The stressosome: molecular architecture of a signalling hub

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Abstract

The stressosome co-ordinates the response of Bacillus subtilis to the imposition of a variety of physical and environmental insults. These stresses include fluctuations in salt concentration, the presence of ethanol, changes in pH and even the level of UV light. Despite the obvious and significant differences between these quite different physicochemical stimuli, the result is the same: the stressosome is phosphorylated by a key kinase to initiate the σ^B cascade. The phosphorylation of the stressosome initiates a signal transduction system that up-regulates the expression of stress-responsive genes so that the Bacillus can survive the imposition of stress. Hence the stressosome acts as a hub, receiving manifold different stimuli to effect a single outcome. Using single-particle analysis of cryo-electron micrographs, we have been able to reconstruct a series of molecular envelopes of the stressosome. These maps have been interpreted at near-atomic resolution with crystal structures of the individual components of the stressosome to provide the first visualization of this unique signalling hub. The macromolecular structure adopted by the stressosome provides the signallng cascade with the potential for co-operative behaviour, which we have also measured in live bacteria. These experiments are consistent with the tuning of the response of B. subtilis to stress relative to the magnitude of the insult.

Introduction

The established model of Gram-positive micro-organisms is the soil-dwelling rod-shaped Bacillus subtilis. This organism has long been exploited in studies of adaptive responses, which are mechanisms that underpin the ability of the organism to respond appropriately to external pressures. Examples of such responses include the ability to swim towards attractants and away from repellents, i.e. chemotaxis [1]; the secretion of scavenging enzymes to provide nutrients [2]; the uptake of foreign DNA, to provide potential competitive advantage over other bacteria, called competence [3]; and the biosynthesis of antibiotics, to kill bacteria competing for the same scarce nutrients [4]. Arguably, the most extreme example of an adaptive response is sporulation [5], the process whereby endospore-forming bacteria divide asymmetrically to produce a mother cell that lyses in order to release a hardy spore that can lie almost indefinitely in the environment until favourable growth conditions are restored. However, in the present paper, we consider another strategy that is utilized by B. subtilis and close relatives when responding to the imposition of various general stress stimuli, such as changes in temperature [6], pH [7] or osmolarity [8], increase in ethanol [9] or Mn^{2+} [10] concentration, the cyanide-precursor sodium nitroprusside [11], blue light [12] and cell wall stress, caused by antibiotics such as bacitracin, a Bacillus-secreted cyclic peptide-based antibiotic [13]. Although part of the pathway that is responsive to these environmental stresses is shared with another pathway that co-ordinates the response of this bacterium to starvation [9,14], or the inhibition of ATP synthesis [15], this latter pathway is not be considered further in the present paper.

The response of B. subtilis to the imposition of environmental stress is to up-regulate the activity of an alternative σ factor subunit of RNA polymerase [8], σ^B. The key regulators of the σ^B pathway are depicted schematically in Figure 1. σ factors are required for the specific recognition of target DNA sequences at the promoters of genes, or operons, that they regulate [16]. The σ^B regulon comprises a large group of disparate gene products that function in concert to provide the cell with global resistance to the imposed stress [17,18]. Under normal growth conditions, the key regulator, σ^B, is held in an inactive protein complex with its cognate anti-σ factor, RsbW (where Rsb is regulator of σ^B) [19,20]. The bifunctional RsbW also acts as an ATP-dependent serine/threonine kinase towards its substrate, the anti-anti-σ factor, RsbV [19–22], a member of the STAS (sulfate transporter/anti-σ factor antagonist) domain superfamily [23]. Phosphorylated RsbV, RsbV-P, is a substrate for the Mg^{2+}/Mn^{2+}-dependent PP2C phosphatase RsbU [22,24], whose specific activity in the absence of stress is maintained at a low level [22,24,25]. The stressosome is found upstream of RsbU in the σ^B regulatory pathway. The stressosome is a large protein complex comprising multiple copies of RsbR (and at least three of its paralogues in the B. subtilis genome) and the STAS domain protein, RsbS [12,26–29]. RsbR (and paralogues) contain a C-terminal STAS domain, preceded by N-terminal domains with no clear
Figure 1 | The \( \sigma^B \) pathway of \( B. \) subtilis

