Molecular BioSystems

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Cite this: *Mol. BioSyst.*, 2012, **8**, 1806–1814

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PAPER

Proteolysis of beta-galactosidase following SigmaB activation in *Bacillus subtilis*†

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Received 25th January 2012, Accepted 20th March 2012
DOI: 10.1039/c2mb25031d

In *Bacillus subtilis* the σ^B mediated general stress response provides protection against various environmental and energy related stress conditions. To better understand the general stress response, we need to explore the mechanism by which the components interact. Here, we performed experiments in *B. subtilis* wild type and mutant strains to test and validate a mathematical model of the dynamics of σ^B activity. In the mutant strain BSA115, σ^B transcription is inducible by the addition of IPTG and negative control of σ^B activity by the anti-sigma factor RsbW is absent. In contrast to our expectations of a continuous β-galactosidase activity from a *ctc::lacZ* fusion, we observed a transient activity in the mutant. To explain this experimental finding, we constructed mathematical models reflecting different hypotheses regarding the regulation of σ^B and β-galactosidase dynamics. Only the model assuming instability of either *ctc::lacZ* mRNA or β-galactosidase protein is able to reproduce the experiments *in silico*. Subsequent Northern blot experiments revealed stable high-level *ctc::lacZ* mRNA concentrations after the induction of the σ^B response. Therefore, we conclude that protein instability following σ^B activation is the most likely explanation for the experimental observations. Our results thus support the idea that *B. subtilis* increases the cytoplasmic proteolytic degradation to adapt the proteome in face of environmental challenges following activation of the general stress response. The findings also have practical implications for the analysis of stress response dynamics using *lacZ* reporter gene fusions, a frequently used strategy for the σ^B response.

Introduction

Bacteria adapt to environmental challenges by altering their gene expression program. These gene expression adjustments involve sensory networks, signal integrators of the environmental and cellular states, eventually regulating the activity of transcription factors. An important transcription factor of *B. subtilis* is σ^B, which is activated by a variety of environmental factors including acids, ethanol, heat, and salt as well as oxidative stress and low temperature growth but also desiccation, energy depletion, and light.[1–3] σ^B regulates at least 150 genes,[4–6] encoding proteins involved in functions like multidrug efflux, non-specific oxidative stress resistance, acid stress resistance, membrane integrity, and transport.[1,7]

The processes activating the σ^B dependent general stress response have been studied in great detail.[1,2,8] σ^B is activated *via* the 'partner switch' mechanism.[9] The anti-sigma factor RsbW binds and hence reduces the free sigma-factor concentration.[10] Release of σ^B from RsbW, and thus activation of general stress response, is initiated if RsbW binds the anti-anti-sigma factor RsbV. RsbV and σ^B have overlapping binding sites on RsbW resulting in binding competition. Since RsbV has a higher affinity to RsbW, an increase in RsbV leads to a release of σ^B constituting the partner switch.[11] During non-stress conditions phosphorylation of RsbV results in a reduced affinity to RsbW, and most RsbW is associated with σ^B.[11–13] Energy limitation and environmental stress induce the activation of the two phosphatases of RsbV: RsbP and RsbU, respectively, thus initiating the partner switch.[9,13–15] Homologous mechanisms of σ^B activation can also be found in related bacteria although RsbP is confined to *B. subtilis*.[1,2,16] Moreover, this control mechanism seems to have been analogously developed for general stress response in *Methylobacterium extorquens*[17] and is used during activation of sporulation.[18,19]

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† Electronic supplementary information (ESI) available: SBML-files and graphics. See DOI: 10.1039/c2mb25031d

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However, the picture of σ^B activation *via* partner switching is incomplete. For example *B. subtilis* mutants lacking the anti-anti-sigma factor RsbV should normally be insensitive to stress activation, yet if *B. subtilis* grows continuously at low temperature strong induction of σ^B-dependent transcription was still observed in a *rsbV* mutant.[20] Also the mechanisms restricting σ^B-activity and accomplishing the transient nature of the σ^B response are not fully understood.

σ^B-activity tests commonly rely on the use of *ctc::lacZ* reporter gene fusions which use the activity of β-galactosidase as an approximation of σ^B-activity. β-galactosidase has a long history as a reporter enzyme and provides valuable insights into many cellular processes.[21,22] *Escherichia coli* and many other organisms tolerate extremely high protein levels of β-galactosidase, up to 20% of total protein while still displaying robust and reliable activities.[22,23] However, the amount of protein and enzymatic activity do not strictly correlate, *e.g.* after cessation of protein synthesis the enzyme-specific activity might still increase.[24] In *B. subtilis* heat shock results in a quick drop in β-galactosidase activity, most probably caused by proteases like Lon and ClpCP that recognize the *E. coli* β-galactosidase as foreign.[25,26]

