Two-component systems of the myxobacteria: structure, diversity and evolutionary relationships

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Two-component systems (TCSs) are a large family of signalling pathways characterized by the successive transfer of phosphoryl groups between the histidine and aspartate residues of paired histidine kinase and response regulator proteins. With the availability of genome sequences for four genera of myxobacteria it has become possible to assess the genomic complements of myxobacterial TCS genes and to characterize features of their organization and evolutionary heritage. In this study we have compiled lists of the TCS genes within myxobacterial genomes and characterized their domain architecture, gene organization and evolutionary relationships. In order to provide an appropriate context for our conclusions, where possible we have compared myxobacterial TCSs with those found in 316 other completely sequenced bacteria. Myxobacteria have the largest number of TCSs of any organisms. An unusually low proportion of TCS genes are paired in myxobacterial genomes, and myxobacterial histidine kinases also seem to sense internal signals to an unusual degree. Phylogenetic evidence has allowed us to suggest homologous relationships of proteins across the myxobacteria, and it appears that myxobacterial TCS evolution has been dominated by duplications, gene rearrangements and changes in sensory domain complements. The systematic classification of the TCS proteins of the myxobacteria presented here should also provide a framework for future experimental studies on two-component regulation in these organisms.

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INTRODUCTION

Myxobacteria are members of the Deltaproteobacteria and form an important part of the soil community (Dawid, 2000). They are copious producers of antibiotics and other biologically active secondary metabolites (Gerth *et al.*, 2003). However, the most striking behaviours of the myxobacteria are social: they exhibit co-ordinated motility, predating other members of the soil microfauna, and they possess a communal response to starvation. Under nutrient limitation a population of cells aggregates, forming a multicellular fruiting body within which cells differentiate into myxospores (for recent reviews see Kaiser, 2004; Whitworth,

2007). Genome sequences have become available for four myxobacteria, including the model myxobacterium *Myxococcus xanthus* DK1622 (Goldman *et al.*, 2006). *Sorangium cellulosum* So ce56 and *Stigmatella aurantiaca* DW4/3-1 are both capable of forming fruiting bodies (Schneiker *et al.*, 2007; Pradella *et al.*, 2002; Neumann *et al.*, 1993), while *Anaeromyxobacter dehalogenans* 2CP-C exhibits an unusual degree of metabolic versatility (Sanford *et al.*, 2002). A second *Anaeromyxobacter* genome (*Anaeromyxobacter* sp. Fw109-5) has recently become available, but was not included in this study. To date, more than 40 two-component system (TCS) proteins have been experimentally characterized and implicated in motility and/or development in the model organism *M. xanthus* (Whitworth & Cock, 2007). Sequence analysis shows that the genome sequences of myxobacteria contain an unparalleled number of TCS genes, making them important model organisms for studies of complexity in TCS signalling.

The first component in a typical TCS is a sensor kinase comprising an N-terminal input domain and a C-terminal transmitter domain. The second TCS component is a response regulator, which usually possesses an N-terminal

Abbreviations: CDS, coding sequence; HK, histidine kinase; HY, hybrid kinase; P, phosphotransfer protein; RR, response regulator; TCS, two-component system.

Three supplementary tables, of *Stigmatella aurantiaca* ORFs containing transmitter/receiver domains not identified as CDSs in GenBank, bacterial genomes surveyed and TCS proteins from the four myxobacterial genomes, and three supplementary figures showing phylogenetic trees for myxobacterial receiver domains, transmitter domains and Hpt domains are available with the online version of this paper.

receiver domain and a C-terminal output domain. Sensor kinase input domains are capable of sensing changes in their environment and, under appropriate conditions, trigger the activation of the transmitter domain. Activated transmitter domains then autophosphorylate on a conserved histidine residue. The phosphorylated transmitter domain is capable of binding to the response regulator receiver domain and in the resulting complex the phosphoryl group is transferred to an aspartate residue within the receiver domain. Phosphorylation of the response regulator alters the activity of the output domain, resulting in an appropriate response to the initial environmental change (for recent reviews see Bijlsma & Groisman, 2003; West & Stock, 2001). Often, the response regulator output domain has DNA-binding activity and phosphorylation of the response regulator leads to changes in DNA-binding activity and therefore gene expression.

More complex versions of TCSs exist with multiple phosphotransfer reactions. Phosphorelays are relatively common examples where there is successive transfer of phosphoryl groups from a transmitter domain to a receiver domain, to a His-containing phosphotransfer domain (usually a Hpt domain) and finally onto a second receiver domain (Appleby *et al.*, 1996). The four TCS domains involved can be found on separate polypeptides (for example, the sporulation phosphorelay of *Bacillus subtilis*: Hoch & Varughese, 2001) or as part of multi-domain proteins (for example, ArcBA of *Escherichia coli*: Peña-Sandoval *et al.*, 2005).

Input and output domains are diverse in sequence due to the variety of different stimuli to which TCSs respond and the different responses they elicit. However, transmitter and receiver domains retain high levels of sequence conservation, reflecting their common functions in the phosphotransfer scheme (Parkinson & Kofoid, 1992). Typically the genes for sensor kinases are found adjacent to the genes for their partner response regulators. However, many 'orphaned' TCS genes are isolated in the genome, while others lie in clusters of many TCS genes. In such cases, the partner proteins and signalling pathways of the encoded TCS proteins are unclear.

Of the TCS proteins described in the model myxobacterium *M. xanthus*, most are involved with the regulation of development or motility (Whitworth & Cock, 2007). However, the high proportion of orphan TCS genes in *M. xanthus* has hindered the description of entire TCS pathways. Orphan proteins with defined phenotypes include the sensor kinase SdeK and the response regulators ActA, ActB, Nla4, Nla6, FruA, FrzS and FrzZ, which all have roles in regulating motility and/or development (Pollack & Singer, 2001; Gronewald & Kaiser, 2001; Caberoy *et al.*, 2003; Ellehaug *et al.* 1998; Ward *et al.*, 2000; Trudeau *et al.*, 1996). The partner signalling proteins of these orphan proteins remain to be identified.

