macrolide antifungal pimaricin in *Streptomyces*
288 *natalensis,* a strain extremely sensitive to media phosphate concentration, although the *pim*
289 biosynthetic genes contains no PHO boxes (67).

290 ## AbrC1/2/3: vnz_21260/vnz_21255/vnz_21250

291 The branched TCS encoded by the *abrC123* operon encodes two histidine kinases (AbrC1
292 and AbrC2) and one response regulator (AbrC3*)* in *S. coelicolor*. Deletion of the *abrC123*
293 genes, or overexpression on a high-copy number plasmid, resulted in an *S. coelicolor* strain
294 with reduced ability to produce the ACT, RED and CDA antibiotics. These strains also
295 displayed decreased differentiation rates (68). The aberration of antibiotic production has been
296 linked to *abrC1* via a single deletion mutant. The SK genes are separated by a 114bp region
297 and DNA regions of this size can contain promoter regions but qRT-PCR experiments did not
298 detect any differential expression and *abrC12* was consistently expressed as single transcript
299 (69). Differential RNA-seq of *S. coelicolor* A3(2) confirmed a single transcriptional start site
300 preceeding *abrC3* (*SCO4596)* and no others within the operon (70). Contrary to this the RR
301 *abrC3* was found to be expressed independently and regulates its own promoter but not that
302 of abrC2 (69). The true number of promoters present still remains undefined.

303 DNA microarray analysis and qRT-PCR revealed that 16 of the 32 significantly downregulated
304 genes in an Δ*abrC3* mutant corresponded to the entire *act* biosynthetic gene cluster.
305 Chromatin immunoprecipitation followed by microarray (ChIP-chip) analysis of AbrC3 showed
306 binding of the regulator to its own promoter, a mechanism which is not uncommon in RRs,
307 and that of the *act* cluster-situated regulator *actII-ORF4* (71).

308 ## EsrSR: vnz_22090/vnz_22095

309 This is homologous to a TCS characterised in *Corynebacterium glutamicum* and named
310 EsrSR (envelope stress response) when it was found to be homologous to the cell envelope
311 stress sensing three component system LiaFSR from *Bacillus subtilis.* One major system

312 induced by cytoplasmic membrane disruption is the phage shock protein (Psp) system, found
313 widely distributed through bacteria as a stress response mechanism and LiaFSR is a three-
314 component system which regulates this Psp response (72,73). The *esrSR* operon is
315 divergently transcribed with the third component, the integral membrane protein EsrI. Both
316 EsrS and EsrI contain PspC domains, important for the Psp response via the PspC protein,
317 and the same holds true for the *Streptomyces venezuelae* homologues (EsrS: vnz_22090 and
318 EsrI: vnz_20095) (73). Interestingly streptomycetes do not seem to encode any part of the
319 Psp system other than PspA which is an essential protein for growth and survival under
320 membrane attack stress conditions (74). Transcription of *esrISR* in *C. glutamicum* can be
321 induced with cell-wall active antibiotics such as bacitracin and vancomycin as was
322 demonstrated using β-galactosidase reporter assays with the *esrI* and *esrSR* promoter
323 regions. EsrR binding domains were identified and subsequently confirmed using EMSA
324 experiments. No β-galactosidase activity was observed when the same experiment was
325 performed using an *esrSR* deletion mutant thus transcription is reliant on EsrSR. However, in
326 the *esrI* deletion mutant there was a two-fold increase in the transcription of *esrSR* suggesting
327 EsrI may act as a repressor of *esrSR* transcription. Whilst EsrSR showed no role in
328 vancomycin resistance, deletion of *esrSR* resulted in a strain that was much more susceptible
329 to bacitracin, which could be complement with plasmid-encoded EsrSR (73). Two EsrR target
330 genes in *C. glutamicum* are ABC transport systems, and these systems have been previously
331 been shown to play an important role in bacitracin resistance in *S. coelicolor* (75). Thus, it
332 appears that the EsrISR system senses and responds to cell envelope stress, orchestrating a
333 broad response including induction of ABC transporter systems (73).

334 AfsQ1/2: vnz_22605/vnz_22600

335 AfsQ1/2 TCS is co-encoded with a partner lipoprotein AfsQ3, just like the MtrAB, EsrSR and
336 CseBC TCSs. The operon encoding AfsQ1/2/3 is divergently transcribed from a gene
337 encoding the ECF sigma factor SigQ and this is reminiscent of CseABC which are co-encoded
338 in an operon with the ECF sigma factor SigE (76,77). AfsQ1 was first described after