In the study presented here, we used an *rsbW* mutant (BSA115) in which expression of σ^B is solely controlled by the IPTG inducible promoter P_{SPAC} and thus independent of the autoregulatory loop of wild type *B. subtilis* strains in which expression of *rsbV*, *rsbW*, *sigB*, and *rsbX* is driven by a σ^B-dependent promoter upstream of *rsbV*. In strain BSA115, due to the lack of RsbW, all σ^B produced from P_{SPAC} in the presence of IPTG should be active and allow constitutive expression of a *ctc::lacZ* fusion. We used this strain to test the consistency of our σ^B model that we constructed based on a model by Igoshin *et al.* (2007).[19] Surprisingly, we found similar transient induction patterns of β-galactosidase activity in the BSA115 derivative compared to a wild type strain with an intact autoregulatory loop controlling σ^B-activity (BSG56). Mathematical modeling suggested increased protease or RNase activities as the most likely explanation for the observations. Subsequent Northern and Western Blot experiments then proved that β-galactosidase protein degradation was responsible for the transient σ^B response pattern observed in BSA115.

Results

σ^B controlled β-galactosidase activity is transient even in the absence of the negative regulator RsbW

Using the *B. subtilis* strain BSA115 we measured the dynamics of an artificially induced general stress response. In BSA115 σ^B is under control of the IPTG inducible P_{SPAC} promoter and its primary negative regulator RsbW is not produced due to a frameshift mutation. Addition of IPTG to this strain releases the P_{SPAC} promoter from repression by LacI and σ^B is induced in a dose-dependent manner which is determined by the concentration of the inductor IPTG. All σ^B produced is active because RsbW, the only known direct negative regulator of σ^B activity, is absent. The positive autoregulatory loop of σ^B expression upstream of *rsbV* is disrupted by deletion of the σ^B-dependent promoter. Therefore, the strain is viable in contrast to *rsbW* mutants, in which unrestricted σ^B-activity is deleterious.

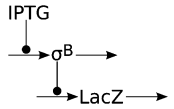

Fig. 1 Genetic basis of the reporter system. IPTG induces the *de novo* synthesis of σ^B which is in turn activating the expression of *lacZ*. Concentrations of proteins are reduced by dilution due to growth or proteolysis. Upstream regulation of σ^B is dysfunctional in BSA115 caused by a frameshift mutation in *rsbW*. Lines ending with a circle denote activation, the arrows denote synthesis and degradation.

We performed experiments using three IPTG concentrations (low: 0.1 mM, medium: 0.2 mM and high: 1 mM). The process diagram in Fig. 1 captures the genetic background of our experimental system. Addition of IPTG activates *de novo* expression of σ^B, which then induces the transcription of *lacZ* of the σ^B-dependent *ctc:lacZ* fusion. Employing a *ctc:lacZ* fusion as a reporter, the induction of σ^B-activity with high IPTG levels (1 mM) was approximately six times stronger compared with an induction in the wild type following the addition of ethanol (not shown). This course of events implies that different IPTG concentrations will cause different maximum β-galactosidase activities. Maximal β-galactosidase activity should be maintained depending on the stimulus level. However, we observed a transient pattern of β-galactosidase activity (Fig. 2b). Activity increased to reach a maximum, with higher IPTG concentrations causing a faster accumulation of β-galactosidase. After the peak, β-galactosidase activity declined rapidly and all experiments displayed a similar low activity 275 min after addition of IPTG.

A σ^B dependent protein leads to the adaptive behavior

Since our *a priori* assumption about the dynamics of the β-galactosidase activity differed with the observations, we hypothesized three mechanisms to explain the data. All hypotheses assume the expression of a σ^B dependent regulatory protein. The decrease in the observed β-galactosidase activity seems to be independent of the growth phase because two cultures with identical growth characteristics (Fig. 2a) had distinct β-galactosidase peaks (0.1 and 0.2 mM IPTG) (Fig. 2b). Addition of high IPTG levels (1 mM) slightly retarded growth and transition to stationary phase but maximum β-galactosidase activity occurred still within the exponential phase of growth. These observations indicate that the decrease in β-galactosidase activity is not concurrent with the transition to stationary phase and is therefore not caused by the changing availability of RNA-polymerase or ribosomes during stationary phase. We also tested the stability of the IPTG induction system by adding IPTG at regular intervals (Fig. S1, ESI†). Additionally, we generated a model for proteolytic β-galactosidase degradation independent of σ^B but dependent on the optical density. In this model, the time of protease synthesis was chosen to correlate with the transition to stationary phase. The σ^B independent model is not consistent with the experimental data (Fig. 2, ESI†). These results led us to conclude that the transient induction of β-galactosidase activity relies on a member of the *sigB* regulon. Three different mechanisms and the respective models that might account for the observations made are shown in Fig. 3, and can be biologically interpreted as follows.

