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REPORT

A quantitative study of the benefits of co-regulation using the *spoIIA* operon as an example

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The distribution of most genes is not random, and functionally linked genes are often found in clusters. Several theories have been put forward to explain the emergence and persistence of operons in bacteria. Careful analysis of genomic data favours the co-regulation model, where gene organization into operons is driven by the benefits of coordinated gene expression and regulation. Direct evidence that coexpression increases the individual's fitness enough to ensure operon formation and maintenance is, however, still lacking. Here, a previously described quantitative model of the network that controls the transcription factor σ^F during sporulation in *Bacillus subtilis* is employed to quantify the benefits arising from both organization of the sporulation genes into the *spoIIA* operon and from translational coupling. The analysis shows that operon organization, together with translational coupling, is important because of the inherent stochastic nature of gene expression, which skews the ratios between protein concentrations in the absence of co-regulation. The predicted impact of different forms of gene regulation on fitness and survival agrees quantitatively with published sporulation efficiencies.
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Introduction

The available genome sequences demonstrate that many genes are clustered on chromosomes according to their function. Genes in bacteria are clustered but can also be organized into operons such that the expression of a group of genes is regulated by the same genetic control element. When operons were first discovered, it was assumed that the benefit of co-transcription led to operon assembly (Jacob and Monod, 1961). Other models have since been proposed, and these belong to one of three classes, the natal model, the Fisher model, or the selfish operon model (Lawrence, 1997). According to the natal model, clustering of genes is the consequence of gene duplication. However, as operons comprise genes that belong to very distant families and the majority of paralogues do not cluster, this model is insufficient to explain operon origin (Lawrence, 1997; Dandekar *et al*, 1998). A recast of the Fisher model, adapted to prokaryotes, proposes that clustering of genes reduces the likelihood that co-adapted genes become separated by recombination. However, this does not explain how operons can emerge, as recombination is as likely to generate clusters as to disrupt them. According to the selfish operon model, operons facilitate the horizontal transfer of functionally related genes (Lawrence and Roth, 1996). The physical proximity of genes thus does not necessarily provide a selective advantage to the individual organism but rather to the gene cluster itself, because it can be efficiently transmitted horizontally as well as vertically. Recent studies have, however, failed to observe the gene cluster pattern predicted by the model, and this strongly suggests that the selfish operon model does not explain the emergence and persistence of operons (Pal and Hurst, 2004; Price *et al*, 2005). So what drives operon assembly?

The idea that co-transcription of genes provides a selective advantage to the individual organism has never been contradicted. It has been questioned only because it remains unclear whether the benefits of co-transcription could be strong enough to drive the assembly of operons by rare recombination events (Lawrence and Roth, 1996; Lawrence, 1997). A genotype that confers higher fitness will dominate in a population with bounded total population size only if selection acts on a timescale that is substantially shorter than the timescale on which recombination and mutation events could negate the benefits.

There are a number of potential selective advantages given by co-transcription. In the case of operons that code for multiprotein complexes, co-transcription enables co-translational folding (Dandekar *et al*, 1998), it limits the half-life of toxic monomers (Pal and Hurst, 2004), and it reduces stochastic differences in gene expression (Swain, 2004). Operons that do

not code for interacting proteins may be advantageous because of the co-regulation of protein expression (Price *et al*, 2005). Many examples of this class of operons are associated with metabolic operons (Lawrence and Roth, 1996) where co-regulated expression is likely to optimize the flux and to facilitate the regulation of functions, especially if these are required only under certain environmental conditions, or if complex regulatory structures are employed (Price *et al*, 2005).

Evidence in favour of any of these proposed driving forces has so far largely been obtained from comparative genomics. Here we use a previously derived quantitative model for the network that controls the transcription factor σ^F during sporulation in *Bacillus subtilis* (Iber *et al*, 2006) to quantify the benefits of coexpression. Spore formation in *B. subtilis* is a response to nutrient deprivation at high cell density and involves asymmetric septation and compartment-specific initiation of gene expression (Hilbert and Piggot, 2004). The different gene programs in the larger mother cell and the smaller prespore are both directed by the transcription factor σ^F, which, although only active in the smaller prespore, affects the transcriptional programs across the septum also in the mother cell, a phenomenon that is referred to as criss-cross regulation (Losick and Stragier, 1992). Successful sporulation therefore requires the rapid septation-dependent and prespore-specific activation of σ^F. σ^F is kept inactive by binding to SpoIIAB and is released upon binding of SpoIIAA (Figure 1). SpoIIAA is phosphorylated by SpoIIAB (Min *et al*, 1993) and reactivated by the serine phosphatase SpoIIE (Duncan *et al*, 1995). The balance between kinase and phosphatase activity thus determines whether or not σ^F is released from its inactive complex with SpoIIAB. SpoIIE accumulates on both sides of the asymmetrically positioned septum and therefore has an increased activity in the smaller compartment (Arigoni *et al*, 1995). A quantitative model of the regulatory network predicts that because of the low turnover rate, most SpoIIE is bound by its substrate such that enzyme and substrate increase together in the smaller compartment (Iber *et al*, 2006). According to the model, this combined increase is sufficient to trigger the formation of micromolar concentrations of σ^F holoenzyme in the prespore.