Here we present a survey of the TCS genes encoded by the four available myxobacterial genomes. In addition to describing major features of the TCSs we provide an

analysis of their evolutionary heritage, enabling identification of paralogous and orthologous relationships.

METHODS

Identification of TCS genes. TCS genes were identified using an automated procedure as described previously (Cock & Whitworth, 2007a). Myxobacterial genomes surveyed were *Anaeromyxobacter dehalogenans* 2CP-C (NC_007760), *Myxococcus xanthus* DK1622 (NC_008095), *Sorangium cellulosum* So ce56 (EMBL accession number AM746676), and *Stigmatella aurantiaca* DW4/3-1 (AAMD01000000). We also identified 12 ORFs encoding receiver and transmitter domains in the *St. aurantiaca* genome (downloaded from <http://www.tigr.org>) that had not been identified as coding sequences (CDSs) in the GenBank accession. We have given these ORFs CDS designations STIAU_X### and provided the translated ORF sequences as supplementary information (see Supplementary Table S1, available with the online version of this paper). In addition TCS complements of 316 completed bacterial genomes were surveyed (these genomes are listed in Supplementary Table S2, available with the online version of this paper).

Characterization of input/output domains, transmembrane helices and TCS gene organization. An automated assessment of input and output domains was undertaken by performing RPS-BLAST searches of all TCS CDSs against the Pfam database (Sonnhammer *et al.*, 1997) with an expectation value cut-off of 10^{-4} . Many domains in addition to transmitter and receiver domains were identified in this manner, and where possible these were classified manually as input or output domains. The presence/absence of transmembrane helices was assessed using TMHMM v2.0 (Sonnhammer *et al.* 1998). The presence of DNA-binding domains was determined by RPS-BLAST hits to a manually compiled list of PFAM domains (pfam00126, 00165, 00196, 00249, 00440, 00447, 00486, 01022, 01381, 02954, 04397, 04545 and 04967) using an expectation value cut-off of 10^{-4} . Gene organization of TCS genes was determined using a classification scheme based on proximity to other TCS genes. If a TCS gene was separated from other TCS genes by >5000 bp it was considered to be orphan; paired TCSs were defined as two TCS genes on the same strand of DNA and separated by <100 bp, encoding a total of one transmitter and one receiver domain. Any other gene organization was defined as complex, including gene clusters encoding multiple TCS genes containing more than one transmitter and/or more than one receiver domain. Orphan hybrid kinases were considered to be orphans regardless of the number of transmitter and receiver domains they encoded. The *St. aurantiaca* genome is incomplete and therefore TCS genes close (<3 kb) to the ends of contigs were annotated as having an uncertain gene organization.

Phylogenetic analysis. Multiple sequence alignments were generated for transmitter and receiver domains using CLUSTAL W (Thompson *et al.*, 1994) with default parameter settings. Phylogenetic trees were constructed with 1000 bootstraps using the neighbour-joining algorithm implemented in QuickJoin (Mailund & Pedersen, 2004), and 100 bootstraps using a parsimony algorithm, with PHYLIP (Felsenstein, 1989).

RESULTS

Myxobacteria have extremely large numbers of TCSs

Complete genome sequences are publicly available for three myxobacteria, *M. xanthus* DK1622 (9.1 Mbp), *So. cellulo-*

sum So ce56 (13.0 Mbp) and *A. dehalogenans* 2CP-C (5.0 Mbp). In addition, a partial genome sequence is available for *St. aurantiaca* DW4/3-1. This sequence consists of 61 scaffolds comprising a total of 10.1 Mbp of DNA. An automated screen was employed to extract TCS genes from the myxobacterial genome sequences and from 316 other completely sequenced bacterial genomes in GenBank (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>) as described previously (Cock & Whitworth, 2007a). Putative TCS genes were then classified as response regulators (RRs; proteins encoding receiver domains but no transmitter domains), histidine kinases (HKs; transmitter domains but no receiver domains), hybrid kinases (HYs; transmitter and receiver domains) or phosphotransfer proteins [Ps; Hpt or HisKA domains but no histidine ATPase (HATPase) or receiver domains].

Fig. 1 displays the number of TCS genes found in an organism against the genome size of that organism, for 320 bacteria (a similar distribution is seen when considering the number of receiver/transmitter domains; data not shown). At one extreme of the distribution are three of the myxobacterial genomes, with the largest numbers of TCS genes and receiver/transmitter domains of any organisms (Fig. 1). *M. xanthus* possesses 278 TCS proteins (337 receiver/transmitter domains), comprising 134 RR, 99 HK, 41 HY and 4 P proteins. *So. cellulorum* encodes 267 TCS proteins (335 receiver/transmitter domains), consisting of 119 RRs, 98 HKs, 49 HYs and 1 P. *St. aurantiaca* has 326 TCS proteins (421 receiver and transmitter domains), 137 RRs, 120 HKs, 66 HYs and 3 Ps. The smallest myxobacterial genome (*A. dehalogenans*) has the lower numbers of TCS proteins (174 proteins, with 195 receiver and transmitter domains), including 92 RRs, 65 HKs and 17 HYs, but still more than most organisms of comparable genome size (the mean number of TCS genes for bacteria

with genome sizes between 4 and 6 Mbp is 81.7 ± 35.3). Lists of the myxobacterial TCS proteins, with descriptions of their genetic and domain architecture and whether they are predicted to possess transmembrane helices, are available as supplementary information (see Supplementary Table S3 available with the online version of this paper).

For most bacteria there is an approximately linear relationship between the number of encoded TCS proteins and genome size (Fig. 1). Organisms that have small numbers of TCSs for their genome size appear to live in relatively constant environments, as observed previously (Galperin, 2005), and include many pathogens. Several bacteria (mostly Delta/Epsilonproteobacteria) possess many more TCSs than most organisms of similar genome size, for example *Wolinella succinogenes* DSM 1740 (83 TCS genes, 2.1 Mbp), *Desulfovibrio vulgaris* Hildenborough (136 TCS genes, 3.8 Mbp), *Geobacter sulfurreducens* PCA (183 TCS genes, 3.8 Mbp), *Nostoc* sp. PCC 7120 (215 TCS genes, 7.2 Mbp) 'Dechloromonas aromatica' RCB (201 TCS genes, 4.5 Mbp) and the myxobacteria (Fig. 1). It has been proposed that the number of TCS genes within genomes correlates more with sophistication of lifestyle than with variability of environment (see Galperin, 2005 for a more involved discussion), a suggestion supported by the behavioural complexity of the myxobacteria.