339 heterologous expression in *S. lividans* from *S. coelicolor* and designated *afs* (A-factor
340 synthesis) as it regulated A-factor production. Mapping of the sequence subsequently
341 revealed the SK gene *afsQ2*. Phenotypically it was shown that expression of *afsQ1* activated
342 ACT and RED production in *S. lividans* but disruption in *S. coelicolor* seemed to have no
343 relatable effect (78). It was later shown that on minimal media supplemented with glutamate
344 an Δ*afsQ1/2* mutation in *S. coelicolor* repressed ACT, RED and CDA alongside a
345 morphological change: increased aerial hyphae growth. However, disruption of *sigQ* proved
346 to have the opposite effect, suggesting an antagonistic activity. RT-PCR data showed that
347 expression of *sigQ* was significantly reduced in the *afsQ1/2* mutant but transcription of *afsQ1/2*
348 was unchanged in the *sigQ* mutant. Consistent with the phenotypic appearance, expression
349 of *actII-ORF4*, *redD* and *cdaR* were all reduced in the *afsQ1/2* mutant and increased in the
350 *sigQ* mutant (79). Interestingly it was shown that AfsQ2 does not seem to detect glutamine
351 nor glutamate even though the TCS seems to respond to glutamate supplementation. In the
352 presence of excess nitrogen AfsQ1 will modulate nitrogen assimilation by competing with the
353 orphan RR GlnR for the promoter regions of *glnA* (glutamine synthase) and *nirB* (nitrogen
354 reductase). This highlights possible cross-talk between AfsQ1/2 and GlnR. Elucidation of the
355 AfsQ1 binding sequence (GTnAC-n_6-GTnAC) allowed the mapping of the regulon to the
356 genome and revealed a global range. Putative binding sites include the developmental genes
357 *bldM* and *whiD* (76). Heterologous constitutive expression of a gain-of-function
358 phosphomimetic mutant of *afsQ1* in a range of wild streptomycete isolates led to lethality in
359 8/11 strains whilst the wild-type gene was not lethal in any of the 11 strains. An alternate
360 thiostrepton-inducible promoter was used and, through titration, non-lethal levels of induction
361 could be achieved. This led to the isolation of the novel class-I lasso peptide siamycin-I from
362 WAC00263 which showed promising activity against both methicillin-resistant *Staphylococcus*
363 *aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE). When aligned to *S. nodosus*
364 the probable siamycin-I cluster contains a TCS (SNOD34175-SK, SNOD34170-RR;(80)).

365 OsaABC: vnz_26710/vnz_26715/vnz_26705

366 The function of this TCS was discovered by transposon mutagenesis in *S. coelicolor* and *S.*
367 *lividans* (81). Deletion of the RR *osaB* resulted in strains unable to form aerial hyphae under
368 osmotic stress. Additionally, the *osaB* mutant produces three to five times more actinorhodin
369 and undecylprodigiosin in *S. coelicolor.* Surprisingly, the hybrid SK OsaA does not seem to be
370 essential for osmoadaptation, reflected in a much less severe phenotype of delayed
371 osmoadaptation and development compared to the *osaA* mutant. The increased production of
372 secondary metabolites in the deletion mutants is most likely caused by the disruption of the
373 complex osmotic stress response rather than direct regulation of antibiotic synthesis. The
374 authors speculate that the phosphorylation of OsaA could be controlled by a second hybrid
375 orphan SK SCO7327 (vnz_01755) (81). Also, the downstream RR and SK are not
376 transcriptionally coupled underlining the independence of the RR which interacts with other
377 proteins rather than DNA due to its atypical coiled-coil output domain. The hybrid SK OsaC is
378 divergently transcribed from *osaA.* This regulatory protein consists of SK kinase components
379 (HATPase_c_2, PAS, 2 GAF domains) whereas the HATPase domain shares homology with
380 RsbW-like-anti-sigma factors. OsaC is required to return *osaB* and *sigB* expression back to
381 constitutive levels after osmotic stress (82). Disruption of *osaB* or *osaC* in the industrial strain
382 *S. avermitilis* led to increased production of oligomycin and avermectin and lack of aerial
383 hyphae formation under osmotic stress (83). This indicates that the involvement of OsaABC
384 in the regulation antibiotic production in response to osmotic stress is conserved throughout
385 *Streptomyces*.