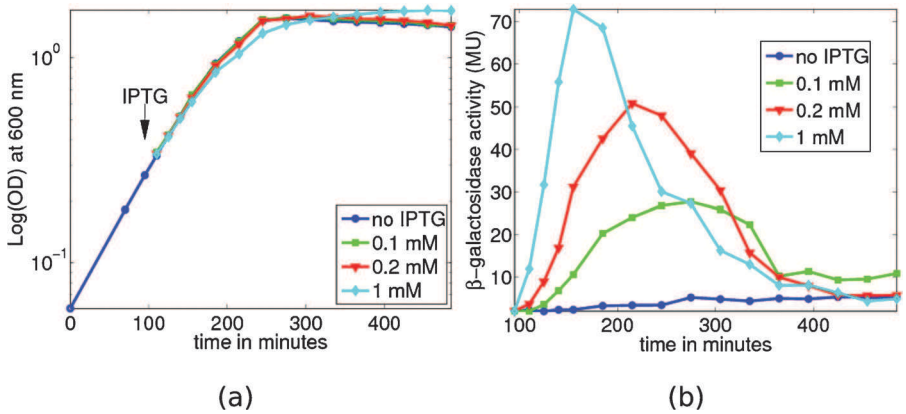

Fig. 2 Shake flask culture of BSA115. Shown are the OD_{600} (a) and the activity of β-galactosidase per cell (b). Induction of expression of σ^B occurs at the time indicated when cells reached an OD_{600} of approximately 0.3.

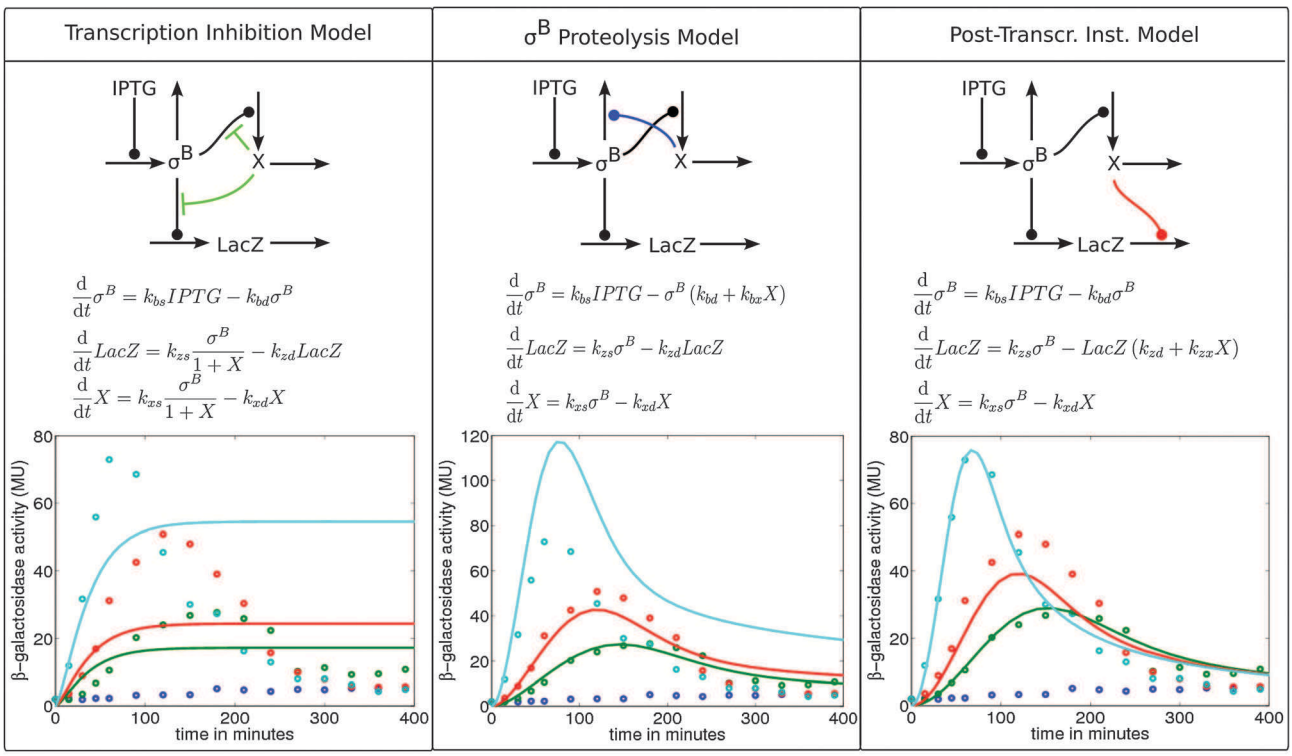

Fig. 3 Comparison of the models showing their process diagrams, their mathematical representation and their ability to fit optimally to the given experiments. Only the 'post-transcriptional instability' model can reproduce the observations sufficiently. Therefore, we assume this model forms the basis of an explanation for the data. Northern blot experiments of *lacZ* mRNA and Western blot experiments with β-galactosidase (Fig. 4) narrow the instability down to proteolytic degradation of the β-galactosidase protein. Lines finishing with a bar denote inhibition of the associated reaction. Induction by IPTG is assigned to time 0 in the concentration-time plots.