For nearly all organisms (including the myxobacteria) there was a constant receiver domain:transmitter domain ratio ($\sim 3:2$), which was independent of genome size or the number of TCSs within each genome (data not shown). This suggests that in most organisms there are more divergent (one HK phosphorylating >1 RR) than convergent (one RR being phosphorylated by >1 HK) TCS pathways. For those organisms with ≥ 50 TCS genes no examples were found with a receiver:transmitter ratio >1.9 , while only nine had a receiver:transmitter ratio <1.0 (three *Bacillus* strains, three *Bacteroides* strains, *Methanosarcina acetivorans*, *Haloarcula marismortui* and *Salinibacter ruber*).

A large proportion of myxobacterial TCS genes are orphaned or in complex clusters

Typical TCSs consist of paired HK and RR genes (often translationally coupled). However, TCS genes can be found either isolated in the genome (orphaned) or in complex gene clusters. An orphan or complex organization may be indicative of multiple partnerships between TCS proteins (for example CheA, CheY and CheB of *E. coli* are found in a complex gene cluster, and the HK CheA phosphorylates both CheB and CheY, while the proteins of the sporulation phosphorelay of *Bacillus subtilis* are orphaned). We classified the gene organization of all TCS proteins in our lists as either paired, orphan or complex (see Methods). The numbers and proportions of orphan, paired and complex systems varied widely between genomes. For organisms with >50 TCS genes, the proportion of paired

Fig. 1. Numbers of TCS genes found in bacterial genomes, as a function of genome size. Data from four myxobacteria (black) and 316 other bacteria (grey) are shown. A linear trendline is presented for non-myxobacterial organisms. Myxobacteria have significantly more TCS genes than would be predicted from their genome size. *Ad*, *Anaeromyxobacter dehalogenans*; *Mx*, *Myxococcus xanthus*; *Sa*, *Stigmatella aurantiaca*; *So*, *Sorangium cellulosum*.

Fig. 2. Proportion of paired TCS genes within a genome with respect to the total number of TCS genes within that genome. Data plotted for four myxobacteria (black) and 316 other bacteria (grey). Organisms with larger numbers of TCS genes tend to have a smaller proportion of paired TCS genes. *Ad*, *Anaeromyxobacter dehalogenans*; *Mx*, *Myxococcus xanthus*; *Sa*, *Stigmatella aurantiaca*; *So*, *Sorangium cellulosum*.

TCS genes varied between 0.8 (*Clostridium tetani* E88) and 0.0 [two *Cyanobacteria* (Yellowstone A and B), *Haloarcula marismortui* and *Methanosarcina acetivorans*]. A plot of the proportion of paired TCS genes against the number of TCS genes in a genome (Fig. 2) demonstrates a reduced proportion of paired TCS genes as the number of TCS genes increases (compensated for by larger proportions of orphaned genes and complex gene clusters). This observation suggests that with increasing numbers of TCS genes comes increased communication between proteins. Table 1 provides a summary of the TCS gene organization of myxobacteria and, for comparison, the model organisms *Escherichia coli*, *Bacillus subtilis*, *Streptomyces coelicolor* and *Synechocystis* sp. PCC 6803. Compared to the model organisms, the myxobacteria have an unexpectedly large number of TCS genes within complex gene clusters, even when compensating for their larger numbers of TCSs.

Five TCS gene clusters of *M. xanthus* are particularly complex, encoding at least two histidine-containing

phosphotransfer domains (transmitter and Hpt domains) and at least three receiver domains (Fig. 3). One of these clusters (*redCDEF*) has been characterized and shown to regulate the timing of multicellular development (Higgs *et al.*, 2005); however, the role of the other clusters has yet to be determined. These gene clusters are currently being investigated experimentally in our laboratory.

An unexpectedly large proportion of myxobacterial kinases are predicted to be cytoplasmic

As a general rule the presence of transmembrane helices in a HK or HY would suggest a transmembrane localization and imply in turn that the kinase was responding to an extracytoplasmic stimulus. Conversely, soluble kinases would be expected to sense changes to the internal milieu. We assessed what proportion of sensor kinases possess predicted transmembrane helices and thus would be expected to respond to extracellular stimuli (see Methods). Fig. 4 shows how the number of sensor kinases (both HK and HY) predicted to be transmembrane varies as a function of the total number of sensor kinases within a genome. For all bacteria with ≥ 10 sensor kinases, the mean proportion of transmembrane kinases is $\sim 71.7\%$ ($\pm 20.2\%$), while the numbers are much lower for the myxobacteria. The large numbers of cytoplasmic HKs may explain the large proportion of HYs in myxobacterial genomes (Cock & Whitworth, 2007b). Other organisms displaying unexpectedly low numbers of transmembrane sensor kinases include *Nostoc* sp. (39 of 133, 29%), *Anabaena variabilis* (38 of 128, 30%), *Methanosarcina acetivorans* (10 of 49, 20%), *Haloarcula marismortui* (14 of 58, 24%) and *Methanospirillum hungatei* (24 of 76, 32%). Presumably these organisms and the myxobacteria predominantly assess their environment by monitoring changes in their internal state. Within the myxobacterial lineage, the absolute number of transmembrane sensor kinases does not increase significantly with increasing genomic complement of TCSs, suggesting that the different myxobacteria respond to a similar number of extracellular factors (Fig. 4).

Table 1. Organization of the TCS genes of four myxobacteria and four model bacteria

TCS gene organizations are classified as paired, orphan or complex, as described in the text. Because the *Stigmatella aurantiaca* genome comprises 492 separate contigs an 'uncertain' gene organization indicates genes found towards the ends of contigs. *Myxa*, *Myxococcus xanthus* DK1622; *Soce*, *Sorangium cellulosum* So ce56; *Stau*, *Stigmatella aurantiaca* DW4/3-1; *Ande*, *Anaeromyxobacter dehalogenans* 2CP-C; *Esco*, *Escherichia coli*; *Basu*, *Bacillus subtilis* (str. 168), *Stco*, *Streptomyces coelicolor* A3(2); *Syn.* 6803, *Synechocystis* sp. PCC 6803.