Before stress, the anti-\( \sigma \) factor RsbW sequesters \( \sigma^B \) and prevents it from directing RNA polymerase to \( \sigma^B \)-controlled promoters. In this state, RsbV is phosphorylated (RsbV-P) by the kinase activity of RsbW and hence RsbV is inactivated. Under stressful conditions, RsbV becomes dephosphorylated by one of two phosphatases and attacks the RsbW-\( \sigma^B \) complex and liberates \( \sigma^B \). Under energetic stress, the phosphatase RsbP is activated by RsbQ and dephosphorylates RsbV-P to allow it to form complexes with RsbW. In the environmental stress pathway, the stressosome sequesters the RsbU phosphatase activator RsbT. Under environmentally stressful conditions, RsbT phosphorylates the STAS domains of the stressosome proteins and disassociates, because of a reduced affinity for the phosphorylated proteins. RsbT switches its binding partner from the stressosome to the phosphatase RsbU. The RsbT-RsbU complex activates RsbV by its dephosphorylation. The phosphatase RsbX acts to remove phosphoryl groups from the stressosome and to mediate the duration of the stress response by ‘resetting’ the system.

Structural biology of the stressosome

The presence of large proteinaceous complexes containing RsbR and RsbS was first described by Haldenwang and colleagues, who found that these proteins co-eluted with ribosomes in gel filtration of \( B. \) subtilis cell lysate [29]. The initial conclusion was that the transcription of the \( \sigma^H \) regulon was linked to that of protein synthesis. Subsequently, using recombinant purified proteins, Yudkin and co-workers were able to demonstrate by gel filtration that RsbR and RsbS were capable of spontaneously forming large complexes that could also recruit RsbT [26]. These complexes were of sufficient size to be eluted in the void volume of the gel-filtration column used. The co-fractionation of ribosomes and stressosomes would thus appear to be a coincidence of their great size, rather than an indication of direct functional links between \( \sigma^H \) activity and protein synthesis. The use of TEM (transmission electron microscopy) of purified stressosomes stained with heavy atom salts revealed that stressosomes are widely distributed and are presumably engaged in multiple and varied sensing roles. In order to understand the assembly of the stressosome, and to probe its biochemical properties, we have embarked on a multi-disciplinary research program to establish the relationships between the stressosome’s structure and its function.

biochemical functions. One of the stressosome’s functions appears to be to sequester multiple copies of the switch kinase RsbT [26], until the imposition of physical stress, at which point RsbT phosphorylates conserved serine and threonine residues in RsbS and RsbR [30,31] to cause the release of RsbT from the stressosome [26]. The disassociation of RsbT leads to a ‘partner-switching’ event, where RsbT binds preferentially to the N-terminal domain of RsbU [32], leading to an increase in the phosphatase activity of the C-terminal catalytic domain [22,24,25,32]. This stimulation of RsbU accompanies \( \sigma^B \) availability; the increase in the rate of dephosphorylation of RsbV-P catalysed by RsbU [22,24,25] results in release of RsbV to attack the \( \sigma^H \)-RsbW complex, causing its disassociation as RsbW also switches partner, to bind preferentially to RsbV [20,21]. The \( \sigma^B \) that is liberated is bound by core RNA polymerase [33] and the holoenzyme then directs the expression of the \( \sigma^B \) regulon to provide the cell with stress-resisting factors [17,18].