1. Transcription inhibition model. The expression of a hypothetical protein, denoted with X, is induced by σ^B. X can be interpreted as a hypothetical transcription factor that inhibits the activity of σ^B. Therefore, following induction of σ^B activity with IPTG, higher levels of X are generated and protein synthesis is subsequently inhibited. In this scenario, we assume X to be an unspecific protein expression inhibitor, but we also tested specific inhibition of *lacZ* expression which gives comparable results (not shown). This hypothesis resembles findings related to Spx, a protein involved in the regulation of disulfide stress response. Spx binds to the α-subunit of RNA-polymerase thereby regulating expression rates for genes related to disulfide stress.[27,28] MgsR (YqgZ) is a Spx paralogue in *B. subtilis* implicated in the regulation of general stress response.[29]

2. σ^B proteolysis model. In this model the hypothetical protein X is assumed to be a protease involved in σ^B degradation. This mechanism is biologically inspired by the regulation of the general stress response sigma factor σ^S in *E. coli* by RssB (SprE). RssB binds to σ^S and delivers it to the ClpXP proteolytic complex for degradation.[30]

3. Post-transcriptional instability model. The decrease in the β-galactosidase activity can also be caused by degradation events acting directly on β-galactosidase either at the mRNA or protein level. The hypothetical protein *X* then represents an RNase or a protease. Within our modeling framework we cannot distinguish between RNase and protease because we combine mRNA and protein production into a single step assuming a quasi-steady-state approximation for mRNA. Thus, the corresponding variable in the equations can either represent mRNA or protein. However, we focus on protein instability as recombinant β-galactosidase has been indicated to be subjected to degradation in response to heat shock.[25,31,32]

Although we assume in our models a direct control of σ^B regarding the regulator, this control might as well be indirect. There may be one or more intermediary σ^B-dependent factors activating the regulator. Direct *versus* indirect regulation is indistinguishable within our modeling framework. In the following section the three models are compared and verified with the experimental observations.

The 'post-transcriptional instability' model, representing proteolytic degradation of β-galactosidase, can optimally capture our observations

The process diagrams (Fig. 1) were used to formulate systems of coupled ordinary differential equations. We then estimated parameter values of the models to reproduce our experimental results. The resulting fits are shown in Fig. 3 and further described in the Materials and Methods section. The 'transcription inhibition' model fails to explain the observed β-galactosidase dynamics, because the model prediction differs qualitatively from the observations (Fig. 3). We also tested a model for inhibition of translation. Its results are similar to the transcription inhibition model (not shown). The 'σ^B proteolysis' model is able to capture some characteristics of the measured dynamics, notably the transient, adaptive nature of the response. However, this model fails to reproduce the observation at 275 minutes after induction with the different IPTG additions approaching a comparable low β-galactosidase activity. High IPTG stimulation results in a disproportional high β-galactosidase activity in the σ^B proteolysis model. Therefore, also the σ^B proteolysis model does not provide a plausible explanation for our experimental observations. In contrast, the 'post-transcriptional instability' model successfully reproduces all aspects of the experimental observations, *i.e.* transience of the dynamics and comparable β-galactosidase activity at the end of the experiment. The model is even able to reproduce the inverted activity results for the three IPTG concentrations at 245 min. At that time low IPTG addition causes highest activity while high IPTG addition results in the lowest signal. The following sections provide a more detailed analysis of the 'post-transcriptional instability' model.

lacZ mRNA remains stable and protein concentration drops after σ^B induction in BSA115

Model simulations and their analysis allowed us to identify instability of either mRNA or β-galactosidase protein as an explanation for the observed transient dynamics of β-galactosidase activity in BSA115. However, mRNA and protein instability is indistinguishable within the model because we assumed a rapid and direct correlation between mRNA and protein level to limit the number of unknown parameters. In effect, we arrive at a variable that combines information about mRNA and protein. To distinguish between mRNA and protein level we performed Northern blot and Western blot experiments to measure *lacZ*-mRNA and β-galactosidase-protein levels, respectively. mRNA levels during IPTG activation are shown in Fig. 4A and display persistent high *lacZ*-mRNA levels. The smear besides the detected main transcript is explained by specific hybridization of the probe to exo- and endonucleolytically truncated degradation intermediates of the full-length mRNA as well as still nascent *lacZ*-mRNA molecules. The Western-blot experiments mimicked the transient nature of the β-galactosidase activity. Remarkably, in the absence of IPTG, and hence at a low basal level of σ^B-activity, β-galactosidase remained stable (Fig. 4B lower panel). The faint bands of β-galactosidase detected in the absence of IPTG induction are likely a reflection of low basal level expression from the leaky P_{SPAC} promoter.