It is obvious from the above that the protein concentration ratio is important. An excess of σ^F or SpoIIAA compared to SpoIIAB will result in free σ^F and σ^F-dependent gene expression, whereas an excess of SpoIIAB will prevent

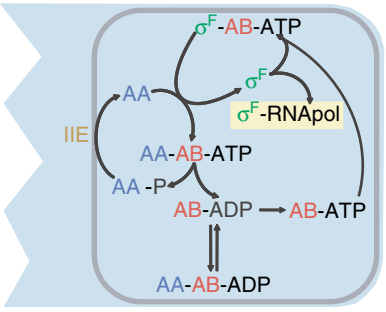

Figure 1 An overview of the interactions in the network that controls σ^F activity in *B. subtilis*. For details, see text. The figure is a reproduction of Figure 1 of Iber *et al* (2006).

SpoIIAA-dependent σ^F release. In the vegetative cell, the sporulation proteins are not detectable, and septation is preceded by 90–120 min of gene expression, dependent on the exact experimental conditions (Magnin *et al*, 1997; Lord *et al*, 1999; Lucet *et al*, 1999). Limiting the stochastic noise inherent in protein expression can be expected to be crucial for avoiding variations in the relative protein concentrations and the resulting sporulation defects. Three of the four proteins in the network are transcribed from genes in the *spoIIA* operon (Figure 2A). These genes are not only co-transcribed into a single mRNA but are also most likely to be coexpressed, as the translation of the three proteins appears to be coupled, at least to some degree. This system therefore offers an excellent opportunity to analyse the influence of transcriptional and translational co-regulation of the sporulation genes on an individual's survival and fitness.

Coupled translation is achieved when two genes are translated by the same ribosome. Reinitiation of translation at a nearby start codon after termination at the upstream gene is possible because ribosome dissociation from the mRNA is a slow and energy-dependent process (McCarthy and Gualerzi, 1990). There is currently no direct experimental evidence for coupled translation of the *spoIIA* operon. Such coupling can, however, be postulated based on the arrangement of genes (Fort and Piggot, 1984). The first two genes in the *spoIIA* operon (encoding SpoIIAA and SpoIIAB) overlap by 4 bp, whereas the genes for SpoIIAB and σ^F are interspaced by 11 bp (Figure 2A); coupled translation has been documented for intercistronic distances of more than 60 bp (McCarthy and Gualerzi, 1990). The majority of genes that are organized in operons are separated by distances comparable to those found in the *spoIIA* operon (Salgado *et al*, 2000), so that the studied system can be considered as representative of operons in general. The efficiency of reinitiation depends on the distance as well as the strength of the Shine–Dalgarno sequence (Adhin and van Duin, 1990; McCarthy and Gualerzi, 1990), which is, in general, located 5–13 bp upstream of a start codon and which binds to the homologous 3′ end of the 16S rRNA, a component of the 30S ribosomal subunit. Moreover, the secondary structure of the mRNA can affect lateral diffusion of the ribosomes (Adhin and van Duin, 1990).