The genes encoding the central stressosome components, RsbR, RsbS and RsbT, are found adjacent on the chromosome of \( B. \) subtilis, at the 5′-end of the eight gene \( rsbR-rsbS-rsbT-rsbU-rsbV-sigB-rsbX \) operon [34]. The \( rsbR-rsbS-rsbT \) triumvirate, the RST module [34], is also found in operons present in other bacteria and the archaea, representing a wide range of microbial phyla. However, the genes downstream of the RST module are not always associated with \( \sigma \) factor subunits, but other communication systems such as two-component histidine kinases and response regulators, and the biosynthesis of secondary messenger signalling molecules, such as cyclic di-GMP [34]. Thus stressosomes are widely distributed and are presumably engaged in multiple and varied sensing roles. In order to understand the assembly of the stressosome, and to probe its biochemical properties, we have embarked on a multi-disciplinary research program to establish the relationships between the stressosome’s structure and its function.
solved in this way (Figure 2): the first of the binary complex between RsbR and RsbS; a second of the ternary complex that also includes RsbT; and the third of the C-terminal STAS domain of RsbR (C-RsbR) in complex with the single STAS domain of RsbS [36]. The structural analyses were aided, and at the same time somewhat hindered, by the presence of pseudo-icosahedral symmetry in the core of the stressosome particle and a reduction of the point group symmetry in the periphery of the stressosome, the arrangement of which is only compatible with $D_2$ point group symmetry [36]. The icosahedral symmetry of the core of the stressosome, comprising the STAS domain from RsbR, and RsbS, is consistent with the crystal packing of *B. subtilis* RsbS alone. Limited diffraction from these crystals, to only $\sim 8$ Å (1 Å = 0.1 nm), was sufficient to reveal peaks in the self-rotation function (Figure 2) on section $\varphi = 180^\circ$, $120^\circ$ and $72^\circ$, their relationships being consistent with the presence of icosahedral particles in the unusually large I23 unit cell of $a = b = c = 360$ Å (J. Marles-Wright and R.J. Lewis, unpublished work).

The molecular envelopes determined by the single-particle analysis of cryo-electron micrographs were interpreted at quasi-atomic resolution by docking crystal structures, or homology models, of the individual domains into the electron density (Figure 2). The crystal structure of the N-terminal domain of RsbR was solved in 2005 [37]. The crystals of RsbS did not diffract beyond 8 Å, so we instead solved the crystal structure of RsbS from *Moorella thermoacetica, MtRsbS*, which shares 41% sequence identity with its orthologue in *B. subtilis* [36,38]. For a model of the C-terminal STAS
domain of RsbR, we also used the structure of MrRsbS, which is 29% identical in sequence with C-RsbR. Finally, no structural information exists for any RsbT orthologue, but a homology model was created from the SpoIIAB component of the crystal structure of a homologous serine/threonine kinase, SpoIIB, in complex with the SpoIIB STAS domain [39]. SpoIIB, the anti-σ factor of σE [40], regulates stage II sporulation in B. subtilis [5], and is 28% identical in sequence with RsbT. These structures were ‘ docked’ into the molecular envelope using a combination of procedures. For the core, the structures were fitted using algorithms within Situs [41], which are similar in function to crystallographic molecular replacement calculations. The N-terminal domain of RsbR was fitted by aligning the local 2-fold symmetry axis of the atomic model with that of the molecular envelope. For RsbT, the TEM-derived maps suggested that RsbT associated with the RsbS component of the stressosome in a similar fashion to that of SpoIIB with SpoIIBA [39], and so this complex helped to guide the manual fitting of RsbT.

The stressosome core has a diameter of ∼180 Å, which is increased to ∼300 Å by the presence of the N-terminal domains of RsbR that decorate the periphery of the stressosome [36]. Overall, the stressosome comprises 40 copies of RsbR and 20 copies of RsbS, and the 2:1 molar ratio is consistent with densitometry of SDS/polyacrylamide gels of stressosome preparations. The mass of the stressosome is thus 1.5 MDa, similar to that estimated previously by sedimentation velocity analysis [26]. The N-terminal domains of RsbR project away from the core of the stressosome in such a way as to be ideally located to interact with potential small-molecule ligands and perhaps other protein components, which may act to funnel stress signals to the stressosome. The N-terminal domains of RsbR also provide access routes to the phosphorylatable residues in RsbS. Indeed, in the ternary RsbR–RsbS–RsbT complex, every copy of RsbS is in contact with a copy of RsbT, such that RsbT and RsbS are equimolar in the stressosome, again consistent with densitometry of purified preparations analysed by SDS/PAGE [36]. RsbS is central to the correct formation of the stressosome. It not only acts as a chaperone for the homogenous formation of the complex, but also provides the opportunity to sequester RsbT before stress. RsbR, and its paralogues, are capable of forming icosahedra without RsbS [26,28]; only in the presence of the RsbS scaffold do the RsbR-like proteins form homogenous complexes that can direct the response to environmental stress. RsbS-free stressosome-like particles do not bind RsbT, because RsbT is physically occluded from the stressosome by the peripherally distributed signalling domains [J. MarlesWright and R.J. Lewis, unpublished work]. If RsbT is freely available in the cell, it will bind to and activate the RsbU phosphatase, and high RsbU activity is inextricably linked to σB activation [14]. Consequently, deletion of rsbS, or its mutation to mimic the phosphorylated state (S59D), is particularly deleterious to the cell as it results in constitutive σB activation [24], a state that is not conducive to vegetative growth of the cell.

