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Stationary phase reorganisation of the *Escherichia coli* transcription machinery by Crl protein, a fine-tuner of σ^S activity and levels

Athanasios Typas, Claudia Barembruch, Alexandra Possling and Regine Hengge*

Institut für Biologie, Mikrobiologie, Freie Universität Berlin, Berlin, Germany

Upon environmental changes, bacteria reschedule gene expression by directing alternative sigma factors to core RNA polymerase (RNAP). This sigma factor switch is achieved by regulating relative amounts of alternative sigmas and by decreasing the competitiveness of the dominant housekeeping σ^{70}. Here we report that during stationary phase, the unorthodox Crl regulator supports a specific sigma factor, σ^S (RpoS), in its competition with σ^{70} for core RNAP by increasing the formation of σ^S-containing RNAP holoenzyme, Eσ^S. Consistently, Crl has a global regulatory effect in stationary phase gene expression exclusively through σ^S, that is, on σ^S-dependent genes only. Not a specific promoter motif, but σ^S availability determines the ability of Crl to exert its function, rendering it of major importance at low σ^S levels. By promoting the formation of Eσ^S, Crl also affects partitioning of σ^S between RNAP core and the proteolytic σ^S-targeting factor RssB, thereby playing a dual role in fine-tuning σ^S proteolysis. In conclusion, Crl has a key role in reorganising the *Escherichia coli* transcriptional machinery and global gene expression during entry into stationary phase.

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Introduction

Bacteria are able to massively reprogram gene expression when confronted with changes in their environment. An efficient way to accomplish this is by competition of promoter-specific sigma subunits for the RNA polymerase (RNAP) core enzyme (for review see Nyström, 2004). Control of expression, stability and/or availability of alternative sigma factors define the conditions under which an alternative sigma factor is able to substantially compete with the vegetative σ^{70} for limiting amounts of core RNAP. However, as σ^{70} is abundant throughout the growth cycle and shows the highest affinity for core RNAP *in vitro* (Jishage *et al*, 1996; Maeda *et al*, 2000), the cell obviously uses additional strategies beyond simple competition in order to ensure the switch between σ^{70} and appropriate alternative sigma factors in the RNAP holoenzyme in response to physiological stresses.

The alarmone ppGpp plays a major role in sigma factor competition for core RNAP upon entry into stationary phase (Jishage *et al*, 2002; Laurie *et al*, 2003; Magnusson *et al*, 2003; Costanzo and Ades, 2006). DksA protein was recently shown to act synergistically with ppGpp (Paul *et al*, 2004, 2005; Perederina *et al*, 2004). As rRNA transcription employs 70% of the σ^{70}-containing RNAP holoenzyme (Eσ^{70}) during exponential growth (Raffaelle *et al*, 2005), factors like DksA and ppGpp, which actively dissociate Eσ^{70} from rRNA loci upon entry into stationary phase, provide more free core RNAP for alternative sigmas (Bernardo *et al*, 2006). Furthermore, overexpression of Rsd, a protein with affinity for σ^{70} and core RNAP (Ilag *et al*, 2004), whose cellular level increases in stationary phase (Jishage and Ishihama, 1998), has similar effects as ppGpp with respect to 'holoenzyme switching' (Jishage *et al*, 2002; Laurie *et al*, 2003). Finally, 6S RNA, a conserved small RNA (Barrick *et al*, 2005; Trotochaud and Wassarman, 2005), is active in stationary phase and structurally mimics an open promoter complex that can 'fool' only Eσ^{70} to recognise it (Wassarman and Storz, 2000). Its presence ensures downregulation of activity of the housekeeping RNAP holoenzyme, thus allowing alternative RNAPs to take over (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004).

The common characteristic of these factors is that all are active upon entry into stationary phase and that their main target of action is σ^{70} effectiveness; by decreasing it, they make room for alternative sigma factors to act. However, stationary phase is mainly the territory of the master regulator for stress responses, σ^S. Eσ^S is actively engaged in the transcription of more genes than any other alternative sigma factor, with the majority of them being also activated in stationary phase (Weber *et al*, 2005). Despite the strong increase in its protein levels upon entering stationary phase (Hengge-Aronis, 2002), σ^S only reaches about one-third of the σ^{70} levels under these conditions (Jishage *et al*, 1996) and exhibits the lowest affinity for core RNAP of all sigma factors *in vitro* (Maeda *et al*, 2000; Colland *et al*, 2002). Therefore, we reasoned that apart from factors that decrease σ^{70} effectiveness in stationary phase and thereby give a collective advantage to all alternative sigmas, there should also be mechanisms dedicated to specifically increase the performance of σ^S and thereby allow Eσ^S to gain its dominant role in stationary phase and several other stress conditions.

Crl protein was initially identified as an activator of genes for curli fimbriae formation (Arnqvist *et al*, 1992). Later, its role was extended to that of an auxiliary factor for Eσ^S activity at certain genes (Pratt and Silhavy, 1998). Recently, Crl was shown to bind specifically to free σ^S, and proposed to

*Corresponding author. Institut für Biologie, Mikrobiologie, Freie Universität Berlin, Königin-Luise-Str. 12-16, 14195 Berlin, Germany. Tel.: +49 30 838 53119; Fax: +49 30 838 53118; E-mail: rhenggea@zedat.fu-berlin.de

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increase the affinity of $E\sigma^S$ for certain promoters at low temperatures (30°C; Bougdour *et al*, 2004). In this study, we show that Crl positively regulates a large subset of σ^S-dependent genes that do not share a common promoter motif, and its action strongly depends on σ^S availability. In *in vitro* transcription assays, Crl aids σ^S-dependent transcription, especially when σ^S is competing with σ^{70} for limiting amounts of core RNAP. Consistently, during entry into stationary phase, crl^- mutant cells possess relatively lower levels of $E\sigma^S$, but enhanced amounts of $E\sigma^{70}$. Owing to its role in controlling the partitioning of σ^S between RNAP core and the proteolytic targeting factor for σ^S, RssB, Crl also plays a complex role in controlling σ^S levels. We conclude that the common basis of all these effects is the ability of Crl to specifically aid σ^S in sigma factor competition for core RNAP during stationary phase.