386 GluRK: vnz_26890/vnz_26895

387 The GluRK TCS can be found located divergently from the glutamate uptake encoding operon
388 *gluABCD.* This genomic organisation is highly conserved and widely distributed throughout
389 Actinobacteria. Interestingly there is no GluRK homologue in *C. glutamicum*, the industrial
390 producer of glutamate, even though it encodes the *gluABCD* uptake system. The operon
391 encodes ATP and glutamate binding proteins (GluA and GluB respectively) and two glutamate

392 permeases (GluC and GluD). RT-PCR and EMSA analysis of *S. coelicolor* Δ*gluRK* and purified
393 His_6-GluR protein confirmed direct activation of the transcription of the *gluABCD* operon. GluK
394 was subsequently shown to be a glutamate sensor with biolayer interferometry (BLI). The
395 extracellular domain of the sensor kinase GluK was his-tagged, immobilised on a Ni-NTA
396 sensor and analysed for glutamate dissociation where it showed a significant shift (0.44 nm)
397 whilst no such shift was observed for the glutamine control. When grown on complex media
398 such as MS and R2YE there was no distinct phenotype of *S. coelicolor* Δ*gluRK.* However, on
399 MM supplemented with low (10 mM) glutamate, the mutant strain had reduced RED and
400 increased ACT production compared to M145 wild-type strain. In high (75 mM) glutamate
401 supplementation the mutant had reduced yCPK and increased ACT alongside significant
402 growth impairment. This regulation is likely to be indirect as EMSAs revealed His_6-GluR could
403 not bind to *actII-ORF4, redZ/red* and *kasO,* all of which are key regulators of ACT, RED and
404 yCPK biosynthesis respectively (84).

405 CutRS: vnz_27390/vnz_27395

406 Discovered in *Streptomyces lividans* in 1991 CutRS was the first TCS to be described in the
407 *Streptomyces* genus. Originally, and erroneously as admitted by the authors, it was thought
408 to be involved in the regulation of copper transport/metabolism and supressed melanin
409 production *melC1* mutations (85). Deletion of *cutRS* from *S. lividans* TK64 caused accelerated
410 and increased production of ACT. The mutants also displayed a premature onset of ACT from
411 7 days to 3-4 days on solid media equivalent to a 15 hours acceleration in liquid. These could
412 be complimented by the introduction of *cutR*. Temporal transcriptional analysis of *S. lividans*
413 TK64 in liquid cultures detected *cutRS* transcripts during transition (19h) and onset (40h)
414 phases but was unable to detect transcripts during the exponential phase. Consequently,
415 actinorhodin biosynthesis (*act)* repression by *cutRS* mRNA lasted approximately 21 hours
416 after transcription (86).

417 ## Other TCSs of interest

418 ### CseBC: vnz_15850/vnz_15855

419 The ECF sigma factor, σ^E, is encoded by the gene *sigE* and plays a role in stabilising the cell
420 envelope in *S. coelicolor*, with null mutants becoming more sensitive to cell wall lytic enzymes
421 (77,87). The CseBC TCS is named for its control of *sigE* (control of *sigE*) with null mutants
422 displaying the same phenotype as the *sigE* null mutant. It is encoded downstream of *sigE*
423 along with *cseA,* a gene encoding a lipoprotein. Determining the function of TCSs associated
424 lipoproteins has historically proven to be challenging, however it is clear that loss of CseA from
425 the cytoplasmic cell membrane induces upregulation of the *sigE* promoter (88). S1 nuclease
426 mapping of the *sigE* promoter showed transcription was reliant on the presence of the
427 response regulator CseB (89). Further studies, using a kanamycin-resistance reporter gene
428 fused to the promoter of the *sigE* operon, were used to investigate the role of CseBC in
429 responding to various antibiotics. This was in response to work done in *B. subtilis* where the
430 σ^W regulon was induced in response to cell wall-specific antibiotics (90,91). In *S. coelicolor*
431 the *sigE* promoter was transiently induced by the addition of the glycopeptide antibiotic
432 vancomycin, increasing 6-fold 30 minutes after exposure to 10 µg/ml of vancomycin but
433 subsequently reducing to uninduced levels by 90 minutes. It seems likely that CseBC is
434 detecting and responding to cell wall disruption, specifically to an intermediate in
435 peptidoglycan degradation/biosynthesis or a physical characteristic of cell wall disruption (91).

436 ### EcrE1/2: vnz_16945/vnz_16940

437 Named EcrE1/2 (vnz_16945/16940) due to having expression coordinated with RED from
438 DNA microarrays in *Streptomyces coelicolor* there were in fact two TCSs found to be
439 expressed, EcrA1/2 being the other but is not present in *S. venezuelae* or many other
440 streptomycetes (92). The SKs EcrE1 and EcrA2 share 33.33% amino acid identity whilst the
441 RRs EcrE2 and EcrA1 share 56.46%. Disruption of *ecrE1/2* in *S. coelicolor* revealed no
442 morphological or ACT production variation from the wild-type however RED production was
443 shown to be ~40% reduced. Northern blotting confirmed that transcription of the transcriptional

444 regulator genes *redD* and *redZ* were markedly reduced in the *ecrE1/2* disruption mutant
445 compared to the wild-type control suggesting that EcrE1/2 plays a role in the transcriptional
446 activation of RED biosynthesis (93). EcrA1/2 were previously shown to be playing a similar
447 role in *S. coelicolor,* reducing RED production by 60% and displaying no difference in
448 morphology or ACT production (92). No work has yet been done to investigate the interplay
449 between two analogous TCSs. It is possible that their activities are interchangeable, detecting
450 different signals but resulting in the same response: positively regulating RED biosynthesis.
451 However, the presence of only EcrE1/2 in a more diverse range of streptomycetes suggests
452 it may play a more important role.