The observed β-galactosidase instability is independent of ClpCP

One of the primary suspects responsible for degradation of β-galactosidase was the ClpCP protease.[31] This complex is activated by stress and is also σ^B sensitive. We used the *clpP*

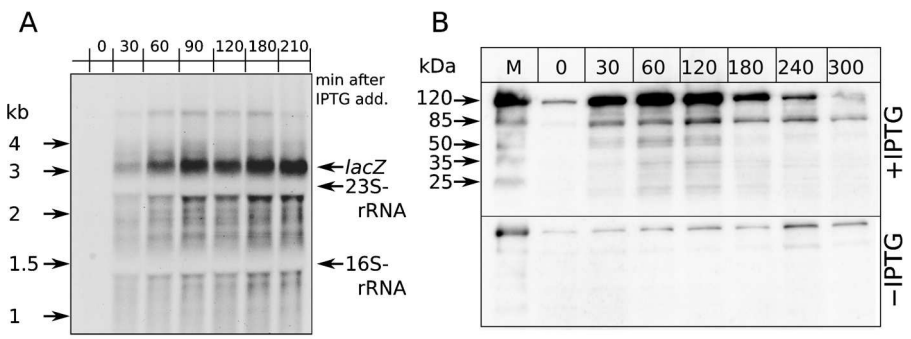

Fig. 4 (A) Northern blot results for *lacZ* mRNA. Induction of σ^B expression and activity of BSA115 with 1 mM IPTG started at 0 min. Induction took place in early exponential phase as indicated in Fig. 2a. The blot was exposed for 30 min. (B) Western blot results for the β-galactosidase protein which was detected at 120 kDa with respect to the marker (lane-M) used in this experiment. The upper panel reflects β-galactosidase levels after induction of σ^B by the addition of IPTG to a final concentration of 1 mM at 0 min. After 120 min the protein level declines, while the negative control without IPTG induction (lower panel) displays a continued low β-galactosidase level. The blot was exposed for 30 s.

deletion strain BSG115 and followed the stability of β-galactosidase. The reporter protein signal was still transient comparable to the signals in the *clpCP* wild type strain as shown in Fig. 2b indicating that the ClpCP protease is not responsible for the observed β-galactosidase instability (Fig. 3, ESI†).

Proteolytic degradation of β-galactosidase can mask σ^B activity measurements even in cells with normal regulation of σ^B activity

In experiments with BSG56, a wild type strain with respect to σ^B regulation, σ^B dependent gene expression first increases during transition into stationary phase, subsequently followed by a decrease in the level of the σ^B dependent reporter protein, often a β-galactosidase reporter system, close to pre-stimulus activity (compare β-galactosidase measurements in, for example, ref. 20 and 33–36). Our presumption for the *rsbW* mutant strain BSA115 was to observe an initial increase in the β-galactosidase activity, with a sustained high and IPTG specific β-galactosidase activity. Negative feedback control mechanisms can explain transient responses and Igoshin *et al.* proposed an RsbW-mediated negative feedback.[19] Indeed, the model of σ^B regulation presented there can reproduce the adaptive β-galactosidase response shown in Fig. 5, where we fitted the model σ^B feedback ('sigB fbck.' model) to an experimental course of general stress induction in a culture experiment with strain BSG56 (blue-dashed line). We did not observe an ongoing β-galactosidase expression in our experiments with BSA115 (Fig. 2), instead the response was similar to the transient response known from an *rsbW* wild type strain. The σ^B dependent proteolytic activity that explains our observations in BSA115 was probably also present in previous experiments using BSG56. Thus, we sought to test how much of the transient nature of σ^B activation in BSG56 could be attributed to proteolytic degradation of β-galactosidase. The continuous red line in Fig. 5 represents a fit of the proteolysis model ('post-translational instability' model in Fig. 3) using experimental data from BSG56. Only two parameters, both not associated with the properties of the hypothetical protease, were used for parameter estimation, namely the stimulation of σ^B synthesis by IPTG, which is now replaced by the cellular energy level (approximated by medium glucose concentration, not shown), and the constant synthesis rate of β-galactosidase, to allow for fitting of the different absolute Miller unit maxima in the two experiments. While not necessarily being the only explanation for the transient dynamics, the mathematical proteolysis model (prot. model) in Fig. 5 suggests an involvement of proteolytic degradation of β-galactosidase.

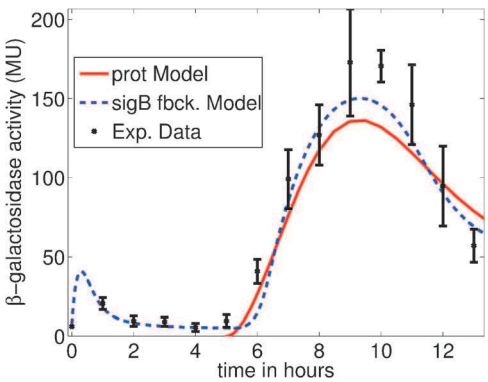

Fig. 5 Illustration of the induction of general stress response during starvation in BSG56. The activity of the general stress response transcription factor σ^B was measured with a *ctc::lacZ* fusion. Black points represent experimental data. The blue-dashed line represents a fit of a σ^B model that includes the regulation of σ^B activity by Rsb-proteins. The red-continuous line represents the fit of the 'post-translational instability' model for the transient activation of the general stress response.