Results

The Crl regulon

As existing studies had monitored the role of Crl for a limited number of genes known to be controlled by σ^S, we evaluated its global function in transcriptional regulation in *E. coli* by genome-wide transcriptional profiling. Using similar conditions as for microarray analyses previously conducted in our laboratory for the σ^S regulon (Weber *et al*, 2005) allowed us to directly compare the results. The *E. coli* K12 strain MC4100 and its isogenic *crl*::*cat* mutant were grown in rich medium at 30°C, as the stationary phase-induced curli genes are only expressed at such reduced temperatures, which had also previously been suggested to play a significant role in Crl activity (Bougdour *et al*, 2004). Total RNA was extracted at an $OD_{578\,nm}$ of 4.0 (i.e. during entry into stationary phase) and further processed for genome-wide microarray analysis (see Materials and methods for details). Genes with expression ratios in MC4100 and its *crl* mutant derivative of >2-fold or <0.5-fold (average of three independent experiments) were considered relevant and are presented in Table I. The results indicated that all of these genes were either part of the known σ^S regulon at 37°C (denoted by an asterisk in Table I; Weber *et al*, 2005) or were shown to be expressed under the control of σ^S only at lower temperatures (denoted by an asterisk in parentheses in Table I; H Weber and R Hengge, unpublished microarray results). In addition, all of the genes positively controlled by Crl showed lower ratios of Crl dependency than previously observed ratios of σ^S dependency, consistent with the assumption that Crl is not essential for but modulates the activity of σ^S, and the effects of the latter are epistatic to those

Table I The Crl regulon (30°C)[a]

Gene name	ID	Average of ratio of medians	Member of the RpoS regulon	Function
Crl	b0240	139.7		Regulatory protein for curli, transcriptional regulator
psiF	b0384	2.2	*	Pho regulon member, requiring PhoRB system
ybaY	b0453	2.2	*	Glycoprotein/polysaccharide metabolism
allR	b0506	2.2	(*)	AllR transcriptional regulator
ybgS	b0753	2.5	*	Putative homeobox protein
ycaC	b0897	2.4	*	Putative cysteine hydrolase
ycdF	b1005	3.0	*	Hypothetical protein
csgB	b1041	2.9	(*)	Curlin, minor subunit precursor
csgA	b1042	3.5	(*)	Curlin, major subunit
csgC	b1043	2.1	(*)	Putative curli production protein
ymdA	b1044	2.0	(*)	Conserved hypothetical protein
ymfE	b1138	2.4	(*)	Hypothetical protein
narU	b1469	2.3	*	MFS nitrite transporter
gadC	b1492	3.1	*	Putative glutamate:gamma-aminobutyric acid antiporter (APC family)
gadB	b1493	3.6	*	Glutamate decarboxylase B subunit
yeaH	b1784	2.4	*	Conserved hypothetical protein
ybeV	b1836	2.4	*	Stimulates the ATPase activity of Hsc62, possibly component of a new Hsp70 chaperone system
luxS	b2687	2.5	*	Quorum sensing, autoinducer II synthase
tdcC	b3116	2.3	(*)	TdcC threonine STP transporter
bfr	b3336	2.2	*	Bacterioferritin monomer
hdeB	b3509	2.3	*	10K-L protein, related to acid resistance protein of *Shigella flexneri*
hdeA	b3510	2.3	*	Acid-resistance protein, possible chaperone
hdeD	b3511	2.4	*	Protein involved in acid resistance
gadE	b3512	3.2	*	Transcriptional regulator, activates glutamate decarhboxylase-dependent acid resistance
gadW	b3515	2.4	*	Transcriptional regulator (AraC/XylS family)
gadA	b3517	3.6	*	Glutamate decarboxylase A subunit
yhjR	b3535	2.1	*	Hypothetical protein
yiaG	b3555	2.8	*	Putative transcriptional regulator
yjbJ	b4045	2.5	(*)	Highly abundant nonessential protein
ydhY	b1674	0.4		Putative oxidoreductase, Fe-S subunit
iadA	b4328	0.5		Subunit ofisoaspartyl dipeptidase
yjiG	b4329	0.5		Putative membrane protein

[a]Genes are ordered according to their chromosomal position, which is reflected in their b-number. The complete data sets can be found at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-720. Genes associated with an asterisk are part of the σ^S regulon at 37°C (Weber *et al*, 2005), whereas those associated with an asterisk in parentheses are part of the σ^S regulon at lower temperatures, that is, 28°C (H Weber and R Hengge , unpublished microarray results).

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of the former (Pratt and Silhavy, 1998). It should also be noted that many genes known to be under the control of σ^S are not listed in Table I, but exhibited ratios just below the cutoff mentioned above (data not shown). Thus, among the genes (approximately 55) with expression ratios between 1.60 and 2, the vast majority (∼90%) was also under the control of σ^S (data not shown). Furthermore, our microarray analysis identified the *csgBA* operon as part of the Crl regulon and could also detect the previously reported (Pratt and Silhavy, 1998; Robbe-Saule *et al*, 2006) relatively modest effects of Crl on the expression of *bolA*, *katE* and *csgD* (data not shown; all three genes exhibited ratios of 1.5–2 at both 30 and 37°C).

To further confirm that the role of Crl in global gene expression in stationary phase is mediated exclusively by σ^S, we extended our genome-wide transcriptional profiling approach to an *rpoS*$^-$ background. Here, Crl did not control any significant regulon (it only slightly repressed the expression of the *paa* operon, responsible for phenylacetic acid degradation; Ferrandez *et al*, 1998; see Supplementary Figure S1 and Supplementary Table S1).

