Mini-Review

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A pivotal role for the response regulator DegU in controlling multicellular behaviour

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Bacteria control multicellular behavioural responses, including biofilm formation and swarming motility, by integrating environmental cues through a complex regulatory network. Heterogeneous gene expression within an otherwise isogenic cell population that allows for differentiation of cell fate is an intriguing phenomenon that adds to the complexity of multicellular behaviour. This review focuses on recent data about how DegU, a pleiotropic response regulator, co-ordinates multicellular behaviour in *Bacillus subtilis*. We review studies that challenge the conventional understanding of the molecular mechanisms underpinning the DegU regulatory system and others that describe novel targets of DegU during activation of biofilm formation by *B. subtilis*. We also discuss a novel role for DegU in regulating multicellular processes in the food-borne pathogen *Listeria monocytogenes*.

Multicellular behaviour in single-celled prokaryotes

It is easy to forget that when bacteria are grown in the laboratory the conditions used to generate such rapidly dividing free-living cells are very different from those in the natural environment. Most bacteria exist in their natural habitats as sessile multicellular communities called biofilms in which the bacterial population differentiates as part of the development process (Costerton *et al.*, 1995; Vlamakis *et al.*, 2008). That most bacterial species can live and grow within a shared habitat, and co-ordinate complex group behaviour that benefits the whole community, is still a relatively new concept (Shapiro, 1998). Examples of such behaviour leading to the propagation of bacterial spores include fruiting body formation in *Myxococcus xanthus* (Kaplan, 2003), biofilm formation and fruiting-body-like assembly in *Bacillus subtilis* (Branda *et al.*, 2001) and aerial hyphae development in *Streptomyces coelicolor* (Claessen *et al.*, 2006).

Experimental advances provide insight into multicellular behaviour

B. subtilis, like other bacterial species, is capable of manifesting different multicellular processes. Studies have focussed on understanding how this species is able to co-ordinate processes that include sporulation (Piggot & Hilbert, 2004), the uptake of exogenous DNA (genetic competence) (Hamoen *et al.*, 2003), social motility (swarming) (Kearns & Losick, 2003), extracellular protease production (Dahl *et al.*, 1992), and biofilm formation/complex colony development (Branda *et al.*, 2001; Hamon & Lazazzera, 2001). Investigations on multicellular beha-

viour in *B. subtilis* have advanced through three main approaches. First, investigations using 'wild' isolates led to the discovery that laboratory strains had lost their ability to express some of the multicellular behaviours described above (Branda *et al.*, 2001; Kearns & Losick, 2003; Stanley & Lazazzera, 2005). *B. subtilis* laboratory isolates such as 168 (Spizizen, 1958) were derived from a progenitor strain that was treated with X-rays or chemical mutagens to make the cells more tractable (Srivatsan *et al.*, 2008; Zeigler *et al.*, 2008). The genes in which the mutations were introduced are now recognized as important for social behaviour and their identification helped to elucidate the genetic circuitry underpinning multicellular responses to the environment (Kearns & Losick, 2003, 2005; Kearns *et al.*, 2004; Stanley & Lazazzera, 2005). Second, developments in the use of fluorescent reporter fusions enable gene expression to be monitored readily within the single cell by flow cytometry. Third, advances in microscopy techniques allow the detection of cell differentiation in an otherwise isogenic bacterial population (Veening *et al.*, 2004, 2005; Vlamakis *et al.*, 2008).

Co-ordination of multicellular behaviour processes by DegS–DegU

Two-component signal transduction systems are the major family of signalling proteins by which bacteria sense and respond to changes in the environment. They typically consist of a membrane-associated histidine kinase (DegS) and a cytoplasmic response regulator (DegU). The former detects the signal or stress, whereas the latter controls cellular response, predominantly through gene transcription (Mascher *et al.*, 2006). DegU plays a key role in

regulating post-exponential-phase processes in *B. subtilis*, including activation and inhibition of genetic competence (Dubnau *et al.*, 1994; Kunst *et al.*, 1994; Ogura & Tanaka, 1996), activation and inhibition of motility (Amati *et al.*, 2004; Kobayashi, 2007; Verhamme *et al.*, 2007), activation of degradative enzyme production (Dahl *et al.*, 1992; Msadek *et al.*, 1990), activation of poly- γ -glutamic acid production (Stanley & Lazazzera, 2005) and also the activation and inhibition of biofilm formation (Kobayashi, 2007; Verhamme *et al.*, 2007). DegU has regulatory activity in both its unphosphorylated and phosphorylated states. This initially led to its designation as a 'molecular switch' dependent on DegU phosphorylation by DegS (Dahl *et al.*, 1992). However, recent studies using wild isolates of *B. subtilis* indicate that DegU~P functions as a 'rheostat' that senses and responds to changes in the environment. This, in turn, enables the integration of genetic competence, swarming motility, biofilm formation and exoprotease production along an increasing gradient of DegU phosphorylation (Kobayashi, 2007; Verhamme *et al.*, 2007).