Discussion

Our results show an activation of proteolytic degradation of β-galactosidase following IPTG induction of σ^B. We adapted the model of σ^B response by Igoshin *et al.* by including glucose starvation as a trigger for activation and fitting it to existing experiments. This model can reproduce experimental observations of σ^B activation and deactivation as represented by the blue-dashed line in Fig. 5. We then performed an experiment in which we used the *B. subtilis* strain BSA115 characterized by stable IPTG-mediated induction of σ^B expression and lack of negative regulation of σ^B activity, due to a frameshift mutation in *rsbW* (see Material and Methods section). Unexpectedly, an induction of σ^B with IPTG resulted in a short-term pulse of β-galactosidase activity as indicated in Fig. 2. Using mathematical modeling to compare different hypotheses leads to the conclusion of an instability of either mRNA or protein as potential causes for the transient activity as demonstrated in Fig. 3. Subsequent Northern and Western blot experiments confirmed proteolytic decay as the cause for the decrease in β-galactosidase signal (Fig. 4). The transient activity in BSA115 resembles the transient activity observed in experiments in a wild type *B. subtilis* strain (BSG56) containing an intact σ^B regulation. We tested whether proteolytic decay is at least partially responsible for transient β-galactosidase dynamics in the wild type by adapting the 'post-transcriptional instability' model to experimental observations in BSG56. Indeed, we find σ^B induced β-galactosidase instability is a process with potential contributions to the adaptive behavior of β-galactosidase in wild type cells. Two questions remain open: (i) is the increase in β-galactosidase proteolysis specific to the recombinant β-galactosidase? and (ii) to which extent different σ^B activation routes lead to differential expression of σ^B dependent genes?

The research focus with respect to σ^B mediated general stress response has been on its activation whereas less information is available about mechanisms of the shut-off of σ^B activity. Using β-galactosidase to investigate σ^B deactivation is complicated since this protein is prone to degradation particularly in the context of overproduction (this study) and heat shock.[25] Several mechanisms can contribute to the transient response of σ^B-dependent transcription: (i) silencing of σ^B by subsequent response strategies, (ii) adaptivity caused by negative feedback, and (iii) increased proteolytic instability after σ^B activation. Silencing of σ^B by subsequent response strategies could be an inevitable event of differentiation and adaptation processes of *B. subtilis* following deteriorating environmental conditions. Activation of processes, like sporulation or biofilm formation, could inhibit the activity of σ^B much like they modulate the activity of competence and chemotaxis.[18,37] Subsequently, the transcriptional activity of σ^B declines. Several studies have promoted the hypothesis that

negative feedback loops within the partner switch regulation of σ^B result in its adaptive behavior.[19,33,38] Activation of σ^B leads to increased expression of the anti-sigma factor RsbW and the phosphatase RsbX resulting in an increase in RsbV phosphorylation. This in turn releases RsbW to sequester and mute σ^B. This mechanism can result in a decline of σ^B dependent transcriptional activity as well. Locke *et al*. (2011) studied how the σ^B system uses noise in combination with a kinase–phosphatase pair (RsbW-RsbP) to adjust stress response activation frequency.[39] They are able to explain their pulse like dynamics only with the RsbW negative feedback and they disregard changes in YFP stability. Pulse like dynamics are generated in our β-galactosidase system *via* increased degradation following σ^B activation, and this offers an additional mechanism to generate or exaggerate pulses.

Results by Reeves *et al*. (2007) point to the importance of the ClpP protease for the regulation of σ^B activity.[40] A *clpP* mutant strain results in lasting σ^B activation measured with β-galactosidase activity from a *ctc::lacZ* fusion.[40] This leads to the assumption that ClpP could be associated with the turnover of σ^B dependent components that result in a deactivation of σ^B in the wild type.[40] However, further experiments performed by us revealed that ClpP did not affect the stability of β-galactosidase. Hence, different proteolytic mechanisms may lead to a reduction in σ^B dependent β-galactosidase activity in our experimental setup. Similarly, our results stress the importance of induced proteolysis, albeit now it is the reporter protein β-galactosidase being targeted for degradation. In our experiments, the transcriptional activity of σ^B is not diminished while the reporter signal still decreased. The previous list of possible mechanisms that could explain the transient nature of the σ^B response is neither complete nor are those mechanisms mutually exclusive. Further work is required to quantify and discriminate each contribution and to uncover new modes of regulation.

The mechanisms we study to explain the experimentally observed transient adaptive-like dynamics are derived from established biological processes associated with bacterial stress response. An analysis about all possible topologies that can result in adaptive dynamics was performed by Ma *et al*.[41] The authors found only two configurations for robust adaptation given suitable parameter combinations: integral feedback (buffered negative feedback), and incoherent feedforward. The 'transcription inhibition' and the 'σ^B proteolysis' models are examples for integral feedback, while the 'post-transcriptional instability' model is an example for an incoherent feedforward loop. Ma *et al*. state there are no clear biological cases where incoherent feedforward loops are used to achieve adaptation.[41] Although we do find such a motif explaining our observations, the wild type regulation of σ^B mediated general stress response is better known for its use of integral feedback regulation *via* anti-sigma factor RsbW[10] and phosphatase RsbX.[38] Thus, the observation by Ma *et al*. remains valid: adaptation generated by incoherent feedforward loops is rare.