As some effects of Crl on σ^S-dependent gene expression were previously observed at 37°C (Pratt and Silhavy, 1998), we performed an analogous microarray analysis at this temperature and found that Crl exerted effects similar to those at 30°C on an overlapping subset of σ^S-dependent genes (data not shown). Thus, Crl effects seemed to be temperature independent, in contrast to a recent report that proposed a thermosensitive function of Crl in regulating σ^S activity (Bougdour *et al*, 2004). Assaying Crl protein levels at different stages of growth revealed only slightly higher expression of Crl at 30°C than at 37°C (∼70% more Crl at 30°C), and weak stationary-phase induction at both temperatures (∼2-fold; Supplementary Figure S2). In a study published during the revision of this manuscript, it was shown that a similar accumulation of Crl takes place in *Salmonella typhimurium* during growth at 28°C (Robbe-Saule *et al*, 2006). To summarise, we propose that Crl modulates the activity of σ^S in stationary phase and thereby plays a global role in the control of σ^S-dependent genes in a temperature-independent manner.

Crl acts on σ^S activity by affecting sigma factor competition for core RNAP

The moderate and rather uniform differences in the expression ratios observed for all genes controlled by Crl suggested that Crl might not have specific sequence requirements for exerting its action. Indeed when aligning the known promoters of the positively regulated genes shown in Table I, no sequence pattern specific for this group of σ^S-dependent genes emerged (data not shown). In addition, assaying synthetic promoters carrying different *cis* features known to contribute to σ^S promoter selectivity (Typas *et al*, 2007) revealed no correlation between those *cis* elements and Crl dependency of the promoter (Supplementary Figure S3); all the promoters exhibited a similar reduction of expression in the *crl* mutant strain. The fact that Crl could activate σ^S-dependent synthetic promoters that do not require any additional transcription factors for their maximal expression also excluded the possibility that Crl facilitates Eσ^S function by optimising its cooperation with *trans*-acting factors (Bougdour *et al*, 2004). All these data suggest that Crl does not aid Eσ^S in the *specific* recognition of promoters (see also Discussion).

When monitoring the role of Crl in the expression of various synthetic and natural promoters *in vivo*, we noticed an inverse correlation between σ^S levels and the ability of Crl to activate σ^S-dependent promoters. When σ^S levels were kept relatively low by using various combinations of genetic backgrounds and/or growth conditions, Crl had a more pronounced role in the expression of these σ^S-controlled promoters (Supplementary Figure S4). On the contrary, when the intracellular σ^S concentration was relatively high, Crl did not stimulate σ^S-dependent promoter activity. This correlation of low intracellular σ^S levels with Crl function is also consistent with Crl exerting its role before promoter recognition by Eσ^S.

To clarify this role at the molecular level, we directly monitored the effect of Crl on σ^S-dependent transcription *in vitro*. A synthetic promoter with strong σ^S preference was chosen (synp9) and single-round and multiround transcription assays were performed (Figure 1). Using an excess of sigma-saturated RNAP over the supercoiled DNA template (20:1) enabled us to distinguish between initial recruitment of RNAP to the promoter and later stages of transcription (under such conditions, the multiround transcription assays are more sensitive in detecting effects on initial recruitment). In both assays, preincubation of increasing amounts of Crl with σ^S before RNAP holoenzyme reconstitution had only a marginal effect in promoter utilisation by Eσ^S alone. Also Eσ^{70}-derived transcription was not influenced by the addition of the maximal amount of Crl (10-fold more than RNAP; note that these ratios of Crl/RNAP are within the physiological range; see also Discussion), which was expected as Crl does not interact with Eσ^{70} (Bougdour *et al*, 2004). Thus, Crl does not seem to significantly affect the ability of Eσ^S *per se* to recognise a promoter sequence and initiate transcription, at least when core RNAP is saturated with sigma (five-fold excess of sigma).

Using the same concept as for the single-round *in vitro* transcription assays above, we established an *in vitro* competition assay for the two sigma factors. Different molecular ratios of σ^{70} and σ^S (and each sigma factor on its own for

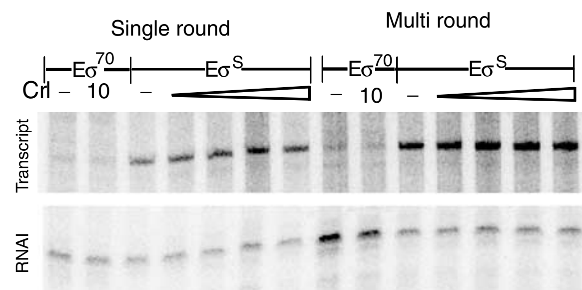

Figure 1 Crl does not alter significantly *in vitro* transcription mediated by Eσ^S alone. Single-round and multi-round *in vitro* transcription assays using synthetic promoter 9 (synp9, with an *rrnB* (T_1,T_2) terminator cloned in the place of *lacZ*; see Materials and methods) were performed at 30°C. RNAP reconstituted with a five-fold excess of either σ^s or σ^{70}, and increasing amounts of Crl (0.5-, 1-, 2- and 10-fold more than core RNAP; only 10-fold more for the experiment with σ^{70}), were used to transcribe synp9 (upper panel). The RNA I transcript encoded by the vector (lower panel, obtained from the same gel) was used for normalisation in the quantification of the transcripts (data not shown).