Gene organization	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>	<i>Esco</i> *	<i>Basu</i> †	<i>Stco</i> ‡	<i>Syn.</i> 6803§
Paired	80	62	78	56	44	60	112	22
Complex	93	91	97	44	9	0	25	12
Orphan	105	114	121	74	9	10	55	52
Uncertain	–	–	30	–	–	–	–	–
Total	278	267	326	174	62	70	192	86

*, †, ‡, §Numbers derived from Mizuno (1997), Fabret *et al.* (1999), Hutchings *et al.*, (2004) and Mizuno *et al.* (1996), respectively.

Fig. 3. Complex TCS gene clusters of *Myxococcus xanthus*. Genes are represented as arrows, with the domain architecture of the predicted protein product as text within each arrow (presumed input/output domains are in small type). Arrow length is proportional to gene length. Each cluster contains at least two histidine-containing phosphotransfer domains (T or Hpt) and at least three receiver domains (R).

TCS input and output domains are unevenly distributed across bacteria

Bacterial predicted proteomes were assessed for the presence of conserved domains by searching against the Pfam database (see Methods). The most common Pfam domain was the ABC transporter (pfam00005), with 21 364 such domains identified. The second most common domain was the receiver domain (pfam00072, 10 171 cases), while the third most frequent domain was the HATPase domain (pfam02518, 8834 cases). Amongst the set of TCS proteins the most common non-phosphotransfer domain was the DNA-binding domain Trans_reg_C

(pfam00486, 2807 cases), which is found for example in PhoB and OmpR. Ten output domains (eight DNA-binding associated domains [Trans_reg_C, GerE, Sigma70_r4, HTH_Fis, HTH_10, Sigma54_activator/AAA_ATPase, LytTR and HTH_AraC], CheB methyltransferase and GGDEF domains) and five input domains (PAS, PAC, GAF, HAMP and CheW domains) were each found in >200 cases amongst the bacterial TCS proteins. The percentages of TCS proteins of the 320 bacteria containing these 15 common domains are presented in Table 2.

The distribution of the 15 common domains among the bacteria was assessed by comparing the numbers of a particular domain found in one organism's TCS proteins as a proportion of the total number of such domains found in the TCS proteins of all 320 bacteria, restricting analysis to genomes with ≥ 100 TCS proteins (57 organisms). Organisms that encoded >5% of all TCS proteins containing a particular domain are listed in Table 3. *St. aurantiaca* was found to contain large numbers of TCS proteins containing GAF domains. The cyanobacteria *Nostoc* and *Anabaena* also possessed large numbers of GAF domains, while *Bacteroides thetaiotaomicron* contained exceptionally large numbers of HTH_AraC domains. GAF domains are found in phytochromes and cGMP phosphodiesterases in addition to sensor proteins, but in the vast majority of cases the signals sensed by GAF domains are unknown. The proportion of the TCS proteins of an organism that contained each domain was also determined. In three cases >40% of the TCS protein complement of an organism was found to contain a particular domain. *Anabaena variabilis* and *Methanospirillum hungatei* each possessed a large proportion of PAC domain-containing TCSs (43% and 64% respectively, 88 and 89 cases), while *Methanospirillum*

Fig. 4. Number of sensor kinases (HKs and HYs) predicted to be transmembrane, as a function of the total number of sensor kinases. Data plotted for four myxobacteria (black) and 316 other bacteria (grey). For all bacteria with ≥ 10 sensor kinases, the mean proportion of transmembrane kinases is $\sim 71.7\%$ (± 20.2), while the myxobacteria have substantially fewer transmembrane sensor kinases. Ad, *Anaeromyxobacter dehalogenans*; Mx, *Myxococcus xanthus*; Sa, *Stigmatella aurantiaca*; So, *Sorangium cellulosum*.

Table 2. Abundant input and output domains: percentage of TCS proteins that contain particularly common input and output domains (found in >1 % of all TCS proteins of 320 bacteria)

Domain	% of TCSs
Input domains	
PAS	16.7
HAMP	15.7
PAC	11.7
GAF	5.4
CheW	2.5
Output domains	
Trans_reg_C	17.1
GerE	8.0
Sigma70_r4	6.2
HTH_8	5.0
Sigma54_activator	4.7
GGDEF	1.9
HTH_10	1.7
LytTR	1.6
HTH_AraC	1.6
CheB_Me-esterase	1.3

hungatei also possessed a large proportion of PAS domain-containing TCSs (92 %, 129 cases). PAS and PAC domains are often associated with one another within proteins and act as signal sensor domains for a wide range of stimuli. Although the unexpectedly large numbers of certain accessory domains within the TCS proteins of specific organisms is intriguing, we have not been able to correlate the presence of those domains with particular physiological behaviours.

Classification of myxobacterial response regulators

Response regulators can be classified according to their domain architecture (Galperin, 2006). Most examples comprise an N-terminal receiver domain and a C-terminal output domain, although more complicated geometries exist. Table 4 groups RRs by domain architecture for the myxobacterial proteins and those of *Escherichia coli*, *Bacillus subtilis* and *Synechocystis* sp. PCC 6803. RR families

are named after well-characterized proteins where possible, or by domain architecture (see Table 4).

Myxobacterial RRs exhibit unusually large numbers of AAA_ATPase, HTH_Fis domains as found in NtrC homologues. Additionally they possess a large number of RRs that consist entirely of a single receiver domain, resembling CheY of *E. coli*. CheY homologues are involved in chemotactic regulation (phosphorylated by CheA homologues) and possess single receiver domains. CheA homologues are easily identified by the presence of an Hpt domain within their transmitter domains, and in most organisms *cheA* is encoded in the proximity of *cheY*. The proximity of a receiver-only RR gene to a *cheA* homologue enables us to classify receiver-only proteins as CheY homologues or not (a division that is supported by phylogenetic evidence; our unpublished data). In the myxobacteria the vast majority of receiver-only RRs are not CheY homologues (Table 4). Presumably they effect regulation by protein–protein contacts, act to modulate phosphoryl transfer to effector RRs, or participate in phosphorelays. The only other organisms which possess large numbers of receiver-only RRs are cyanobacteria, particularly *Nostoc* sp. PCC 7120 and *Anabaena variabilis*.