453 CagRS: vnz_18555/vnz_18560

454 Clavulanic acid (CA) is a potent β-lactamase inhibitor used in the clinical treatment of □-
455 lactam-resistant microbial infections. *Streptomyces clavuligerus* is an industrialy important
456 producer of CA (94). Originally designated *orf22* (SK) and *orf23* (RR) this TCS was found
457 adjacent to the clavulanic acid biosynthetic gene cluster (95). These were later renamed *cagS*
458 and *cagR* respectively (96). Disruption of *cagS* slowed growth and sporulation on solid and
459 liquid media and decreased CA production whilst the opposite proved true during
460 overexpression of *cagS* (95). Later investigation exposed *cagRS* as a global regulator. ChIP-
461 seq revealed CagR primarily regulates genes involved in CA biosynthesis, fatty acid
462 degradation, arginine metabolism and glycerol-3-phosphate production. The latter two are
463 important CA biosynthesis precursors whilst it was shown CagR could bind the promoter
464 region of the γ-butyrolactone biosynthesis protein *BB341_RS25520 (avaA2)* through which
465 CA production can also be regulated highlighting a complex and multiplex control of CA
466 production at various metabolic levels (96).

467 RagKR: vnz_18850/vnz_18845

468 This TCS was identified using DNA microarrays as being part of the operon *ragABKR*
469 regulated by the orphan RR RamR, thus named RagK and RagR (*ramR*-activated genes).
470 Unlike the *ramR* mutant, the *S. coelicolor* Δ*ragABKR* mutant did not display a *bld* phenotype,

471 however there was an obvious and significant delay in sporulation on R2YE solid media. It
472 also presented with long, straight, undifferentiated aerial hyphae lacking commitment to
473 sporulation, similar to the *whiG* developmental mutant. Overexpression of *ragK* and *ragR* in
474 developmental mutants showed that both were essential for recovery of *ramR*, *ramS* or *bldK*
475 mutant phenotypes. In addition to this, complementation was possible with RagR(D53E), a
476 constitutively active version of RagR. The *ramR* mutant is also unable to synthesize SapB, a
477 small secreted surfactant which can act at the air-water interface to release surface tension
478 (97,98). SapB synthesis could be restored by RagR(D53E) suggesting that *ragAB* are not
479 required for SapB production and it is in fact *ragR* regulon elements which are key.
480 Interestingly, it has long been thought that SapB is the ultimate inducer of aerial hyphae
481 formation following an extracellular signalling cascade as there is impaired SapB production
482 in all the *bld* mutants. Whilst SapB synthesis could not be restored via the phosphorylation-
483 negative RagR(D53N) construct, it was still able to rescue the *ram/bld* developmental mutants
484 suggesting a SapB-independent induction of morphogenesis. This diffusible extracellular
485 signalling factor could be similar to the γ-butyrolactone A-factor of *S. grisues* or paramycin in
486 *S. alboniger* (98).

487 ## SenSR: vnz_19885/vnz_19880

488 SenSR was first described in 2005 in *Streptomyces reticuli,* a cellulose degrading species of
489 *Streptomcyes* which exhibits catalase and manganese-peroxidase activity through the
490 mycelia-associated haem-containing enzyme CpeB (vnz_36165) (99). This peroxidase activity
491 is controlled by the FurS protein which binds to its cognate operator upstream of the *furS* gene
492 (*vnz_36170*) when in its thiol state (100). Using a *senSR* disruption mutant in *S. reticuli* and
493 *S. lividans* transformants it was shown that SenSR reduces *cpeB, furS* transcripts and that of
494 a downstream gene, *hbpS* (*vnz_29470*). The disruption mutant exhibited increased sensitivity
495 to haemin, an iron-containing porphyrin with antibacterial activity, and plumbagin, another
496 redox-cycling compound known to generate reactive oxygen species (ROS). It is thought that
497 SenSR could be involved in the sensing of redox-triggered event signals. The sensitivity to

498 H_2O_2 and diamide was increased in the *senSR* mutant but relatively less than the sensitivity
499 to haemin and porphyrin (26% and 12% compared to 66% and 41% respectively). Further
500 protein-protein interactions studies revealed that SenS interacted with an extracellular protein
501 revealed to be HbpS (101). This heme-binding protein was proposed to be of importance in
502 the SenS signal-transduction cascade leading to increased CpeB synthesis as *S. reticuli*
503 mutants lacking active HpbS had reduced CpeB levels (102). Further studies propose the
504 HpbS-SenS complex acts by HbpS binding and degrading heme in a H_2O_2-dependant manner
505 consequently displaying iron on its surface, disrupting the HpbS-SenS extracellular interaction
506 and triggering the signaling cascade (103). In addition to this, the ability of HbpS to oxidise
507 ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}) prevents the formation of the highly reactive products
508 of the Fenton reaction, protecting *S. reticuli* from hazardous haem concentrations via iron-
509 sequestration (104).