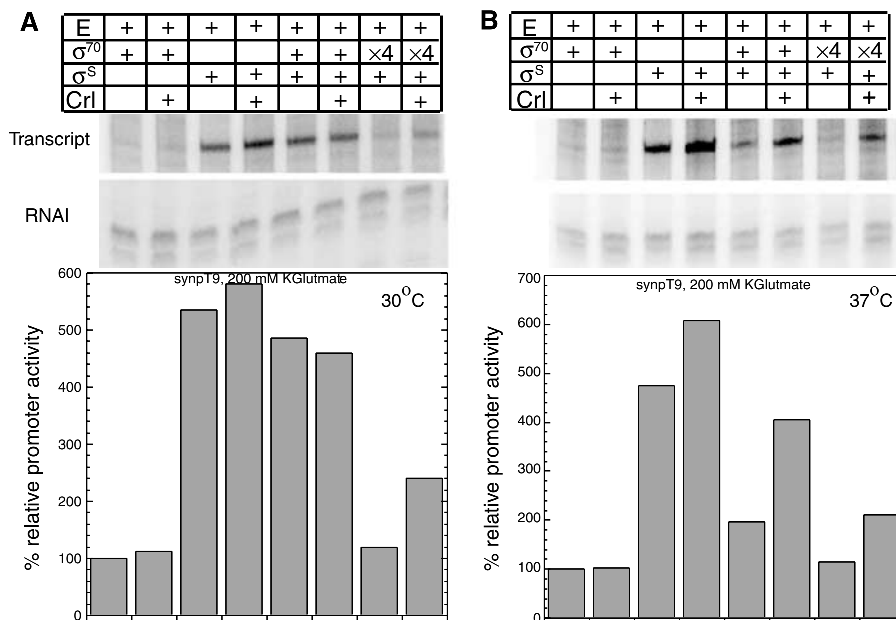

Figure 2 Crl shifts sigma factor competition for core RNAP in favour of σ^S. *In vitro* sigma competition and single-round transcription assays were performed in the presence of 200 mM potassium glutamate at different temperatures: (**A**) 30°C and (**B**) 37°C. Purified σ^s and/or σ^{70} were added in equimolar amounts to core RNAP (σ^{70} also in four-fold excess where stated in the figure) for holoenzyme reconstitution, in the presence or absence of excess Crl (10-fold more than σ^s and core RNAP; note that the sigma factors were preincubated with Crl for 10 min at 30°C before addition to core RNAP). The mixture was used to transcribe synp9 as in Figure 1 (upper panel). The RNA I transcript (lower panel), also encoded by the template plasmid, was used for normalising quantification of synp9-derived transcripts (presented below the corresponding gel). For each gel, the amount of $E\sigma^{70}$-derived transcript was set to 100%.

control) were preincubated with Crl or buffer alone and then added to limiting amounts of core RNAP for reconstitution. The resulting RNAP holoenzyme mixtures were used to transcribe the synthetic promoter synp9. As shown in Figure 2, Crl shifts the competition balance in favour of σ^S, that is, the presence of Crl can counteract the reduction in synp9 expression caused by the presence of σ^{70}. The supportive effect of Crl on $E\sigma^S$ and its output was especially pronounced when competition for core RNAP was harsher for σ^S, for example, when σ^{70} was present in four-fold excess, whereas in the absence of Crl, synp9 expression was as low as with σ^{70} alone (i.e. no $E\sigma^S$ is present at all; Figure 2A and B). In addition, the effect of Crl was at least as pronounced at 37°C as at 30°C (Figure 2B). Again, in the absence of competition, that is, with either σ^S or σ^{70} alone, Crl had no or only minor effects (Figure 2A and B). It should be noted that the positive effect of Crl on $E\sigma^S$-derived transcription—upon σ factor competition—might seem relatively small ($\sim$2-fold in most cases), but this reflects the fact that the synp9 promoter retains a basal $E\sigma^{70}$-dependent transcription that can reach up to 20% of that of $E\sigma^S$. In any case, the main difference between the 'simple' and the competition *in vitro* transcription assays was that in the latter case core RNAP was not saturated with σ^S alone, and therefore the effects of Crl on $E\sigma^S$ holoenzyme formation in a sigma factor competition situation could be monitored.

In order to test whether Crl also has an impact on sigma factor competition *in vivo*, thereby facilitating the formation of $E\sigma^S$, we measured the relative *in vivo* amounts of σ^S and σ^{70} bound to core RNAP in wild-type and *crl* mutant strains during the onset of stationary phase. After harvesting the cells, whole-cell extracts were fractionated by gel filtration and the amounts of sigma factors (σ^S and σ^{70}), Crl and the β' RNAP subunit in each fraction were quantified by immunoblot analysis. Both sigmas were found to elute in two separate sets of the fractions collected (Figure 3). σ^S coeluted with the β' subunit, as part of the RNAP ($E\sigma^S$), in fractions A1–A3, whereas fractions A7–A9 contained σ^S in its free form. On the other hand, $E\sigma^{70}$ eluted mostly in fractions A2–A4, whereas free σ^{70} was found in fractions A7–A9. The fractionation pattern was also verified by experiments using purified free σ^S and σ^{70} or their reconstituted RNAP forms (data not shown). It is apparent that a significantly larger fraction of σ^S was bound to RNAP in the wild-type strain compared with the *crl* mutant strain, whereas the opposite tendency can be observed for σ^{70} (Figure 3). This result verified that Crl supports σ^S in its competition with σ^{70} for core RNAP *in vivo*, and stimulates the formation of $E\sigma^S$ at the expense of formation of the vegetative holoenzyme, $E\sigma^{70}$.

In conclusion, both *in vitro* and *in vivo* data shown above indicate that Crl plays a role in σ^S-dependent transcription by affecting sigma factor competition for limiting amounts of core RNAP in favour of σ^S, and that this role becomes particularly important at low σ^S levels, that is, before σ^S reaches its maximal level in stationary phase.