Over-represented RR families (even compensating for the large number of RRs in myxobacterial genomes) also include the CheY, CheB, PleD, RpfG, CyaB and K,R,Cyc families (Table 4). Several RR families appear to be unique to myxobacteria, including the FrzZ family of proteins, which contain tandem receiver domains, and the R,STAS family, which contain C-terminal anti-anti-sigma factor/sulfate transport domains. The only common RR family that is under-represented in myxobacterial genomes is the NarL family, which includes the important regulator of fruiting FruA (Ellehaug *et al.*, 1998).

Myxobacterial histidine kinase input domains

A variety of common input domains can be found in HKs and HYs, typically N-terminal to the transmitter domain. Table 5 lists the numbers of HKs and HYs in myxobacterial genomes that possess HAMP, GAF and/or PAS/PAC input domains. Also described are the numbers of CheA homologues, characterized by Hpt-containing transmitter domains and CheW domains.

Relatively uncommon input domains are also found amongst the myxobacterial TCS kinases (Table 6). Sixteen of the HKs and HYs of *So. cellulosum* (nine from *St. aurantiaca* and one from *M. xanthus*) possess Ser/Thr kinase domains (Table 6), implying an unusual degree of integration of Ser/Thr kinase and TCS signalling networks. The three myxobacteria containing Ser/Thr kinase input domains also contain HKs with FHA (forkhead-associated) domains implicated in transducing signals from Ser/Thr kinases (Jelsbak *et al.*, 2005). All four myxobacteria contain HKs with input domains associated with regulation of potassium uptake/efflux (Kdp and KefB domains), three

Table 3. Abundant input and output domains: organisms that contain large numbers of particular input/output domains (>5 % of all TCS proteins containing those domains, from 320 bacteria)

Domain	Organism	% of all TCSs	Domain-containing TCSs
GAF	<i>St. aurantiaca</i>	5.5	49
GAF	<i>Nostoc</i> sp.	7.3	65
GAF	<i>Anabaena variabilis</i>	7.8	69
HTH_AraC	<i>Bacteriodes thetaiotaomicron</i>	16.7	43

Table 4. Classification of myxobacterial response regulators by domain architecture

R, receiver domain; T, transmitter domain; K, Ser/Thr kinase domain; O, output domain; *Myxa*, *Myxococcus xanthus* DK1622; *Soce*, *Sorangium cellulosum* So ce56; *Stau*, *Stigmatella aurantiaca* DW4/3-1; *Ande*, *Anaeromyxobacter dehalogenans* 2CP-C; *Esco*, *Escherichia coli*; *Basu*, *Bacillus subtilis* (str. 168); *Syn.* 6803, *Synechocystis* sp. PCC 6803.

Family	Domains	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>	Total (myxobacteria)	<i>Esco</i> *	<i>Basu</i> †	<i>Syn.</i> 6803‡
CheY	R	8	7	9	7	31	1	1	
PhoB	R,Trans_reg_C	11	11	10	5	37	14	14	10
NtrC	R,AAA_ATPase,HTH_Fis	30	22	20	27	99	4	—	—
NarL	R,HTH_LuxR	5	3	5	3	16	7	9	7
CheB	R,CheB	6	2	9	6	23	1	—	—
R	R	46	51	60	31	188	—	2	7
FrzZ	R,R	4	4 (1§)	5	2	15	—	—	—
R,STAS	R,STAS	—	4 (1)	—	—	4	—	—	—
PleD	R,GGDEF	8 (2)	1	5	4 (1)	18	—	—	—
RpfG	R,HDc	3	1 (1)	2	2	8	—	—	—
CyaB	R,CyaA	1	2	1	—	4	—	—	—
K,R,Cyc	S/T_Kin,R,CyaA	1	3	3	—	7	—	—	—
RO	R,O (DNA-binding)	4	4	2	1	11	2	3	1
RO	R,O (other)	4	2	2	2	10	3	4	7
OR	O,R	2	1	3	2	8	—	—	6
ORO	O,R,O	—	1	1	—	2	—	—	—
RHRRO	R,H,R,R,GGDEF	1	—	—	—	1	—	—	—
Total		134	119	137	92	482	32	33	38

*, †, ‡Numbers derived from Mizuno (1997), Fabret *et al.* (1999) and Mizuno *et al.* (1996), respectively.

§Protein contains an additional Hpt domain.

||Proteins contain an additional GAF domain.

myxobacterial HYs possess paired CheB,CheR input domains, and three HKs possess PutP domains for Na⁺/proline symport. Both *M. xanthus* and *St. aurantiaca* possess HKs with CAP-effector domains (CAP-EDs), which are implicated in binding cyclic nucleotides, *St. aurantiaca* possesses two HKs with phytochrome input domains, while intriguingly a HK of *A. dehalogenans* has an N-terminal globin-coupled-receptor domain.

Myxobacterial hybrid kinases have diverse and complicated domain architectures

HYs can be classified according to the number and order of their transmitter and receiver domains (Table 7). The most common domain architectures consist of single receiver and transmitter domains (125 of 173); however, 48 HYs have multiple transmitter (T) and/or receiver (R) domains.

Table 5. HK and HY input domains: numbers of myxobacterial HKs and HYs containing PAS, GAF or HAMP domains

Myxa, *Myxococcus xanthus* DK1622; *Soce*, *Sorangium cellulosum* So ce56; *Stau*, *Stigmatella aurantiaca* DW4/3-1; *Ande*, *Anaeromyxobacter dehalogenans* 2CP-C. Numbers of CheA homologues are also presented (characterized by a CheW input domain).