According to the protein expression data for the *spoIIA* operon, it appears that the last gene in the operon which encodes σ^F, is expressed at much lower levels than are *spoIIAA* and *spoIIAB*, whereas SpoIIAB monomers may be expressed at equal or up to three times higher levels compared to SpoIIAA (Magnin *et al*, 1997; Lord *et al*, 1999; Lucet *et al*, 1999). The weaker expression of a downstream gene (as is the case for σ^F) can, in general, be accounted for by a weaker ribosomal binding site, which is removed far enough from the termination codon of the upstream cistron that a considerable fraction of ribosomes dissociate from the mRNA before translation can be reinitiated (McCarthy and Gualerzi, 1990). It should be noted that whereas the transcriptional and translational coupling will reduce the noise in the relative SpoIIAB to σ^F expression levels, the unbinding of ribosomes is necessarily a stochastic process and will therefore add a low level of noise. The stronger expression of a downstream gene (as may be the case for SpoIIAB relative to SpoIIAA) can, in general, only be observed if a strong initiation sequence for the downstream

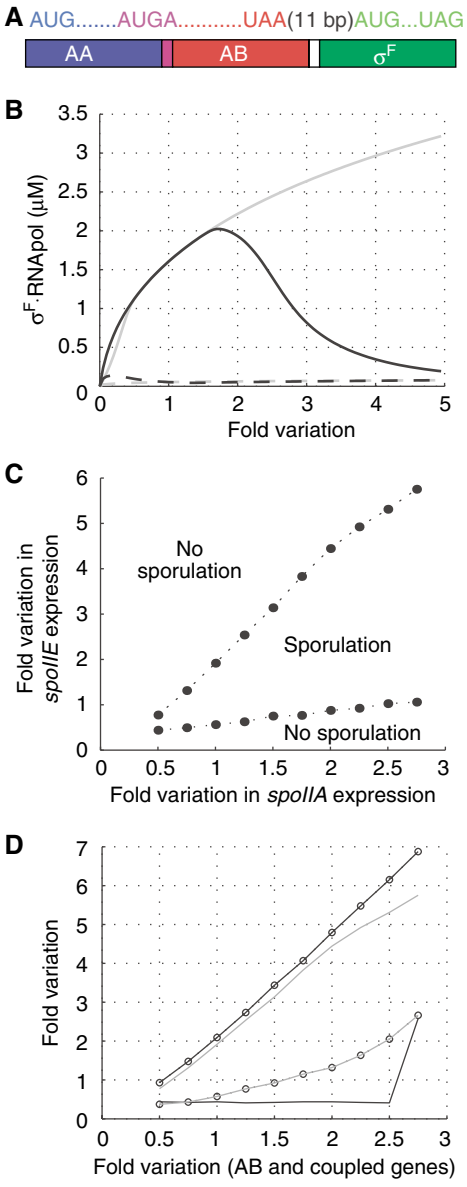

Figure 2 The impact of parallel and random variations in the expression of *spoIIE* and *spoIIA* genes on σ^F release. (**A**) The *spoIIA* operon comprises the genes for SpoIIAA, SpoIIAB, and σ^F. The genes for SpoIIAA and SpoIIAB overlap; the genes for SpoIIAB and σ^F are separated by 11 bp. (**B**) The regulatory network is robust to parallel variations in gene expression. The predicted concentration of σ^F-RNA polymerase holoenzyme before (dashed lines) and after septum formation (continuous lines) if either all (grey lines) or all protein expression rates except that of SpoIIE (black lines) were increased by the factor on the horizontal axis compared to the standard reference rates (6×10^{-9} M s^{-1} for SpoIIAA and SpoIIAB dimers and 2×10^{-9} M s^{-1} for σ^F and SpoIIE; Iber *et al*, 2006). (C, D) The expression rate combinations for which septation-dependent σ^F release is possible (between the lines) or not possible (outside the area marked by lines). (**C**) The impact of differential regulation of *spoIIE* and *spoIIA* expression. The vertical and horizontal axes indicate the fold variation in the *spoIIE* and *spoIIA* expression rates respectively, compared to the standard reference rates. (**D**) The impact of differential regulation of the expression of genes encoded in the *spoIIA* operon. The vertical axis indicates the fold variation in the expression of SpoIIAA (circles), σ^F (black lines), or SpoIIAA and σ^F (grey lines). The horizontal axis indicates the fold variation in the expression of SpoIIAB and of any other protein whose expression is coupled to that of SpoIIAB (which are those genes in the *spoIIA* operon not reported on the vertical axis). The sudden jump observed at a high SpoIIAB to σ^F ratio (lower black line) is the consequence of impaired σ^F release when the relative SpoIIAB concentration is too high.

gene is occluded by mRNA secondary structure, which is melted by the ribosome that transcribes the upstream gene (McCarthy and Gualerzi, 1990). Such a condition does not seem to be met by the gene for SpoIIAB, and more accurate expression data will be necessary to establish whether more SpoIIAB than SpoIIAA is expressed.