Materials and methods

Strain construction

BSG55 was constructed by transforming a prototrophic *B. subtilis* strain (BSB1, 168 Trp^+)[42] with chromosomal DNA of BSM269 selecting for the transfer of the *gsiB::gfp* fusion in *amyE* by screening for chloramphenicol resistant colonies.[20] BSG55 was then used as a recipient for transformation with chromosomal DNA from BSM151[20] selecting for erythromycin resistance and thus transfer of the SPβ located *ctc::lacZ* fusion thus generating BSG56. BSG56 is characterized by the following genotype *amyE::pGK30 gsiB::gfp cat86 SPβctc::lacZ*. In BSA115 (*trpC2 rsbU::kan* P_B 28::P_{SPAC} *rsbW313* pTet-I *SPβctc::lacZ*) *rsbU* is interrupted with a kanamycin cassette, the chromosomal σ^B-dependent promoter upstream of *rsbV* is replaced by P_{SPAC} and *rsbW* is inactivated by introducing a frameshift mutation.[12,38] In addition this strain carries a plasmid pTet-I which delivers LacI and thus allows repression of P_{SPAC} in the absence of IPTG. If IPTG is added σ^B is produced in a concentration dependent manner and always active because its primary negative regulator RsbW is missing due to a frameshift mutation in *rsbW*. Moreover, the autoregulatory σ^B-dependent promoter upstream of *rsbV* is replaced by P_{SPAC}. The *clpP* deletion strain BSG115 was constructed by transforming strain BSA115 with chromosomal DNA of the *clpP* mutant strain QB4316[43] selecting for the transfer of the *clpP::spc* deletion by screening for spectinomycin resistant colonies.

Batch shake-flask experiments for BSA115, BSG115 and BSG56.

M9 media composition was 0.3% glucose, 0.3% malate, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.05 mM $FeCl_3$, 8.5 g l^{-1} $Na_2HPO_4{\cdot}2H_2O$, 3 g l^{-1} KH_2PO_4, 1 g l^{-1} NH_4Cl, 0.5 g l^{-1} NaCl, 1 mg l^{-1} $MnCl_2$, 1.7 mg l^{-1} $ZnCl_2$, 0.43 mg l^{-1} $CuCl_2{\cdot}2H_2O$, 0.6 mg l^{-1} $CoCl_2{\cdot}6H_2O$ and 0.6 mg l^{-1} $Na_2MoO_4{\cdot}2H_2O$. BSG56, BSA115 and BSG115 were grown overnight at 37 °C at 300 rpm in fresh M9 medium for 12–14 h overnight and inoculated into the main culture to a final OD_{600} of 0.05. For BSA115 strain, 100 mM IPTG was added to a final concentration of 1 mM, 200 μM and 100 μM at desired OD (at OD_{600} 0.2). Samples were harvested at appropriate time intervals (see Fig. 2 and 4) for RNA preparation and β-galactosidase activity measurement.

Determination of β-galactosidase activity

For determination of the β-galactosidase activities of the *ctc::lacZ* fusion strains, cultures were propagated as described above. At appropriate time points, 1 ml aliquots were harvested by centrifugation in an Eppendorf tabletop centrifuge at 4 °C. β-Galactosidase enzyme assays were performed as described previously.[14,44]

Northern blot analysis

Preparation of total RNA was carried out as described by Eymann *et al*.[45] Eight cell samples equivalent to 15 OD_{600} units each were sequentially harvested for every 30 minutes. At OD_{600} of 0.2, the BSA115 culture was induced with IPTG (1 mM). High-quality RNA was prepared. Northern analysis was performed as described by Homuth *et al*.[44] using 5 μg of total RNA per lane. Transcript sizes were determined by comparison with an RNA size marker (Invitrogen). The positions of the molecular size markers are depicted on the

Table 1 Parameter values for the three competing models used to fit the experiments as shown in Fig. 3. The dimension for IPTG concentration is μM and reaction rate constants are in Miller units per minute (MU min^{-1}). The adapted parameter values for fitting the 'post-transcriptional instability' model to experimental data in BSG56 in Fig. 5 are shown in brackets. Parameter fitting analyses indicate that the parameters can be estimated independently and have in general a low cross-correlation (Fig. 4, ESI)