510 ## ChiRS: vnz_24860/vnz_24865

511 The polysaccharide of *N*-acetylglucosamine, Chitin, is the most abundant natural biopolymer
512 derived from the exoskeletons of crustaceans, fungi and insects and proves highly insoluble
513 (105). Streptomycetes rely significantly on polymers such as chitin, cellulose and xylan as food
514 sources in their ecological niches, be that soil or sea, due to their worldwide abundance (106).
515 Chitinases hydrolyse the β1,4 linkage to produce chitooligosaccharides which are then
516 converted to GlcNAc by *N*-acetylglucosaminidase. These can be used as a carbon and
517 nitrogen source by the cell. ChiRS was first discovered in *Streptomyces thermoviolaceus*
518 OPC-520 in 1999 as a TCS found upstream of the chitinase locus (*chi40*). Introduction of this
519 *chiRS* operon into *S. lividans* 66 resulted in vastly increased production of Chi40 when induced
520 with chitobiose and chitin whilst GlcNAc had no effect (107). Follow up experiments including
521 EMSAs could not confirm the specificity of ChiR for the chitinase *chiC* promoter in *S. coelicolor*
522 however S1-nuclease mapping showed a significant decrease in promoter RNA-protected
523 fragment between M145 and a *chiR::tsr* disruption mutant suggesting that *chiR,* at least
524 indirectly, influenced the regulation of *chiC* (108). It is suggested, in corroboration with

525 evidence from the chitinolytic system in *Vibrio* spp. that in *S. coelicolor*, DasA, a chitobiose-binding protein, binds to the extracellular domain of ChiS, inactivating it and repressing the chitinolytic genes. Presence of extracellular chitin and thus $(GlcNAc)_2$ creates competition for DasA, and ChiS cannot bind DasA-$(GlcNAc)_2$ resulting in the chitinolytic phenotype via ChiR. Deletion of DasA results in a vastly increased chitinolytic activity which may confirm the ability of DasA to interfere with the start of the ChiS signaling cascade (106).

531 AbsA1/2: vnz_25235/vnz_25240

532 Identified during a screen for global antibiotics regulators in *S. coelicolor* (controlling the four antibiotics ACT, RED, CDA and MMY), four mutants were found to block antibiotic production without adverse morphological development beyond the lack of pigmentation you would expect from disrupted antibiotic production. These mutations were located in the *absA* locus which contains the SK *absA1* and its cognate RR *absA2* (109). Further investigation revealed that all the mutations were located in the histidine kinase sensor-transmitter domain. These strains could spontaneously revert to a pigmented, antibiotic phenotype via *sab* (suppressor of *abs*) mutations. A second phenotype (type-II) was described, also pigmented but with one day earlier expression of ACT and RED, these were known as Pha (precocious hyperproduction of antibiotics; (110)). In *S. coelicolor* the *absA1/2* operon is located within the CDA biosynthetic gene cluster. In *S. venezuelae* only a homolog of the RR *absA2* is present, located within a γ-butyrolactone (GBL) BCG. These are used as autoinducers, an intraspecies quorum-sensing signalling molecule. Deletion of GBL genes in *Streptomyces chattanoogensis* led to abolishment of morphological differentiation and antibiotic production on solid media. Control of GBL biosynthesis is also critical for the correct timing of metabolic switch events (111).

548 KdpDE: vnz_27435/vnz_27440

549 KdpDE is a homeostasis regulating TCS most extensively studied in *E. coli* with homologous systems found almost ubiquitously among bacteria. Potassium is the major intracellular cation and, whilst essential for life, it is also key in regulating cellular electrolyte and fluid balance