Input	HKs				HYs				Total
	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>	
PAS	18	8	21	10	11	14	18	3	103
GAF	7	13	23	3	3	2	11	—	62
HAMP	12	19	13	12	—	8	3	1	68
PAS,GAF	9	7	12	2	—	10	3	—	43
GAF,HAMP	—	—	—	—	2	2	—	—	4
PAS,HAMP	1	1	—	—	—	—	—	—	2
HAMP,CheA	—	1	—	—	—	—	—	—	1
CheA	3	5	5	5	5	1	4	2	30
None/other	49	44	46	33	20	12	27	11	242
Total	99	98	120	65	41	49	66	17	555

Table 6. HK and HY input domains: unusual input domains found in myxobacterial kinases

Myxa, *Myxococcus xanthus* DK1622; *Soce*, *Sorangium cellulosum* So ce56; *Stau*, *Stigmatella aurantiaca* DW4/3-1; *Ande*, *Anaeromyxobacter dehalogenans* 2CP-C.

Input	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>
S/T_Kinase	1	16	9	–
CAP-ED	1	–	1	–
Kdp	1	1	–	1
KefB	–	–	1	–
PhnD	–	–	1	–
CheB/CheR	–	1	1	1
PutP	1	1	–	1
FHA	1	1	1	–
Globin	–	–	–	1
Phytochrome	–	–	2	–

Of these TRT, TRR, TRRR and RTR geometry HYs dominate (39 proteins). These proteins are intriguing as it is likely that multiple routes of phospho transfer exist within each protein (presumably inter-dependently), potentially giving rise to sophisticated dynamic behaviour. One such example is RodK (Rasmussen *et al.*, 2005), a TRRR protein that regulates the timing of fruiting body formation. All four domains of RodK are required for activity and each domain has a distinct phosphorylation-dependent function (Rasmussen *et al.*, 2006). In the complex HYs of *E. coli* (ArcBA, BarA/UvrY, EvgAS and TorSR), signalling domains are located in sequential order N- to C-terminal, reflecting the direction of phosphoryl group transfer during signalling. This does not appear to be the case for the myxobacterial HYs, unless receiver-to-receiver and receiver-to-transmitter phosphotransfer also occurs.

Table 7. Domain architecture of myxobacterial HYs

Myxa, *Myxococcus xanthus* DK1622; *Soce*, *Sorangium cellulosum* So ce56; *Stau*, *Stigmatella aurantiaca* DW4/3-1; *Ande*, *Anaeromyxobacter dehalogenans* 2CP-C. R, receiver domain; T, transmitter domain.

	<i>Myxa</i>	<i>Soce</i>	<i>Stau</i>	<i>Ande</i>	Total
TR	14	24	19	10	67
TRT	1	5	6	–	12
TRTR	–	2	1	–	3
TRTTRT	–	–	1	–	1
TRR	5	1	5	–	11
TRRR	3	1	3	–	7
TRRH	–	1	1	–	2
RT	10	10	21	4	45
RTR	1	4	3	1	9
RTRR	1	–	1	–	2
RRT	1	–	–	–	1
CheAY	5	1	5	2	13
Total	41	49	66	17	173

Phylogenetic relationships and evolution of myxobacterial TCS proteins

In order to assess homology relationships between myxobacterial TCS proteins and gain an understanding of the evolutionary events responsible for the contemporary set of proteins, phylogenetic trees were constructed for myxobacterial receiver and transmitter domains (see Methods). Fig. 5 is a phylogenetic distance tree derived from the DnaA protein sequences of the four myxobacteria and a selection of more distantly related organisms. The tree in Fig. 5 agrees with previously published molecular phylogenies (Sanford *et al.*, 2002), and illustrates the evolutionary distance between the organisms under consideration. *M. xanthus* and *St. aurantiaca* are most closely related to each other, and together they are closer to *A. dehalogenans* than to *So. cellulosum*. TCS trees are available with the online version of this paper (Supplementary Figures S1, S2 and S3). For each leaf of the trees the domain architecture and gene organization is presented and leaves/clades are coloured according to the host organism. Splits were represented in colour/black if bootstrap support was obtained for that split (>70 % of bootstrapped trees contained that split using both neighbour-joining and parsimony methods), otherwise splits were coloured mid-grey if one method gave a bootstrap value >70 %, or pale grey if neither method gave bootstrap support. The trees were then used to identify clades of homologues. Table 8 presents homology relationships for myxobacterial TCS proteins that have been experimentally characterized (based on the list presented in Whitworth & Cock, 2007), using clades with bootstrap support >50 %.

Fewer orthologues were identified with increasing evolutionary distance from *M. xanthus* (57 in *St. aurantiaca*, 25

Fig. 5. Neighbour-joining phylogenetic tree of the DnaA protein of the four myxobacteria and selected non-myxobacterial bacteria. Numbers at nodes represent the number of times out of 1000 bootstrap replicates that the cluster defined by the node was monophyletic. *Basu*, *Bacillus subtilis*; *Nosp*, *Nostoc sp.*; *Esco*, *Escherichia coli*; *Soce*, *Sorangium cellulosum*; *Ande*, *Anaeromyxobacter dehalogenans*; *Stau*, *Stigmatella aurantiaca*; *Myxa*, *Myxococcus xanthus*. *Bacillus subtilis* was defined as the outgroup.

Table 8. Experimentally characterized *M. xanthus* TCS proteins and their homologues in other myxobacterial genomes

Partner HK and RR proteins are presented on the same line where possible, with a description of their domain architecture. Homologues shown in bold text have undergone an alteration in domain architecture relative to their *M. xanthus* counterparts, while those in italics have undergone a change in gene organization.