Interplay between Crl, RNAP and RssB: Crl also regulates intracellular σ^S levels by affecting σ^S proteolysis

In parallel with enhancing σ^S activity in stationary phase, Crl also seems to reduce intracellular σ^S levels (Pratt and Silhavy, 1998). Although at first glance this may seem paradoxical, this means that Crl allows σ^S to be effective at lower levels, such that high levels of σ^S are not needed. We observed that this reducing effect of Crl on σ^S concentration is apparent at all stages of growth both at 30 and 37°C, but it is eliminated

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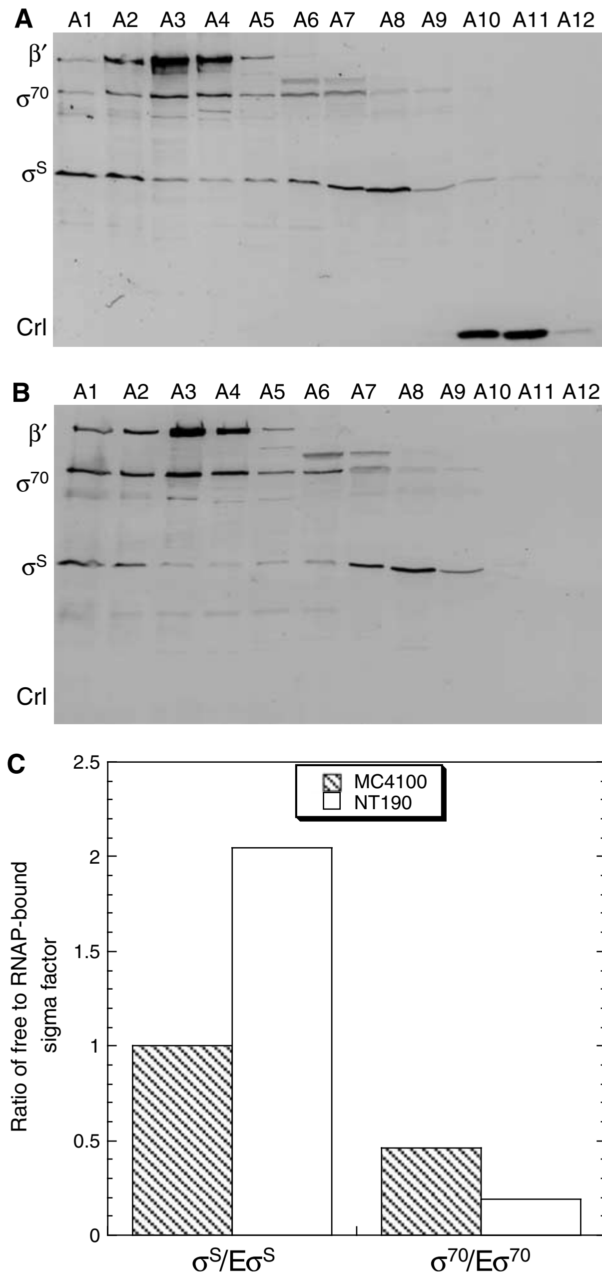

Figure 3 *In vivo*, Crl supports $E\sigma^S$ formation in stationary phase at the expense of $E\sigma^{70}$. Wild-type MC4100 (**A**) and its crl^- mutant (**B**; NT190) were grown in LB at 30°C until the onset of stationary phase ($OD_{578\,nm}=3$; cells growing in rich medium have a wide range of time duration during which they do not completely cease growing, but grow considerably slower: we denote this time as the onset/entry into stationary phase). Cells were harvested and lysed in order to obtain whole-cell extracts, which were further fractionated by gel filtration. Fractions were analysed by SDS–PAGE and visualised by immunoblots using monoclonal antibodies against the σ^S, σ^{70} and β′ subunits of RNAP and a polyclonal antibody against Crl. (**C**) Results of the quantification performed for the two Western blots using the IMAGE GAUGE software. The ratio of free to bound sigma factor was calculated for both σ^S and σ^{70} in the different genetic backgrounds (bound σ^S: in fractions A1–A3; free σ^S: A7–A9; bound σ^{70}: A2–A4; and free σ^{70}: A6–A8). The experiments were performed twice with reproducible results.

in an $rssB^-$ background (Figure 4A and data not shown). RssB, which is the target of complex signal transduction pathways, shows phosphorylation-dependent affinity for σ^S

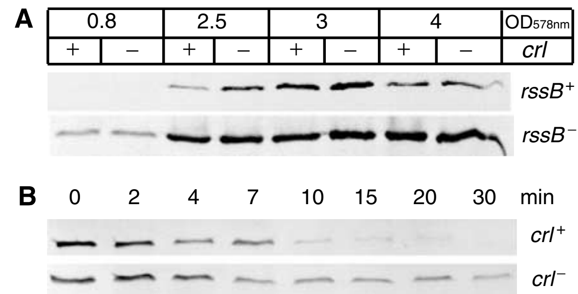

Figure 4 Crl stimulates σ^S degradation *in vivo*. (**A**) Increased σ^S levels in the crl^- mutant are observed only in the presence of RssB. Immunoblots depict cellular σ^S levels at different stages of growth at 30°C, in the presence or absence of Crl and in $rssB^+$ or *rssB*-deficient backgrounds. (**B**) Cellular σ^S levels were monitored in the presence or absence of Crl at 30°C by immunoblot analysis after the addition of bacteriostatic amounts of chroramphenicol at an OD_{578} of 3.0 (identical results were obtained also after addition of spectinomycin). The quantification of σ^S degradation is shown in Supplementary Figure S5.

and serves as its targeting factor to ClpXP protease (Muffler *et al*, 1996; Pratt and Silhavy, 1996; Bouché *et al*, 1998; Becker *et al*, 1999; Klauck *et al*, 2001; Zhou *et al*, 2001; Stüdemann *et al*, 2003; Mika and Hengge, 2005). The observation that Crl exerts its effect on σ^S levels via RssB suggested that Crl influences σ^S degradation. Indeed, σ^S proteolysis (measured during entry into stationary phase) was slowed down in the *crl* mutant (Figure 4B). σ^S half-lives were approximately 4–5 and 10–12 min in crl^+ and *crl* mutant backgrounds, respectively (Supplementary Figure S5).