Mechanism of DegU and DegU~P in response to signalling

In its unphosphorylated state DegU activates genetic competence through the recruitment of ComK (Hamoen *et al.*, 2000). ComK functions as an anti-repressor for the transcriptional repressors Rok and CodY, and thereby allows the expression of its own gene. This permits the establishment of the bistable system pre-required for competence development (Albano *et al.*, 2005; Smits *et al.*, 2007). In contrast, DegU~P recruits RNA polymerase at promoter regions of genes such as *yvcA* and *aprE* to activate biofilm formation and exoprotease production respectively (Ogura *et al.*, 2003; Verhamme *et al.*, 2007). Recent genome-wide transcription and proteomic studies have identified over 170 genes (~4% of the *B. subtilis* genome) that are regulated by DegU~P under various growth conditions (Kobayashi, 2007; Mader *et al.*, 2002; Ogura *et al.*, 2001). Therefore the mechanism by which DegU~P coordinates multicellular behaviour processes in response to signal perception is challenging. One simple model supported by *in vitro* data indicates that DegU~P has specific affinities for different promoter regions. For example, the *flgB* promoter has a higher affinity for DegU~P than the *sacB* promoter. This provides the molecular basis by which swarming motility could be activated prior to other multicellular processes (Kobayashi, 2007; Tsukahara & Ogura, 2008). Variations in promoter binding affinities by transcription factors may represent a common mechanism mediating control over multicellular behaviour responses. High- and low-affinity binding sites in *B. subtilis* are used within the promoter regions of genes controlled by Spo0A. At low levels of Spo0A phosphorylation, biofilm formation and cannibalism are regulated. In contrast, the activation of sporulation occurs at high levels of Spo0A phosphorylation (Fujita *et al.*, 2005). However, the question arises: Why does a process that is activated at lower levels of signal perception not occur in the presence of a high level of the signal? The first possibility is that

when low-affinity promoters are triggered, a negative feedback loop blocks the process that is controlled by the high-affinity promoters. Second, the promoter regions contain multiple binding sites, with specific affinities, that result in the activation of transcription at low levels of signal perception but inhibition of transcription at high levels of signal. Third, multiple environmental conditions need to be sensed and integrated by the cell to activate a physiological response.

A three-tiered regulatory system controls DegS–DegU activity

Given the central role that DegS and DegU have in coordinating multicellular responses in *B. subtilis* it is not surprising that the DegS–DegU regulatory system is finely controlled at the following three stages within the cell: *degU* transcription, DegU phosphorylation and DegU~P activity.

degU transcription

The *degSU* operon contains three promoters (Veening *et al.*, 2008a; Yasumura *et al.*, 2008). The first is upstream of *degS* and drives the expression of *degS* and *degU*. The second is located within the coding region of *degS* and increases the level of *degU* under nitrogen-limiting conditions. The third is located in the *degS–degU* intergenic region and increases the level of *degU* in response to DegU~P. In laboratory isolates, the positive auto-regulation of *degU* transcription (Fig. 1) by DegU~P leads to the heterogeneous expression of *degU* within the population (Veening *et al.*, 2008a, b).

DegU phosphorylation

DegS is a cytoplasmic bifunctional protein that exhibits kinase and phosphatase activities (Tanaka *et al.*, 1991). DegS interacts with the SMC–ScpA–ScpB complex, which controls DNA condensation and repair. The interaction of DegS with ScpA inhibits its kinase activity and results in a decrease in the pool of DegU~P within the cell. As the level of SMC–ScpA–ScpB complex decreases during stationary phase there is an increase in the level of DegU~P in that growth phase (Dervyn *et al.*, 2004). Transfer of the phosphate moiety from DegS to DegU (Fig. 1) is enhanced in the presence of DegQ, a small protein of 46 amino acids (Kobayashi, 2007). Some laboratory isolates of *B. subtilis* contain a point mutation within the promoter of *degQ* that reduces the level of DegQ synthesized (Stanley & Lazazzera, 2005); this in turn, reduces the level of DegU~P in the cell (Kobayashi, 2007; Stanley & Lazazzera, 2005; Verhamme *et al.*, 2007). This single point mutation highlights one of the key genomic differences between wild and laboratory isolates of *B. subtilis* that influences multicellular behaviour (Kearns *et al.*, 2004; Kobayashi, 2008; Stanley & Lazazzera, 2005). Transcription of *degQ* is regulated by the quorum-sensing-responsive transcription factor ComA (Msadek *et al.*, 1991). This ensures that DegU~P in the cell increases alongside an increase in cell density and is maximal at