Domain architecture		<i>M. xanthus</i>		<i>St. aurantiaca</i>		<i>A. dehalogenans</i>		<i>So. cellulorum</i>	
HK	RR	HK	RR	HK	RR	HK	RR	HK	RR
Hpt,HATPase,CheW,R	R,CheB	CheA3	CheB	STIAU_1049	STIAU_1047		<i>Adeh_0614</i>		
Hpt,HATPase,CheW,R	R	CheA4	CheY4	STIAU_X008	STIAU_4795				
Hpt,HATPase,CheW	R	DifE	DifD	STIAU_8663	STIAU_8664	Adeh_4316	Adeh_4317		
Hpt,HATPase,CheW,R	R,CheB	FrzE	FrzG	STIAU_6834	STIAU_6833	<i>Adeh_0613</i>			
	R,R		FrzZ		<i>STIAU_6840</i>		<i>Adeh_0609</i>		
	R,coiled-coil		FrzS		STIAU_6845		Adeh_2380		Sce5864
T	R,R	RedC	RedD	STIAU_3439	STIAU_3440			Sce5167,	Sce5166,
								Sce8798	Sce8799
T	R	RedE	RedF	STIAU_3441	STIAU_3442			Sce5168,	Sce8800
								Sce8797	
T,R,R,R	R	RodK	MXAN_0732	STIAU_5673	STIAU_5674				
PAS,PAS,T	R	TodK	DotR	<i>STIAU_0573</i>	<i>STIAU_6974</i>		Adeh_0606		<i>Sce2424,</i> <i>Sce2206</i>
R,T	R	AsgA	MXAN_2671	STIAU_4780	STIAU_4782			<i>Sce6174</i>	
	R		RomR		STIAU_0910		Adeh_2391		<i>Sce0012</i>
	R,coiled-coil		AglZ		STIAU_7168		Adeh_2705		
	R,GGDEF		ActA						
	R,HTH_Xre		DigR		STIAU_6121				<i>Sce0622</i>
	R,HTH_LuxR		FruA						
T	R,Trans_reg_C	PhoR1	PhoP1	STIAU_5393	STIAU_5394				
T	R,Trans_reg_C	PhoR2	PhoP2	STIAU_0112	STIAU_X001				
T	R,Trans_reg_C	PhoR3	PhoP3	STIAU_0427	STIAU_X002				
	R,Trans_reg_C		PhoP4		STIAU_5390				
	R,AAA_ATPase,HTH_Fis		CrdA		STIAU_1056		Adeh_3338		<i>Sce1691</i>
	R,AAA_ATPase,HTH_Fis		ActB		STIAU_5048		Adeh_2651		
T	R,AAA_ATPase,HTH_Fis	FrgB	FrgC	STIAU_3782	STIAU_3783				
T	R,AAA_ATPase,HTH_Fis	MrpA	MrpB	STIAU_1020	STIAU_1022				
PutP,PAS,T	R,AAA_ATPase,HTH_Fis	MXAN_5852	Nla1				<i>Adeh_4250</i>	<i>Sce2727</i>	<i>Sce2692</i>
	R,AAA_ATPase,HTH_Fis		Nla4		STIAU_4539		Adeh_0688		<i>Sce9222</i>
HisKA	R,AAA_ATPase,HTH_Fis	MXAN_4043	Nla6	STIAU_6745	STIAU_6743		<i>Adeh_1555</i>		
T	R,AAA_ATPase,HTH_Fis	MXAN_5778	Nla23	STIAU_7821	STIAU_7820	Adeh_0638	Adeh_0639		<i>Sce2881</i>
HAMP,T	R,AAA_ATPase,HTH_Fis	MXAN_7439	Nla24						
T	R,AAA_ATPase,HTH_Fis	MXAN_1166	Nla28	STIAU_3702	STIAU_3701	<i>Adeh_0021</i>		<i>Sce0947</i>	
PAS,T	R,AAA_ATPase,HTH_Fis	PilS	PilR	STIAU_7829	STIAU_7828	Adeh_0625	Adeh_0626		
HAMP,T	R,AAA_ATPase,HTH_Fis	SasS	SasR	STIAU_3603	STIAU_3617	Adeh_4246	Adeh_4240		
PAS,GAF,T	R,AAA_ATPase,HTH_Fis	SpdS	SpdR	STIAU_2431	STIAU_2430	<i>Adeh_0418</i>	<i>Adeh_0417</i>		<i>Sce1649</i>
R,T	R,AAA_ATPase,HTH_Fis	HsfB	HsfA	STIAU_6307	STIAU_6308	Adeh_3472	Adeh_3471		
R,GAF,GAF,T		AsgD		<i>STIAU_0817</i>					

Table 8. cont.

Domain architecture	<i>M. xanthus</i>			<i>St. aurantiaca</i>			<i>A. dehalogenans</i>			<i>So. cellulosum</i>		
	HK	RR	HK	RR	HK	RR	HK	RR	HK	RR	HK	RR
FHA,PAS,PAS/PAC,T,R												
PAS,PAC,T,R	EspA				STIAU_8475							
T,R	EspC				STIAU_0700							
LBD,T,R	KfaA				STIAU_5133							
PAS,T	MokA											
PAS,GAF,T	SdeK				STIAU_8399							
	SocD				STIAU_0576							

in *A. dehalogenans*, and 20 in *So. cellulosum*, respectively), presumably due to either sequence divergence or gene gain/loss. Several changes in character were observed among the set of homologues. In 24 cases the gene organization of homologues had changed relative to the *M. xanthus* genes as a consequence of recruitment/loss of neighbouring TCS genes. Four duplications of characterized *M. xanthus* genes were also observed amongst their myxobacterial homologues, but only in *So. cellulosum* (three of the four *red* operon genes, and *dotR*). Additionally, in seven cases the homologues presented in Table 8 also exhibited changes of domain architecture. In five cases the domain changes were recruitment/loss of an input domain within a HK gene. The sixth case is the *A. dehalogenans* FrzZ homologue, which lacks the first receiver domain of the *M. xanthus* and *St. aurantiaca* proteins, while the seventh case is the ApgA homologue of *So. cellulosum*, which has an AAA_ATPase,HTH_Fis domain instead of a transmitter domain.

Phylogenetic trees were also assessed in their entirety for evidence of gross genetic changes. This was achieved by identifying all clades of paired homologous domains from *M. xanthus* and *St. aurantiaca* (with strong bootstrap support, >70 % using both algorithms), and assessing whether there was any change in character between the two proteins. Particularly, we addressed changes in gene organization, gain/loss of input and output domains, and fusion/fission of TCS genes. In the majority of cases (85 protein pairs) we could identify no change in character between orthologous proteins; however, in many cases (25) input/output domains were missing from one orthologue, with changes of input domain (21 cases) far outnumbering changes in output domain (four cases). We identified 25 protein orthologues in which changes of gene organization had occurred, and a further three instances where adjacent TCS genes had apparently fused or split. Finally, we counted all clades in the trees composed solely of paralogous domains, which are suggestive of organism-specific domain duplications. The vast majority of duplicated domains came from *So. cellulosum* (52 paralogous domains), with large numbers also evident in the *St. aurantiaca* (28 domains) and *A. dehalogenans* (15 domains) genomes. Surprisingly, *M. xanthus* seems to have had relatively small numbers of domain duplications since splitting from the common ancestor (two paralogous domains, MXAN_0176 and MXAN_0706).