In the absence of strong stress signals that interfere with RssB activity, the cellular RssB level is the limiting factor in σ^S degradation. Consistently, the control of *rssB* expression by σ^S provides the system with a homeostatic feedback loop that sets the threshold for titration of RssB and therefore for the stabilisation of σ^S by certain stress conditions that rapidly and strongly induce σ^S synthesis (Pruteanu and Hengge-Aronis, 2002). Thus, we reasoned that, Crl could affect, via its effect on σ^S activity, *rssB* expression and thereby σ^S proteolysis (Supplementary Figure S6). Using a transcriptional fusion of the *rssAB* operon promoter to *lacZ*, we could verify that *rssB* behaves like other σ^S-dependent genes, that is, its expression is reduced in the absence of Crl (Figure 5). We conclude that Crl stimulates the expression of the limiting factor of σ^S proteolysis, RssB, and thereby increases σ^S degradation rates. Consequently, cellular σ^S levels are higher in the *crl* knockout strain.

On the other hand, we wondered what would happen if RssB expression was uncoupled from this σ^S/Crl control. We suspected that in such a situation, the effect of Crl on σ^S/σ^{70} competition for limiting amounts of core RNAP might be revealed: as Crl favours σ^S in this competition, more σ^S would be bound to RNAP in the presence of Crl (Figure 3), and thus be protected against proteolysis. In other words, a *crl* mutant strain would be expected to show increased σ^S proteolysis and therefore lower σ^S levels when Crl does not affect the expression of *rssB* (opposite to what is observed when *rssB* is expressed from its chromosomal locus with its natural σ^S/Crl-controlled promoter; see Figure 4)

In order to test this hypothesis, we used a moderate-copy-number plasmid with *rssB* under p_{tac} promoter control (pMP8; Pruteanu and Hengge-Aronis, 2002). RssB expression

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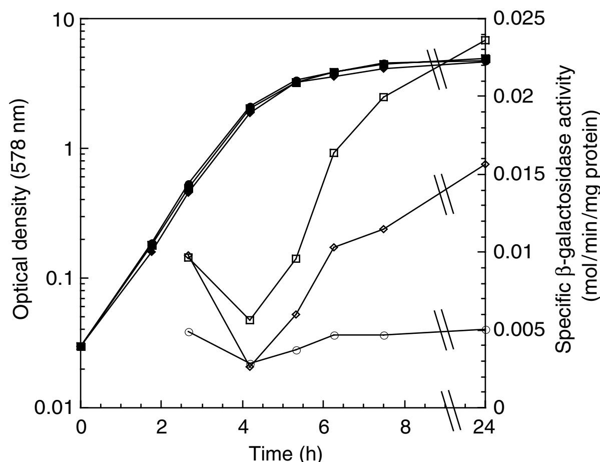

Figure 5 *rssB* expression is reduced in the *crl* mutant. Expression of a single-copy *rssAB:lacZ* operon fusion was determined in wild-type (squares), $rpoS^-$ (circles) and crl^- (diamonds) backgrounds. Cells were grown in LB medium at 30°C and optical densities and specific β-galactosidase activities were measured along the growth curve.

Figure 6 Uncoupling *rssB* expression from Crl/σ^S control results in decreased σ^S levels in the absence of Crl. In the *rssB* mutant background, RssB was expressed ectopically from pMP8 under the control of the p_{tac} promoter (no inducer present; RssB levels obtained are nevertheless slightly higher than those in the wild-type strain). An immunoblot depicting σ^S levels during different stages of growth at 30°C (o/n stands for overnight), in otherwise isogenic crl^+ and *crl* mutant backgrounds, is shown; for reference, σ^S levels at an OD_{578} of 3.0 in the wild-type strain (MC4100) are also shown (last lane).

from this plasmid is only slightly higher than that from its chromosomal wild-type gene, when no inducer is added, which leads to somewhat higher σ^S degradation rates (Pruteanu and Hengge-Aronis, 2002) and therefore lower but still detectable σ^S levels (Figure 6). As hypothesised above, introducing the *crl* mutation in this background resulted in σ^S levels that were below the limit of detection (Figure 6). This correlated perfectly with the expression of a synthetic σ^S-dependent promoter assayed in the same genetic backgrounds, which showed only residual σ^{70}-dependent expression throughout the whole growth curve in the *crl* mutant (data not shown). To summarise, when the negative feedback link between σ^S/Crl and *rssB* expression is eliminated by expressing *rssB* ectopically from a constitutive promoter, the function of Crl in favour of $E\sigma^S$ formation results in increased σ^S stability, which becomes visible as higher σ^S levels in the presence of Crl (Figure 6). This increased stability derives from σ^S being protected within the holoenzyme (Zhou *et al*, 2001).

Next, we were interested to clarify whether Crl can also directly compete with RssB for binding to σ^S, or whether, alternatively, all three proteins can form a ternary complex. Phosphorylated RssB (RssB-P) is known to strongly interact with σ^S (Becker *et al*, 1999; Zhou *et al*, 2001). Crl, on the contrary, seems to exhibit rather weak binding to σ^S (Bougdour *et al*, 2004). To test if and how Crl influences the interaction of σ^S with RssB-P, we used an established coelution protocol (Becker *et al*, 1999; Klauck *et al*, 2001) and gel filtration analysis (Supplementary Figure S7). In both cases, Crl could not compete with RssB for σ^S binding, and also no ternary complex formation was apparent.