552 (112). In *E. coli kdpDE* are part of the *kdpFABCDE* operon where *kdpFABC* encodes a P-type
553 ATPase: a high affinity K^+ transporter. KdpD is an internally orientated SK with four
554 transmembrane domains forming two very short extracellular loops. It senses changes in
555 cytoplasmic K^+ and ATP concentration as well as osmolarity with a C-terminal K^+ sensor
556 (113,114). KdpA is a unique subunit of the ATPase in *E. coli* with no other similar units present
557 in any other P-type ATPase. It does, however, share structural and functional homology to
558 KcsA, an MPM-type potassium channel found in a few streptomycetes including *S. lividans*
559 (115). Our analysis found three streptomycetes that do not contain a KdpDE homologue
560 raising the question of how they sense and regulate K^+ concentration. It is conceivable that
561 systems such as KcsA make *kdpFABCDE* redundant and are regulated by an alternate TCS.
562 This TCS is also a key adaptive regulation system for bacteria virulence with a KdpDE deletion
563 in *M. tuberculosis* displaying hypervirulence in mice. Similar effects have been observed in *E.*
564 *coli* and *Staphylococcus aureus* (116). In *Mycobacterium* KdpE binds to a A-rich 22bp
565 promoter sequence with much greater affinity when phosphorylated. In *M. tuberculosis* KdpD
566 interacts with two lipoproteins, LprJ and LprF, which likely act as ligand-binding proteins or
567 are involved in the phosphor-relay system (117,118).

568 HrrAS: vnz_30150/vnz_30155

569 Little is known about *vnz_30150/55* however it shares the most homology with *DIP2267/8* from
570 the strict human pathogen *Corynebacterium diphtheriae* which encodes a TCS named HrrAS
571 (119). In this organism the heme oxygenase (HmuO) is key for iron uptake and utilisation in
572 the infectious environment, where iron is scarce. This is activated by two TCSs: ChrAS (no
573 clear homologue in *S. venezuelae*) and HrrAS with crosstalk also able to counteract deletions
574 in single KO strains (119). Comparative transcriptome analysis alongside DNA-protein
575 interaction assays revealed that heme-containing subunits of the respiratory chain such as
576 *ctaD* and the *ctaE-qcrCAB* operon are transcriptionally activated by HrrA. Conversely, HrrA
577 represses most of the genes involved in heme biosynthesis including glutamyl-tRNA
578 reductase (*hemA*), uroporphyrinogen decarboxylase (*hemE*) and ferrochetolase (*hemH*)

579 (120). Homologues of these in *S. venezuelae* are *vnz_15635*, *vnz_28830* and *vnz_27380*
580 respectively. The *hrrA* deletion mutant displayed depleted growth rate on solid and in liquid
581 cultures containing heme because of the reduction in activation of *hmuO* (120). HrrSA and
582 ChrAS have both been shown to play crucial roles in the detection and regulation of heme
583 utilisation but they appear to interfere in each other's activities with both HrrA and ChrA directly
584 repressing the transcription of the *chrSA* operon and *hrrA* (121).

585 VanRS: vnz_33685/vnz_33690

586 Vancomycin is a glycopeptide-class antibiotic whose resistance can be found throughout a
587 wide diversity of microorganisms including human pathogens such as *Staphylococcus aureus*
588 and actinomycetes including *S. coelicolor.* Like many other glycopeptide antibiotics,
589 vancomycin inhibits cell wall biosynthesis by binding to extracytoplasmic lipid-attached
590 peptidoglycan precursors via their D-Ala-D-Ala terminus. Resistance arises through two main
591 mechanisms: either the alteration of D-Ala-D-Ala to D-Ala-D-Lac or to D-Ala-D-Aer. Unlike
592 other glycopeptide-resistant actinomycetes such as *Streptomyces toyocaensis* and
593 *Actinoplanes teichomyceticus, S. coelicolor* does not produce a glycopeptide itself (122). In *S.*
594 *coelicolor* resistance arises through seven genes in four transcriptional operons: the TCS
595 *vanRS, vanJ*, *vanK* and *vanHAX*. Interestingly the *van* cluster confers resistance to another
596 glycopeptide, teicoplanin, but only when first induced by vancomycin, in isolation *S. coelicolor*
597 is not resistant to teicoplanin. Transcriptional analysis revealed that the *van* operon is
598 completely dependent on *vanR* and conversely proves its entire regulon, and that the *vanRS*
599 transcript is undetectable without induction via vancomycin. Remarkably there was also a
600 startling increase in the presence of *sigE* (the ECF sigma factor mentioned in regard to
601 CseBC) in the *vanR* mutant. It is proposed that without the cell wall remodelling that occurs in
602 response to vancomycin-induced *vanR* expression, the SigE activating cell wall intermediates
603 build up and induce a response (123,124). In the absence of vancomycin, dephosphorylated
604 VanR can be phosphorylated by the small molecule phosphodonor acetyl phosphate at which
605 point VanS acts as balance, using its kinase activity to remove the phosphate group from

606 VanR. *S. coelicolor* and *S. toyocaensis* have different VanRS induction spectra and simply by
607 swapping the VanRS systems over you can alter their respective spectra suggesting different
608 substrate specificities (124). In *S. coelicolor* it is proposed that VanS is activated by a complex
609 formed of vancomycin-bound vancosamine sugars present in peptidoglycan precursors,
610 initiating the signaling cascade and inducing glycopeptide resistance (125).