The σB response is transient, with maximal up-regulation of σB, and hence the stress-responsive genes, occurring within 30 min of the imposition of environmental stress [8]. The cells quickly revert to a pre-stress state and inactivate σB to reset the signal transduction cascade. The re-setting of the stressosome is achieved by the protein phosphatase RsbX, which acts to dephosphorylate those residues in RsbR and RsbS that were phosphorylated by RsbT [22,42]. This dephosphorylation reverses the partner-switching mechanism that was initiated by the primary stress response, resulting in RsbT-mediated RsbU activation, a prerequisite of σB activation.

**Functional biology of the stressosome**

The structure of the stressosome suggests that it plays a complex role in the cell. The stressosome funnels a wide range of different physicochemical signals into the partner-switching mechanisms that regulate σB activity. How it does this, on a molecular basis, is far from clear. However, the complexity of the structure also suggests that one role the stressosome might play is to ensure the correct scale of response to the magnitude of the input. The σB regulon is the largest in B. subtilis, with over 150 ORFs (open reading frames) [17,18]. Consequently, if low levels of stress are encountered, limited transcription of the σB regulon is sufficient to respond to the environmental insult. Conversely, a more significant stress needs to be countered with a more substantial response. To test this, σB activity was measured from a σB-dependent reporter gene fusion as a function of the concentration of NaCl and ethanol [36]. Both of these compounds are known to produce a σB response in the laboratory, and are likely to enter the cell by different mechanisms. NaCl is likely to be transported across the cell membrane by membrane-embedded transporters, whereas ethanol will probably diffuse directly across the membrane. In the case of both stimuli, the σB activity shows a high degree of co-operativity [36]. In both cases, calculated Hill coefficients exceed 8 (P.R. Race, J. Marles-Wright and R.J. Lewis, unpublished work). In comparison, the Hill coefficient of haemoglobin, the paradigm of co-operativity in the interplay between RsbV–RsbW–σB activation, is impossible to define here because the experiment was conducted on live bacteria. However, by stimulating the branch of the σB pathway that is responsive to changes in ATP concentration and energy levels [43], which converges with the environmental stress signalling pathway on RsbV–P [43], co-operativity in the interplay between RsbV–RsbW–σB can be assessed. This pathway is independent of the stressosome, and σB activation as a function of azide concentration is shown to be parabolic [36]. Hence the positive co-operativity that is seen in the response to environmental stimuli must occur.
upstream of RsbV-P, and the obvious conclusion is that it is provided by the stressosome.

Conclusions
In recent times, we and others have described how the environmental stress response in Bacillus is regulated by a large multi-component signalling complex [21,26,27]. Our high-resolution structural analyses of the components of the stressosome [36–38] laid the foundations for the recent interpretation of cryo-TEM molecular envelopes at quasi-atomic resolution [36]. However, our studies on stressosomes containing only RsbR–RsbS–RsbT require complementation with structures of stressosomes containing the RsbR paralogues that form stressosomes in the cell. The dynamic interplay between these RsbR paralogues needs to be determined in order to understand how the stressosome is built, when other STAS domain-containing proteins do not formicosahedral particles, and how it may adapt its composition depending upon the status of the cell. Further unanswered questions for the future include the following. How can completely unrelated stress stimuli activate the stressosome? Are there regulators upstream of the stressosome? What are the structural changes that accompany stressosome activation? And, finally, with advances in microscopy, can we combine structural and cellular biology approaches to visualize stressosome activation in the cell?

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