Available expression data can best be captured by an expression rate for SpoIIAB dimers and SpoIIAA of 6×10^{-9} M s^{-1} and 2×10^{-9} M s^{-1} for σ^F and SpoIIE (Iber *et al*, 2006); it should be noted that the simulation yields qualitatively similar results if SpoIIAB monomers and SpoIIAA are expressed at equal rates (6×10^{-9} M s^{-1}), as long as the σ^F and SpoIIE expression rate is then reduced to 10^{-9} M s^{-1} (Iber, 2006). As discussed by Iber (2006), the linear increase in the protein concentration assumed here does not fully match the experimental observations. There are, nonetheless, two good reasons to use a linear model. First of all, the data are too inaccurate and, in parts, contradictory to be modelled exactly. Secondly, the chosen rates correspond to the protein concentrations measured at the time of septation (Magnin *et al*, 1997; Lord *et al*, 1999; Lucet *et al*, 1999), the critical time point to judge sporulation success. This is because, in the cell, the IIE concentration increases more slowly than the other protein concentrations and only increases sharply immediately before septation (Feucht *et al*, 2002). As a consequence, the greatest danger of spontaneous uncompartmentalized activation of σ^F is just before septation, and this risk is fully assessed by the linear expression model. As our analysis focuses mainly at what happens minutes before and after septation, individual fluctuations in the global expression rates during the 2 h preceding septation are not important and the linear protein expression rates used should be considered as an averaged protein expression rate per bacterium.

Our quantitative ordinary differential equation model is very detailed—it comprises 50 dependent variables and 150 kinetic constants to describe the dynamics of only four proteins; the reader is referred to a detailed discussion of the model in Supplementary information of Iber *et al* (2006). Given its high level of detail and accuracy, the model predicts the phenotypes of essentially all mutants for which the biochemical effect is known. We can therefore expect that the predicted sporulation efficiencies in response to changes in parameter values are realistic. In the following, we employ the model to quantify how far different levels of stochastic noise in gene expression, as modulated by different degrees of coupling of protein expression (that is by the coupling of both transcription and translation), affect the sporulation efficiency, that is the survival chances.

Results and discussion

In addressing how variations in the protein expression rates affect the sporulation efficiency we will look at the effect of parallel changes in all protein expression rates as well as at the effects of independent changes that skew the ratios of protein concentrations. As the standard, 'wild-type' protein expression rates, we use 6×10^{-9} M s^{-1} for SpoIIAA and SpoIIAB dimers and 2×10^{-9} M s^{-1} for σ^F and SpoIIE (Iber *et al*, 2006). After 120 min of protein expression, the septum forms and SpoIIE

accumulates on both sides of this septum. This is modelled by a four-fold increase in the concentration of SpoIIE, together with its associated substrate (phosphorylated SpoIIAA) in the prespore. As before, we define a successful sporulation event by the requirement that before septation the concentration of σ^F. RNA polymerase holoenzyme does not exceed 0.4 μM, whereas after septation the concentration exceeds 1 μM (Iber *et al*, 2006).

If the protein expression rates are all varied in parallel, that is by a common factor as denoted on the horizontal axis in Figure 2B, we find that the predicted sporulation efficiency is not affected as long as a minimal expression rate is kept to provide sufficient σ^F for binding to the RNA polymerase (Figure 2B, grey lines). If the expression of SpoIIE is kept constant (in order to reflect that this protein is transcribed from a different locus and may therefore vary independently), then an independent 2.5-fold increase in the other sporulation proteins can still be tolerated before the relative activity of the phosphatase becomes too weak (Figure 2B, black lines). An even higher independent increase in the expression of the *spoIIA* genes can be tolerated if we assume that the expression of the *spoIIA* and *spoIIE* genes is at least weakly correlated such that a large increase in the expression of the *spoIIA* genes is accompanied by a small increase in the expression of the *spoIIE* genes (Figure 2C). Such a correlation is not unexpected, considering that variations in gene expression are the result of both intrinsic and extrinsic noise. The latter, which reflects cell-to-cell variation in the concentration of other molecular species such as the RNA polymerase, will affect all genes similarly. We can conclude that the independent regulation of the *spoIIA* and *spoIIE* genes is unlikely to generate a major risk of failed sporulation. Separation of the *spoIIA* and *spoIIE* genes on the bacterial chromosome, on the other hand, has benefits because it ensures that, upon septation, each compartment retains one copy of *spoIIE* while initially (for the first 10–15 min) two copies of *spoIIA* are in the mother cell but none in the prespore (Frandsen *et al*, 1999). This initial transient genetic imbalance may protect the mother cell from a relative increase *of spoIIE* to *spoIIA* gene products (Iber, 2006).