611

612 Unpaired SK

613 The P2RP analysis predicted 27 unpaired SKs in *S. venzuelae* which are all classic SKs
614 except the hybrid vnz_01755. 14 of the unpaired SK are probably incomplete, meaning that
615 they lack an obvious phosphorylatable His residue. The hybrid SK vnz_01755 was suggested
616 for crosstalk with the OsaABC system since the deletion of the hybrid SK *osaA* only displays
617 a mild phenotype (81).

618 The only unpaired SK described in detail is OhkA which is a classic SK containing one PAS
619 domain, the SK typical dimerization domain HisKA and the ATPase domain (Table 3). This
620 structure is similar to the *E. coli* SK NtrB (126). Both SKs do not contain any transmembrane
621 domains, thus they are likely cytoplasmic-sensing SKs. Not only is the structure of OhKA
622 highly conserved throughout *Streptomyces* (see Figure 2, *vnz_05825*) but the genomic
623 context as well (127). The SK OhKA forms an operon with the upstream putative rRNA
624 methylase (127). Downstream are *pheS* (*SCO1595)* and *pheT* (*SCO1594*), which encode
625 alpha and beta chains of a putative phenylalanyl-tRNA synthetase respectively. To investigate
626 the role of OhkA in *Streptomyces* the genes were deleted in *S. coelicolor* and *S. avermitilis*.
627 Both strains showed a similar phenotype of compromised aerial hyphae and spore formation
628 and increased production of ACT and CDA in *S. coelicolor* and oligomycin A in *S. avermitilis*
629 (127). Additionally, the deletion of *ohkA* in *Streptomyces peucetius* led to increased production
630 of doxorubicin and daunorubicin. However, OhkA is not paired with a RR in the DOX or DNR
631 clusters (128).

632 Unpaired SKs can be part of conservons (conserved operons). *S. coelicolor* genome contains
633 13 conservons (*cvn1-13*) each containing four genes *cvnA-D*: the first gene encodes for a
634 membrane sensor kinase, the second and third genes are of unknown function and the fourth
635 encodes for a possible ATP/GTP-binding protein. In four of the conservons in *S. coelicolor* a
636 predicted cytochrome P-450 lies downstream of the operon. *S. venezuelae* contains eight
637 conservons (cvn1-7 and cvn9). In *S. coelicolor cvn1* and *9* display altered formation of aerial
638 hyphae and antibiotic production upon deletion (129,130) The proteins of *cvn9* interact with
639 each other and form a membrane bound heterocomplex in an adenosine phosphate
640 dependent manner. The authors suggest that the Cnv9 complex may act as a signal
641 transducer which transfers an environmental signal to an intracellular response; which, in the
642 case of *cvnA,* is the different expression of the *bld* cascade (129). In a later study all 13
643 conservons in *S. coelicolor* were deleted but only *cvn1* displayed a phenotype under the tested
644 conditions. Aerial hyphae formation and antibiotic production were disrupted not only in the
645 *cvn1* mutant but also in the *cvnA1* mutant. Transcriptional analysis presented that *cvn1*
646 represses the vegetative sigma factor σ^{SigU} (130).
647 Unpaired SKs most likely originate from duplication events (31) and add additional complexity
648 to the gene regulation by TCSs, allowing bacteria to adapt to complex environmental stimuli.
649 Identification of RR partners of unpaired SK is not trivial. Several bioinformatical approaches
650 of identifying interaction partners of unpaired SK have been published (131–133) which are
651 based on chromosomally coupled RR and SK. Therefore, the identification of interaction of
652 distinct unpaired SK is limited (134).

653 # Orphan RR

654 In addition to the 27 unpaired SKs are the 18 orphan RRs of *S. venezuelae* that have been
655 identified by P2RP (Table 4). Orphan RRs are capable of effecting transcriptional regulation
656 independent of a cognate sensor kinase although this does not necessarily imply disregard to
657 phosphorylation state. These fall into two broad categories: typical and atypical with six of the
658 18 falling into the typical category and 12 proven or putative atypical orphan RRs.