Fig. 1. The DegS–DegU regulatory system of *B. subtilis*. The names of the genes are indicated. Open arrowheads indicate a change in the phosphorylation state. The lower left-hand insert provides a symbol key and the lower right-hand insert depicts the domain structure of DegS, with the HisKA_3 and HATPase_c domains identified by Pfam shown in grey. S79 represents serine amino acid number 79; H189 represents the site of autophosphorylation on histidine 189. ‘+’ represents activation and ‘–’ represents inhibition.

stationary phase. DegQ does not affect the stability of DegS~P or block the intrinsic phosphatase action of DegS (Kobayashi, 2007); therefore the mechanism by which DegS controls the balance of phosphatase and kinase activity remains unknown.

DegU~P activity

The response regulator aspartyl phosphatase (Rap) RapG binds to DegU, without affecting the latter’s phosphorylation status. This suggests that RapG inhibits the ability of DegU to bind to target promoter DNA (Ogura *et al.*, 2003). RapG is encoded in an operon with PhrG, a phosphatase regulator (Phr). Although *rapG* and *phrG* share a common

promoter upstream of *rapG*, the transcription of *phrG* is also activated by a σ^H -dependent promoter on entry to stationary phase (Britton *et al.*, 2002). At high levels of PhrG production, PhrG interacts with RapG, lessening RapG inhibition of DegU activity. As the RapG–PhrG system functions as a quorum-sensing system (Fig. 1), DegU~P activity increases in parallel with an increase in cell density (Lazazzera *et al.*, 1999; Ogura *et al.*, 2003).

New roles for DegU activity in *B. subtilis* during biofilm formation

DegU was first identified as a positive regulator of biofilm formation in a study using *B. subtilis* isolate RO-FF1

(Stanley & Lazazzera, 2005). Currently, three targets that are activated by DegU~P are known. The first is poly- γ -glutamic acid, an extracellular polymer that can form part of the extracellular matrix of some isolates of *B. subtilis*, though not in NCIB 3610 (Branda *et al.*, 2006; Morikawa *et al.*, 2006; Stanley & Lazazzera, 2005). This suggests that the specific requirements to form a multicellular community differ in a strain-dependent manner and highlights the value of scrutinizing different natural isolates. The other two targets for DegU are the proteins, YuaB and YvcA. YuaB is a small secreted protein required for pellicle formation and complex colony development in ATCC 6051 and NCIB 3610 (Kobayashi, 2007; Verhamme *et al.*, 2008). YvcA is a putative membrane-bound lipoprotein required for complex colony development in NCIB 3610 but not for pellicle formation in ATCC 6051 (Kobayashi, 2007; Verhamme *et al.*, 2007). A complex genetic circuit that is dependent on activation of DegU and Spo0A ensuring *yvcA* and *yuaB* transcription during complex colony development in *B. subtilis* NCIB 3610 has recently been described (Verhamme *et al.*, 2008).

Heterogeneous *degU* expression and exoprotease production

During biofilm formation by *B. subtilis*, cells differentiate into specific subpopulations that become responsible for matrix production or sporulation (Chai *et al.*, 2008; Vlamakis *et al.*, 2008). This finding suggests that biofilm matrix production altruistically benefits the entire population/community, not just the producer cells. During heterogeneous gene expression, threshold levels of a key regulator controlling cell state need to be attained. This can be mediated by a positive feedback loop at the transcription stage (Losick & Desplan, 2008). Intriguingly it was recently reported that while *degU* transcription is auto-activated it is not sufficient to generate heterogeneous expression in wild-type laboratory isolates. However, when genetic manipulations are used to increase the level of DegU~P, a distinct bistable population was observed (Veening *et al.*, 2008a). In the same study it also was demonstrated that a small population of cells in which *aprE*, the gene encoding the major exoprotease of *B. subtilis*, is copiously transcribed can be established (Veening *et al.*, 2008a). Two criteria must be met for this to happen in the small subset of cells. First, Spo0A~P levels must increase to a threshold that stimulates derepression of *aprE* transcription (but not to such a level that the cells sporulate). Second, the levels of DegU~P in the cell must be high. This dual requirement of DegU and Spo0A activation as part of a genetic logic-AND network might represent a common mechanism for co-ordinating entry into the different multicellular behavioural processes of *B. subtilis* (Veening *et al.*, 2008a, b; Verhamme *et al.*, 2008). It remains to be seen whether intracellular fluctuations of phosphorylated DegU and Spo0A combined with intrinsic differences in promoter binding affinities co-ordinate the different multicellular behavioural processes manifested by *B. subtilis*.