If the expression levels of the genes in the *spoIIA* operon are varied independently of each other, the tolerance of the network to variations in gene expression drops substantially. In particular, if expression of SpoIIAB and SpoIIAA is no longer co-regulated, the network is sensitive to rather small changes (Figure 2D, grey lines and circles). Thus, if the SpoIIAA expression rate remains fixed and the SpoIIAB expression rate increases by 60% (corresponding to the factor 1.6 on the horizontal axis in Figure 2D), then sporulation is predicted to fail; 60% variation from the mean is a noise level observed in bacterial (*Escherichia coli*) expression systems (Elowitz *et al*, 2002). On the other hand, if expression of SpoIIAA and SpoIIAB remains co-regulated but σ^F expression is regulated independently (Figure 2D, black lines), the network is rather robust to variations in gene expression as long as the expression of SpoIIAB is increased more than the expression of σ^F and the overall σ^F concentration remains high enough to form micromolar concentrations of the holoenzyme. The transcriptional coupling together with a strong translational coupling of SpoIIAA and SpoIIAB therefore substantially increases the robustness of the network to fluctuations in gene expression. Stochastic variations in the relative rate of σ^F translation, on the other hand, are not as detrimental as long as the translation efficiency for σ^F is lower than for SpoIIAA and SpoIIAB, as can be achieved by a weaker ribosomal binding site and the resulting (stochastic) dissociation of ribosomes. An advantage of preferential dissociation of the ribosomes before translating the gene for σ^F is that the bacterium saves the energy that would otherwise be required to translate, and subsequently degrade, unnecessary (harmful) copies of σ^F. Considering that σ^F comprises 255 amino acids and linkage of each amino acid requires the equivalent of four ATPs, the energy by not translating and degrading 10 μM σ^F corresponds to more than 10 mM ATP, which is a considerable amount

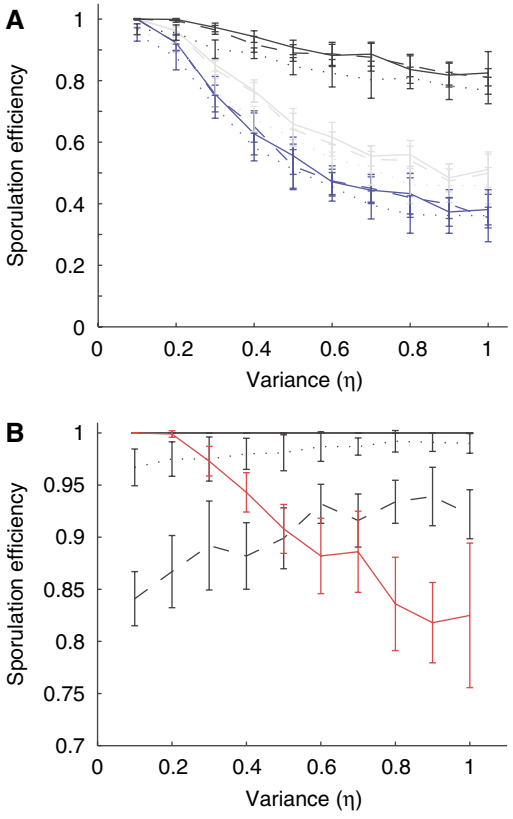

Figure 3 The impact of stochastic variation in gene expression on sporulation efficiency. (**A**) The fraction of successful sporulation events (as defined in the text) dependent on the variance in gene expression if expression of the *spoIIA* genes is either coupled (black lines), the expression of SpoIIAB and σ^F is coupled (grey lines), or the expression of SpoIIAA and σ^F is coupled (blue lines). SpoIIE is expressed throughout at the standard rate of 2×10^{-9} M s^{-1}. The broken lines show the effect of an additional independent normal variation in the rate of σ^F expression with η_S=0.1 (dashed lines) or η_S=0.3 (dotted lines) from the coupled rates. If σ^F is one of the coupled rates, then σ^F expression is varied both together with its coupling partner and additionally independently to reflect the additive levels of noise acting at the initiation of translation and the re-initiation/dissociation step. (**B**) The fraction of successful sporulation events (as defined in the text) dependent on the variance in gene expression if expression of the *spoIIA* and *spoIIE* genes is coupled (to assess the benefits of correlated expression), and an additional noise term η_E is added to the expression *of spoIIE* with η_E=0.1 (black continuous line), η_E=0.3 (dotted line), or η_E=0.6 (dashed line); η_E assesses the effects of independent promoters and spatial heterogeneity in the concentration of transcription and translation factors. The red line is identical to the continuous black line in panel A (noise in coupled *spoIIA* expression, SpoIIE expressed at 2×10^{-9} M s^{-1}). Mean and standard deviation are based on 10 times 100 independent runs.

considering that the bacterial ATP concentration is 1–3 mM (Jolliffe *et al*, 1981; Guffanti *et al*, 1987; Hecker *et al*, 1988) and sporulation is a response to starvation, that is energy deprivation.