DISCUSSION

The myxobacteria as a group include organisms that possess the largest number of TCS genes of all prokaryotes, more even than would be expected for organisms with typically large genomes. This large regulatory potential correlates with an extremely sophisticated and complex lifestyle, characterized by cooperative multicellular development and metabolic diversity. But myxobacteria also

seem to organize and utilize their TCS complement in unusual ways. An unusually large proportion of TCS genes are either orphaned or in complex gene clusters, suggesting more complex action than the typical TCS. Additionally, a disproportionate number of sensor kinases lack transmembrane helices, suggesting that they are responding to internal state changes. This is not particularly surprising, as most experimentally characterized TCSs of *M. xanthus* are known to be involved in regulating the gene transcription network governing fruiting body formation (Whitworth & Cock, 2007).

Unfortunately, not enough is understood about TCS input domains to postulate signals to which myxobacterial TCSs respond; however, domain complements suggest a common mode of sensing, with *St. aurantiaca* particularly favouring GAF domains, and PAS/PAC being unusually common in myxobacterial proteins. More is known about TCS output domains, and a wide variety of such domains are found in the myxobacteria. Some are notably abundant, particularly AAA_ATPase, HTH_Fis domains, which are known to be used in large numbers to regulate fruiting and motility (Caberoy *et al.*, 2003), but response regulators lacking any output domains are also abundant. Presumably these affect responses within the cell through protein–protein interactions, or act as relays within complex phosphotransfer schemes. Such complicated phosphotransfer schemes appear to be encoded within several HYs, often found together in the genome, and possessing multiple transmitter and receiver domains. It has been argued that phosphorelays have evolved because they possess many points at which the phosphotransfer scheme can be regulated, or at which phosphoryl groups can be introduced into the signalling scheme (Burbulys *et al.*, 1991). If this is the case, then the complex architectures of the myxobacterial HYs would suggest that these proteins act as important points for TCS signal integration, worthy of experimental focus.

In order to facilitate experimental analysis beyond the model myxobacterium *M. xanthus*, we have identified homologues of characterized *M. xanthus* proteins in the other myxobacterial genomes. Intriguingly, sets of orthologues seem to have been subjected to a large degree of gene rearrangements. It is tempting to speculate that the multiplicity of conserved domains among the TCS proteins has facilitated homologous recombination within evolving myxobacterial lineages, a hypothesis that requires many more myxobacterial genome sequences to test. Amongst sets of myxobacterial orthologues there are also a surprisingly large number of domain architecture changes, although it is intriguing that the majority of these (84%) involve the gain/loss of input domains, while the minority affect output domains. This may suggest that the evolution of myxobacterial regulatory systems has progressed predominantly by changing which environmental cues are sensed, rather than by altering the responses generated within the cell. We also find a large degree of within-lineage domain duplications.

Another striking observation as a result of our analysis was the similarity of myxobacterial TCSs to those of cyanobacteria, particularly *Anabaena* and *Nostoc*. In terms of cytoplasmic sensing, lack of paired genes, dominance of GAF input domains, abundance of response regulators lacking output domains, and the presence of complex multi-domain HYs, the characteristics that make myxobacterial TCS regulation unusual, are also particularly characteristic of cyanobacteria.

The most remarkable features of myxobacterial biology are the social phenomena of fruiting body formation, sporulation and predation. It appears at first glance that multicellularity requires an increased regulatory complement relative to asocial organisms; however, it is intriguing that the increase in regulatory complexity in myxobacteria seems more profound for systems responding to internal stimuli rather than to extracellular events. Multicellular fruiting body formation has been shown to require just two inter-cellular signals (the diffusible A-signal and cell-contact mediated C-signal). It may be the case that myxobacteria use their internal metabolism as an indicator of extracellular events, thus responding to environmental change indirectly. However, there is no convincing argument why that should necessarily be a requirement for multicellularity. In the endospore-forming bacteria such as *Bacillus subtilis*, there is no dramatic increase in TCS complexity over comparable non-spore formers, therefore spore formation *per se* does not require the level of regulatory complexity seen in the myxobacteria. Similarly, predatory bacteria such as *Bdellovibrio bacteriovorus* manage to predate without regulatory networks as large as those of the myxobacteria. Why then the increase in regulatory complexity observed during the evolution of multicellularity? We propose that evolution of multicellularity provides more of a challenge than just being able to interact with other cells within a population. Myxobacterial strains which defect or cheat during the developmental process have been characterized (Velicer *et al.*, 2000) and it seems likely that the ability to protect the cooperative population from defectors/cheaters would require sophisticated regulatory mechanisms (Velicer, 2005). Similarly in the natural environment many myxobacterial strains co-exist (Vos & Velicer, 2006), which presumably makes the nature of ‘self’ and ‘self-recognition’ important determinants of evolutionary fitness. Thus we propose that the complexity in TCS regulation of the myxobacteria may reflect ‘hard-wired’ regulatory systems that make the multicellular population robust to a changing social milieu. This conjecture suggests that differences in TCS regulation between myxobacterial species/strains may reflect modifications to the basic scheme for multicellular behaviour that provide some element of ‘self’ (or non-‘self’ incompatibility) to a population.

In summary, by categorizing the total set of TCS proteins from four myxobacterial genome sequences we have provided a framework for research into the TCSs of the myxobacteria. In addition to providing lists of gene sets and

mapping homologues, the phylogenetic trees provided here should guide research into the TCSs of myxobacteria beyond *M. xanthus*, and possibly even direct experimental analysis of *M. xanthus* regulation through evolutionary arguments.

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