In addition, the role of Crl in σ^S proteolysis was also assessed more directly by using *in vitro* degradation assays (Figure 7, for a more detailed version of this figure, see Supplementary Figure S8). The presence of a two-fold molecular excess of Crl over σ^S had no effect on the rate of RssB/ClpXP-dependent degradation of σ^S, that is, σ^S half-life remained the same ($\sim$15 min) in the absence or presence of Crl (Figure 7A and Supplementary Figure S8A and C). Thus, Crl on its own could not protect σ^S from being degraded. In addition, Crl itself (with an N-terminal His6 tag) was not a substrate of the ClpXP proteolytic machinery (Figure 7A and Supplementary Figure S8B). However, Crl enhanced the protection provided by core RNAP to σ^S and further slowed down σ^S proteolysis about two-fold (Figure 7B and Supplementary Figure S8D). Binding of σ^S to RNAP polymerase is known to protect σ^S from degradation (Klauck *et al*, 2001; Zhou *et al*, 2001), and even sub-stoichiometric amounts of core RNAP (core RNAP:σ^S = 1:7) were shown here (Figure 7B and Supplementary Figure S8D) to substantially stabilise σ^S (increasing core RNAP to a ratio of 1:5 slowed down σ^S proteolysis even more dramatically, leading to a σ^S half-life of $>$60 min; data not shown). This stabilisation of σ^S was further enhanced by the presence of Crl (Figure 7B and Supplementary Figure S8D), in concert with the role of Crl in increasing the formation of $E\sigma^S$, and thereby, protecting σ^S from degradation. In conclusion, Crl can affect the partitioning of σ^S between RssB and RNAP in favour of the latter and thus rescue σ^S from proteolysis, both *in vivo* and *in vitro*.

Discussion

Increased formation of $E\sigma^S$ is the basis of the opposing effects of Crl on σ^S levels and activity

In this study, we demonstrate that Crl is a global regulatory factor in stationary phase, which functions through σ^S. Crl exerts multiple effects on σ^S activity and levels, and we present evidence that the common basis of all these effects is the ability of Crl to influence sigma factor competition for core RNAP in favour of σ^S and thereby facilitate the formation of $E\sigma^S$.

Crl affects σ^S levels and activity in an opposite manner. This apparently paradoxical behaviour leads to less, but more active σ^S, when Crl is present. On the one hand, by influencing the partitioning of core RNAP between the competing sigma factors, σ^{70} and σ^S, in favour of the latter, Crl positively affects σ^S-dependent expression of RssB, and thereby stimulates σ^S proteolysis and reduces σ^S levels (Figures 4 and 5). On the other hand, by 'driving' σ^S into RNAP, Crl also affects the partitioning of σ^S between RssB and RNAP in favour of the latter; as a consequence, Crl can protect σ^S against degradation (as observed both *in vivo* and *in vitro*; Figures 6 and 7 and Supplementary Figure S8), thus increasing σ^S levels. In the wild-type strain, this proteolysis-protective effect is masked by the dominant first effect, that is, Crl

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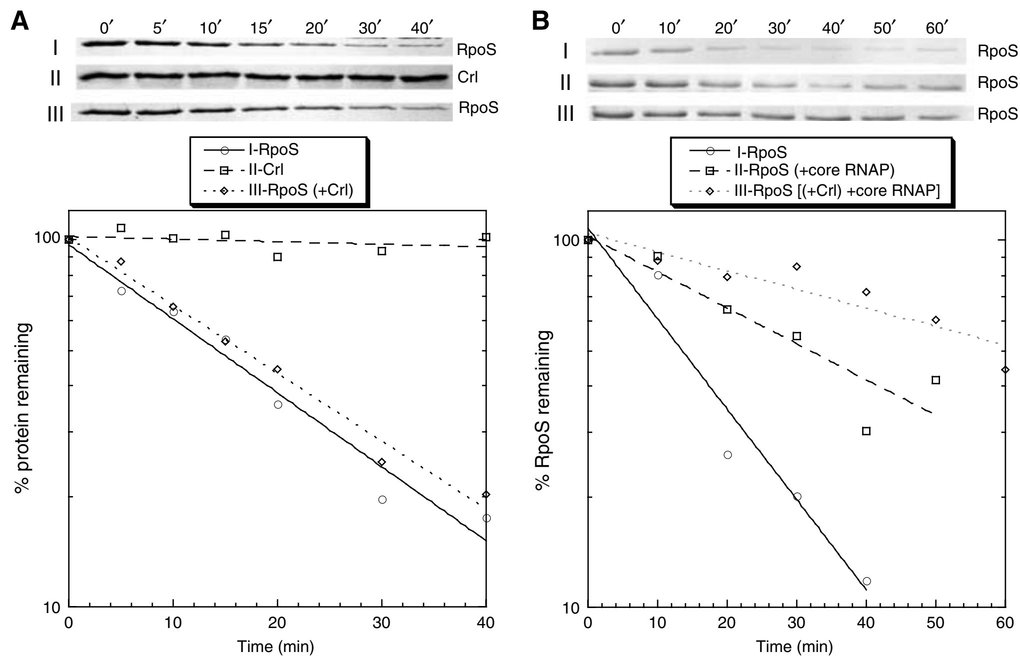

Figure 7 Crl rescues σ^S from RssB/ClpXP-mediated degradation *in vitro*, but only in the presence of core RNAP. *In vitro* degradation of σ^S (**A**, **I** and **III** and **B**, **I–III**) was assayed in reaction mixtures containing 2 μM σ^S, 0.2 μM RssB, 0.2 μM reconstituted ClpXP, 5 mM ATP, 10 mM acetyl phosphate and where applicable 4 μM Crl (A, II and B, III), 0,29 μM core RNAP (B, II, III) or 2 μM BSA (B, I). In panel A, II, a control *in vitro* degradation assay for Crl alone is presented, using the same conditions and reagents as for σ^S (note that Crl was also stable in an *in vitro* degradation assay in which RssB was omitted; data not shown). For more experimental details, see Materials and methods, and for a more complete picture of the stained SDS–PAGE gels, see Supplementary Figure S8. Below the *in vitro* degradation assays, densitometric quantifications of the data are depicted. The intensity of bands representing σ^s (or Crl in panel A, II) was calculated relative to the intensity of bands representing a stable protein that was always present in the assay, that is, ClpX. Each experiment was repeated two or three times with highly reproducible results; a representative of those experiments is shown here. The half-life of σ^S is 14.5 min ($\pm$1.2) in the absence of Crl, 15 min ($\pm$2) in its presence (two-fold excess), 34 min ($\pm$3) in the presence of sub-stoichiometric amounts of core RNAP (1:7 molecular ratio) and 57.5 min ($\pm$3.5) in the presence of both Crl and core RNAP. Note that the presence of BSA (in amounts similar to those of Crl) in the mixture did not influence the degradation rates of σ^S.