In a last step, we can now quantify the impact of gene organization on sporulation efficiency, and therefore fitness. For this, we assume that the gene expression levels in the cell population follow a normal distribution with variance η around the mean value. Given the complex regulation pattern of gene expression, gene expression levels are unlikely to be distributed exactly normally. A normal distribution is, however, still likely to provide an approximation no worse than what could be obtained with a detailed model of the regulatory process in the absence of sufficient data to determine all required parameter values (Swain, 2004). Sporulation efficiency is determined as the fraction of simulation runs for which the concentration of σ^F. RNA polymerase holoenzyme does not exceed 0.4 μM before septation and exceeds one micromolar after septation (Iber *et al*, 2006). For each condition, the mean sporulation efficiency and standard deviation are calculated from 100 independent runs that are carried out 10 times. In each run, the protein expression rates were set randomly such that overall the respective distributions of the protein expression rates were obtained. Determination of the sporulation efficiency for $\eta \in [0,1]$ shows that as long as the sporulation genes are translationally coupled, even high variances hardly affect the sporulation efficiency (Figure 3A, black lines). The sporulation efficiency is even higher at high noise level, η, if *spoIIE* expression co-varies with *spoIIA* expression, at least weakly (Figure 3B). A lengthening of the transcription time (that is a delay in septation), when transcription levels are too low to generate sufficient σ^F until septation, will further increase robustness to fluctuations in the rate of protein expression. Such a dependency of the time point of septation on the protein (and in particular the SpoIIE) concentration is in agreement with experiments (Khvorova *et al*, 1998; Ben-Yehuda and Losick, 2002) and might explain the large variance in the delay between the onset of sporulation and septation that is observed under different sporulation conditions. Low levels of additional stochastic noise in σ^F expression (broken lines), as may arise because of the stochastic dissociation of ribosomes, also has rather little impact and confirms that the weak coupling of SpoIIAB and σ^F translation does not substantially reduce sporulation efficiency. If on the other hand, *spoIIAB* is removed from the operon and controlled independently by the same promoter, then the sporulation efficiency drops rapidly (Figure 3A, blue lines). This is in good quantitative agreement with experiments, which find that the sporulation efficiency drops to 40–80% of wild-type levels (Dworkin and Losick, 2001), especially when considering that $\eta \sim [0.3, 0.6]$ for these expression levels (Elowitz *et al*, 2002). If *spoIIAA* is moved instead, then the effect is reduced (J Clarkson, personal communication), as also predicted by the model (Figure 3A, grey lines).

It should be noted that this drop in sporulation efficiency has previously been accounted for by the loss of the transient genetic imbalance when *spoIIAB* is moved to a chromosomal position close to the origin of replication (Dworkin and Losick, 2001). The transient lack of SpoIIAB expression in the prespore together with accelerated degradation of unbound SpoIIAB (Pan *et al*, 2001) had been suggested to enable σ^F release (Dworkin and Losick, 2001). However, we have shown previously that the transient genetic imbalance does not affect σ^F release on the timescale on which it persists (Iber, 2006), and stochastic effects are therefore a much more likely explanation for the observed phenotype of the mutants.

We conclude from the analysis of this well-studied model system that the protection from stochastic variation in the expression rate of interacting proteins can substantially increase viability, and therefore constitutes a driving force for gene clustering and co-regulation. Although the importance of gene dosage had been recognized before (Veitia, 2002), and underexpression and overexpression of protein complex subunits in yeast had been shown to lower fitness (Papp *et al*, 2003), this study reveals that much smaller variances, as can result from stochastic effects, can already have substantial detrimental effects. The detailed analysis of the expression of the sporulation proteins therefore demonstrates the optimized character of gene regulation and suggests that co-regulation of genes serves to optimize cellular network dynamics in spite of the inherent noise in all biological processes.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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