Motility and biofilm formation regulation by DegU in *L. monocytogenes*

The role of DegU in co-ordinating multicellular behaviour is not restricted to *B. subtilis*. *Listeria monocytogenes* is a Gram-positive facultative intracellular pathogen that causes listeriosis. DegU (encoded by *lmo2515*) regulates motility, biofilm formation and virulence in *Listeria* species (Gueriri *et al.*, 2008b; Knudsen *et al.*, 2004; Williams *et al.*, 2005a, b). The genome sequences of the pathogenic strain *L. monocytogenes* EGD-e and non-pathogenic strain *L. innocua* are remarkably similar to that of *B. subtilis* (Glaser *et al.*, 2001). However, in the region surrounding *degU*, the *degS* homologue is conspicuously absent despite other major genome features being maintained (Gueriri *et al.*, 2008b; Knudsen *et al.*, 2004). The absence of *degS* appears to be specific to the *Listeria* species (Fig. 2), although further evolutionary analyses are needed to establish whether *degS* has been lost from *Listeria* or gained by *Bacillus* (Gueriri *et al.*, 2008b).

Biofilm formation by *L. monocytogenes* is dependent on flagellar motility to propel the cells towards a surface prior to attachment (Lemon *et al.*, 2007). DegU was recently identified as a positive activator of flagellum biosynthesis (Knudsen *et al.*, 2004; Williams *et al.*, 2005a, b). When grown at ≤ 30 °C *L. monocytogenes* has four to six simple peritrichous glycosylated flagella. The flagellum is glycosylated at multiple sites on the flagellin protein FlaA, with up to six glycosylation moieties per monomer. The glycosylation moiety is transferred to FlaA post-translationally by the bifunctional O-GlcNAc transferase GmaR (Shen *et al.*, 2006). The physiological significance of the glycosylation is not yet fully understood but it is proposed that it may be an important factor for environmental adaptation outside the host. At temperatures ≥ 37 °C *L. monocytogenes* is typically non-motile. Inhibition of motility at high temperatures occurs through the tightly controlled repression of *flaA* transcription by the protein MogR, which binds to the *flaA* upstream promoter region (Shen & Higgins, 2006). It was initially proposed that DegU served as a direct activator of *flaA* transcription at low temperature, although later work showed that this role is indirect (Shen & Higgins, 2006). At low temperatures DegU activates transcription of *gmaR*, which encodes GmaR, the transcriptional activator of *flaA*. How DegU activates *gmaR* transcription at low temperatures but not at high temperatures remains unknown. GmaR is a bifunctional protein that first binds to MogR, removes it from the *flaA* promoter, and then glycosylates the FlaA protein. DegU also controls flagellin levels post-transcription, as transcription of *flaA* in a *mogR degU* strain background per se is not sufficient to restore motility (Shen & Higgins, 2006). This unique regulatory mechanism ensures that flagella of *L. monocytogenes* are only synthesized at low temperatures and that the flagella are fully glycosylated.

Fig. 2. Genome context of *degU* on the chromosome of *B. subtilis* with respect to other Gram-positive bacteria. The percentage identity at the protein level is indicated for *degU* and *degS* homologues under the gene. The gene size, position and direction are drawn to the scale indicated at the top of the diagram.

DegU regulates virulence in *L. monocytogenes*

Although the mechanism for DegU-dependent motility is relatively well known, how DegU affects virulence is not yet understood. DegU mutant strains are still able to enter host cells with an efficiency that is comparable to, or even better than, that of wild-type cells (Williams *et al.*, 2005a). However, the bacterial load maintained within the spleen and other organs of the host is lower (Knudsen *et al.*, 2004). DegU-dependent control of virulence is not due to the non-motile phenotype because flagella-minus strains of *L. monocytogenes* remain virulent (Shen & Higgins, 2006). It will be exciting to understand how DegU contributes to virulence and how this is co-ordinated with its other roles in controlling motility and activation of biofilm formation (Gueriri *et al.*, 2008b).