Parameter	Meaning	Transcription inhibition	σ^B Proteolysis	Post-transcriptional instability
IPTG	IPTG conc. for σ^B activation	100, 200, 1000 (28.9)		
k_{bs}	σ^B synthesis	100	100	100
k_{bd}	σ^B degradation	4.4×10^{-2}	5.8×10^{-9}	1.7×10^{-2}
k_{bx}	Regulator mediated σ^B degr.	—	8.4×10^{-5}	—
k_{zs}	*lacZ*/β-gal synth.	4×10^{-4}	1.7×10^{-6}	9×10^{-7} (8.2×10^{-6})
k_{zd}	*lacZ*/β-gal degr.	4.1×10^{-2}	5.2×10^{-2}	1.3×10^{-7}
k_{zx}	Regulator mediated β-gal degr.	—	—	3.2×10^{-3}
k_{xs}	Regulator synth.	7.6×10^{-1}	2×10^{-6}	9.3×10^{-8}
k_{xd}	Regulator degr.	9	1.2×10^{-13}	1.1×10^{-9}

left in the Northern blot image (Fig. 4A). Digoxigenin-labeled *lacZ*-specific RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase, using specific PCR products as templates. Synthesis of the *lacZ* templates by PCR was performed using the following oligonucleotide primer pairs: forward *lacZ*-probe (5′-AGGAAGGCCA-GACGCGAATT-3′) and reverse *lacZ*-probe with T7 Promoter (5′-CTAATACGACTCACTATAGGGAGAAACCACCACG-CTCATCGATA-3′). The blots were hybridized with anti-digoxigenin-AP (alkaline phosphatase) Fab fragments (Roche) and developed with CDP-Star (Tropix) substrate. The blots were scanned with the Vilber Lourmat Imagingsystem (Vilber Fusion-SL, 3500-WL) and analyzed with the software (Fusion-Capt 2.0.4; V15.08).

Protein isolation and estimation

When required, 7 OD_{600} units were harvested by centrifugation at 8000 rpm (5 min, 4 °C) and the pellet was washed with cold TE buffer and again centrifuged. Protein isolation from the frozen pellet and protein estimation were performed according to ref. 44.

Western blot analysis

For each sample 30 μg of protein were separated on a 10% SDS PAGE and transferred to a PVDF membrane (Immobilion-P, Millipore, Bedford, USA) by semi-dry electro blotting. Blocking of the membrane was performed for 1.5 h with TBST buffer (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween-20) containing 5% milk powder. After brief washing in TBST buffer the membrane was incubated overnight with TBST buffer containing 5% milk powder and the primary antibody (dilution 1 : 200) raised against *E. coli* β-galactosidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were detected by incubation with a secondary antibody (dilution 1 : 5000) conjugated to HRP (Jackson Immunoresearch, Suffolk, UK) and detected by enhanced chemiluminescence using Super-Signals West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

Model building, comparison and parameter fitting

We constructed three models and tested their ability to fit experimental data. The network diagrams and differential equations for the three models are given in Fig. 3. The parameter values used to generate the time courses are given in Table 1. For the construction and the analysis of the models the Systems Biology Toolbox2 for Matlab was used.[46] SBML files of the model are uploaded to the JWS-model database and accessible with the following accession numbers: transcription inhibition (bsa_trscrinhib20122131995), σ^B proteolysis (bsa_sigbprlysis201221319839), and post-transcriptional instability (bsa_ptinst201221319512).

The parameters of σ^B synthesis k_{bs} and degradation/dilution k_{bd} are highly correlated. We constrained k_{bs} arbitrarily to 100 min^{-1} to be able to estimate k_{bd}. This procedure is possible since only the ratio of synthesis and degradation determines the β-galactosidase dynamics. Parameter estimation was performed in SBToolbox2 with the particle swarm algorithm that was applied to the model until no fitness improvement could be achieved.[46,47] Measurements took place with three different experimental conditions. Different levels of σ^B were induced *via* three different medium concentrations of IPTG, namely low, medium and high (0.1, 0.2 and 1 mM). The data obtained with low and medium IPTG concentration were used as a training set for parameter estimation, while the data for high IPTG addition served as a test set to evaluate how well the parameters can predict this experiment.

We adapted the model of σ^B by Igoshin *et al.*[19] to reproduce the σ^B dependent expression of β-galactosidase during the transition from exponential to stationary growth phase in BSG56, *cf.* Table 1. The parameter estimation was conducted as explained above. Parameter estimation of the 'post-transcriptional instability' model with this data took place using only the measurements after 5 hours of cultivation since only then σ^B expression was induced. That time therefore represents the addition of IPTG in the BSA115 strain experiments. Only the two parameters IPTG and k_{zs} were allowed to vary during estimation with the particle swarm algorithm. The numerical results of the parameter estimation are shown in brackets in Table 1 and the fit is shown in Fig. 5.

Acknowledgements

The authors acknowledge excellent technical support by Marc Schaffer. The authors acknowledge support by Hendrikje Hildisch for starvation experiments of Fig. 5, and Felix Winter for the parameter characterization. The research was supported by German Federal Ministry of Education and

Research (BMBF) BaCell-(0313978), BaCell2 (0315784), and Cosmic2 (0315782D) SysMO projects.

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