659 Atypical response regulators are so categorised due to a lack of essential phosphorylation
660 residues within the conserved phosphorylation pocket. These RRs include important
661 developmental regulators such as the NarL/FixJ family BldM and WhiI, which are required for
662 the formation of aerial hyphae and spore maturation respectively. Whilst BldM contains the
663 conserved pocket, a D54A mutation at the conserved phosphorylation site is fully functional,
664 hence being categorised as atypical. WhiI lacks a pair of adjacent aspartates and one highly
665 conserved lysine forming a degenerate phosphorylation pocket incapable of binding Mg^{2+}
666 (135,136). The BldM regulon included two distinct groups of genes based on *whi* gene
667 dependency. Group I genes are regulated by a BldM homodimer, independently of WhiI, and
668 are important for early stage development, including *whiB* which is vital for normal aerial tip
669 growth. The BldM-WhiI heterodimer controls Group II genes which include late-stage
670 developmental genes such as the DNA translocase *smeA-sffA.* These target chromosomes
671 into spores during septa formation and the *whiE* operon which encodes the spore pigment
672 (136). BldM and WhiI are both highly conserved in *Streptomyces*. This stringent conservation
673 could suggest interaction with as yet undiscovered partners. Another atypical RR is JadR, the
674 OmpR-family transcriptional activator of jadomycin B biosynthesis in *S. venezuelae*. The dual
675 aspartates of JadR at residues 49 and 50 have been replaced with a glutamate and serine.
676 Instead of being activated by phosphorylation, JadR actually binds the small molecule
677 jadomycin B, leading to the conformational change required for DNA binding. This is similar to
678 the mechanism shown by the NarL-family atypical orphan RR RedZ of *S. coelicolor* which
679 regulates production of the RED antibiotic but is unable to bind the *redD* promoter in the
680 presence of RED (137). It is still an unanswered question as to whether BldM and/or WhiI
681 sense such effector molecules, or they merely exert gene regulation based on their
682 concentration inside the cell.

683 Typical orphan RRs are simply RRs without a partner SK encoded adjacent on the genome.
684 There has been little work done on typical orphan RRs in *S. venezuelae* but the OmpR-like
685 orphan response regulator GlnR of *S. coelicolor* has cross-talk with AfsQ1/2 as previously

686 mentioned. It regulates the expression of the glutamine synthetase (GS) I-b-subtype enzyme,
687 *glnA,* and a putative ammonium transporter *amtB* in *S. coelicolor*. Disruption of *glnR* results in
688 a glutamine auxotrophic phenotype. The transcriptional regulator, a close GlnRI homologue
689 (31% homology), GlnRII recognises the same promoters as GlnR however the *glnRII*
690 disruption mutant proved to glutamine prototrophic. GlnRII lacks many of the conserved
691 residues in the phosphorylation pocket including serine/threonine, tyrosine and the
692 phosphorylatable aspartate. Due to the observable phenotype differences and lack of
693 important residues it is likely that GlnRII is not a true functional RR (138,139). In addition to its
694 role in nitrogen assimilation, GlnR has been implicated in the regulation of cellobiose
695 degradation, upregulating β-glucosidase genes under nitrogen-limited conditions in
696 *Saccharopolyspora erythraea*. The cellobiose cluster in *S. coelicolor* A3(2) (*SCO2795-2798)*
697 was also found to contain putative GlnR-binding boxes in the promoter regions. Similar results
698 were found in other streptomycetes, *Mycobacterium smegmatis* and *Amycolatopsis*
699 *mediterranei* suggesting a level of conservation within Actinobacteria (140).

700 Future prospects

701 In our previous paper (25) there was sufficient knowledge to review eight specific TCSs. Just
702 fifteen years later that number has risen to twenty-two. With a suite of knockout and
703 mutagenesis tools available to use within streptomycetes including the CRISPR-Cas9 system,
704 it is possible to rapidly and flawlessly not only make knockouts but *cis* single nucleotide
705 changes which are vital tools for the study of TCSs (141). Combine this with the plethora of
706 next-generation whole genome sequences and the modern suites of powerful *in silico* tools
707 that can expedite the analysis of such data, the identification of TCSs of interest has become
708 almost trivial. The real challenge lies in two areas: identifying the activating signal(s) of the
709 sensor kinase and translating the basic research into applicable tools and techniques. The
710 industrial and clinical importance of Actinobacteria cannot be understated and the work
711 discussed in this review provides a clear and valuable foundation of TCS knowledge which
712 can be used to uncover and increase antibiotic production in these talented bacteria.

713

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720

Conflicts of interest

721 The authors declare that there are no conflicts of interest.

722

Figure Legends

723 **Figure 1.** A generalised diagram of a two-component system. The sensor kinase can be found
724 in the cytoplasmic membrane, often as a dimer. Autophosphorylation occurs at the conserved
725 histidine. Following activation by a signal the histidine kinase phosphorylates the partner
726 response regulator at the target aspartate residue. Following phosphorylation, the response
727 regulator dimerises in order to bind target DNA sequences and effect gene activation and / or
728 repression.

729 **Figure 2.** Conservation of S. venezuelae TCS genes in other streptomycetes. The TCS genes
730 are sorted according to their conservation and the species are sorted in alphabetical order. S.
731 venezuelae genes represent the reference for each ortholog cluster and those separated by
732 commas share high identity and could be paralogs

733

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