DegU is an orphan response regulator in *L. monocytogenes*

The absence of *degS* in *L. monocytogenes* raises questions about whether DegU can be phosphorylated, and if so, whether DegU~P is required to activate flagella-based motility and/or virulence. Although DegU is an orphan response regulator in *L. monocytogenes*, importantly there are no orphan sensor kinases encoded in its genome (Williams *et al.*, 2005a). It is possible that in the absence of a cognate or orphan sensor kinase, DegU could be phosphorylated by a non-cognate sensor kinase. However, this is very unlikely due to the inbuilt specificity mechanisms in a two-component regulatory system (Laub & Goulian, 2007). Alternatively, phosphorylation of DegU could be mediated via a small molecular phosphate donor such as acetyl phosphate. This is becoming increasingly

recognized as a global regulator of gene transcription (Wolfe *et al.*, 2003, 2008). The presence of an 'orphan' response regulator, or sensor kinase that also has phosphatase activity, suggests that acetyl phosphate may play a role in the signal transduction system (Wolfe, 2005). A study on motility and ethanol resistance by *L. monocytogenes* investigated whether DegU is required to be phosphorylated in order to carry out its regulatory function (Mauder *et al.*, 2008). These authors constructed a strain of *L. monocytogenes* carrying a variant of *degU* with a point mutation in the proposed phosphorylation site. They demonstrated a partial restoration of swimming motility in comparison with the *degU* mutant, though motility was less than that observed in the wild-type. These findings indicate that DegU retains some functionality in its unphosphorylated state, but do not rule out the possibility that DegU~P has some physiological role. Consistent with this suggestion, DegU of *L. monocytogenes* can be phosphorylated by acetyl phosphate *in vitro* and alterations in the level of acetyl phosphate *in vivo* control motility and biofilm formation (Gueriri *et al.*, 2008a). As the level of acetyl phosphate varies with both growth condition and growth phase (Klein *et al.*, 2007; Wolfe, 2005) it is possible that DegU functions as a read-out of the metabolic status of *L. monocytogenes*.

Future studies

Since the advent of single-cell analyses and the observation of multicellular behaviour, the full potential of DegU as a central regulator in both *L. monocytogenes* and *B. subtilis* is becoming apparent. There remain many unanswered questions about how DegU functions in both *Bacillus* and *Listeria* species. A recent phosphoproteome screening of *B. subtilis* found that DegS can be phosphorylated on amino acid serine 79. This is outside the HisKA_3 domain containing histidine 189, which is the site (Fig. 1) of autophosphorylation (Macek *et al.*, 2007). This raises questions about whether phosphorylation on serine 79 has any physiological role in *B. subtilis*. For example, is the phosphorylation of serine 79 and histidine 189 responsible for controlling separate multicellular behaviour processes that are dependent on DegU~P in the face of different environmental stimuli? Additionally, growing evidence indicates that the function of DegU in laboratory isolates differs from that of wild isolates of *B. subtilis* (Kobayashi, 2007; Stanley & Lazazzera, 2005; Veening *et al.*, 2008a; Verhamme *et al.*, 2007). Therefore it remains to be established whether heterogeneous expression of *degU* occurs in wild isolates (e.g. NCIB 3160) of *B. subtilis*. If heterogeneous expression is observed, what influence does this have over the capacity to integrate multicellular behaviour responses? For *L. monocytogenes*, which lacks *degS*, it is important to establish whether a heterogeneous population dependent on DegU can be formed. If so, does the differentiation of tasks within the cell population confer any survival advantage to the bacterium both within and outside the host?

Concluding remarks

It is likely that the DegS–DegU regulatory system will continue to provide an excellent model of a 'systems biology' approach. Thus objectives will be to understand how bacteria co-ordinate the decision-making processes that occur within the cell to ensure that the desired physiological response occurs in the face of different environmental conditions. Our ability to study multicellular behaviour in *B. subtilis*, and other closely related species, will be enhanced upon completion of genome sequences of natural isolates collected from a variety of geographical locations (<http://www.bacillusgenomics.org/bsubtilis/index.html>) (Earl *et al.*, 2007, 2008; Srivatsan *et al.*, 2008). Such data could be used to determine the impacts of genome polymorphisms on behaviour and adaptation.

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