stimulating σ^S proteolysis via RssB. Therefore, in the presence of Crl, overall σ^S levels are decreased. Taking into consideration that the role of Crl is more profound when σ^S levels are low (Supplementary Figure S4 and Robbe-Saule *et al*, 2006), Crl in fact seems to generate the conditions where it gains a significant physiological role. In other words, the opposing effects of Crl on σ^S levels and activity are both necessary for Crl to be able to fine-tune the σ^S output. That Crl indeed significantly affects the *in vivo* output of σ^S is also supported by the finding that a *crl* mutation confers a selective advantage in long-term stab cultures of *E. coli* (Faure *et al*, 2004).

Crl supports Eσ^S formation and thereby stimulates σ^S-dependent gene expression in a way that is independent of a specific promoter motif. Why then does it influence only a specific subset of σ^S-controlled genes in our genome-wide analysis (Table I)? First, many more σ^S-controlled genes seem to be affected by Crl, but their expression ratios are just below the threshold we have set here (among them also *rssB*). Second, as Crl functions by directly aiding σ^S in its competition with σ^{70} and therefore increasing Eσ^S levels, it would be expected to more strongly influence (i) weak promoters, that is, those with a relatively low affinity for Eσ^S (Grigorova *et al*, 2006) or (ii) genes the expression of which is σ^S-controlled at multiple stages, for example, in feedforward loops such as for *gadA/BC* and *csgBA* (Weber *et al*, 2005, 2006). These genes or operons exhibit the strongest regulation by Crl (Table I and Supplementary Figure S4D) and at the same time are extremely sensitive to variations in σ^S levels and activity (A Typas and R Hengge, unpublished data).

Molecular mechanism of Crl action in competition of σ^S and σ^{70} for core RNAP

How exactly does Crl support σ^S-dependent transcription? Our data indicate that the primary effect of Crl is to significantly bias transcription in favour of Eσ^S under conditions where σ^S has to compete with the predominant σ^{70} for binding to limiting amounts of core RNAP (Figure 2). In the presence of σ^{70}, few if any σ^S-containing RNAP is formed without Crl (Figure 2A and B). In the presence of Crl, however, this disadvantage of σ^S to compete with σ^{70} for binding to core RNAP is alleviated, presumably because Crl directly facilitates Eσ^S formation. Other lines of evidence also verify that Crl promotes Eσ^S formation. In the presence of Crl, the Eσ^S holoenzyme is increased at the expense of Eσ^{70} in stationary phase cells (Figure 3), and Crl can protect σ^S against proteolysis *in vivo* and *in vitro*, but only when it can usher σ^S to RNAP (Figures 6, 7 and Supplementary Figures S7 and S8).

Consistent with our results, a publication submitted while our study was under review also proposed that Crl facilitates the formation not only of Eσ^S, but also other holoenzymes to a more modest degree (Gaal *et al*, 2006). Although only preliminary *in vitro* experiments were presented there (using a system with non-physiologically high amounts of Crl), their data together with our *in vitro* and *in vivo* experiments provide strong evidence that the main mechanism of

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action of Crl on Eσ^S activity is at the initial step of holoenzyme formation. Whether the reported modest effects of Crl on the formation of Eσ^{32} (Gaal *et al*, 2006) are physiologically relevant remains to be further tested.

In addition to its major effect on Eσ^S formation under sigma competition conditions, Crl also seems to have a minor positive influence on *in vitro* transcription mediated by Eσ^S alone (Figure 1), which seems to be identical in the single- and multiround transcription assays (which differ in their ability to sense changes in the initial recruitment of the holoenzyme to the promoter). Thus, these data indicate that Crl does not aid Eσ^S in being recruited by its cognate promoters, but may have some minor effect in steps following Eσ^S recruitment to the promoter (open complex formation, abortive initiation) or in the kinetics of the various steps of transcriptional initiation (which are difficult to detect in *in vitro* transcription assays). Consistently, during the revision of this paper, Crl was reported to exert a subtle activation in σ^S-dependent transcription *in vitro*, mainly due to an increase in open complex formation (Robbe-Saule *et al*, 2006). The $\sim$2-fold effects reported in these *in vitro* transcription assays are comparable to our observations with Eσ^S alone (Figures 1 and 2, about 50% activation in the presence of Crl), if we do not normalise against the RNA I transcript (assuming that Crl enhances the performance of Eσ^S with both our test promoter and the RNA I promoter).

The finding that Crl binds to free σ^S, but RssB 'chases' Crl out of a complex with σ^S (Supplementary Figure S7B) has interesting implications for the mechanism of action of Crl. First, RssB binding results in a structural change in σ^S, which exposes an otherwise cryptic binding site for the ClpX hexameric ring (Stüdemann *et al*, 2003) and in parallel may also reduce affinity for Crl. Alternatively, the binding region on σ^S for RssB (i.e. region 2.5/3.0) and that for Crl may partially overlap. However, we consider the latter possibility less likely as K173 in region 2.5/3.0 of σ^S in the RNAP holoenzyme provides an important contact to the promoter (to a C at position −13; Becker and Hengge-Aronis, 2001), which should not be occluded by binding of Crl. In addition, Crl can still aid σ^S-dependent transcription even with the K173E variant of σ^S (data not shown), which is defective for RssB binding (Becker *et al*, 1999). Moreover, taking into consideration that Crl binds only weakly to σ^S and that it is not completely clear if and how it binds to Eσ^S (see also Figure 3, where most Crl is found to be free and not as part of the Eσ^S complex *in vivo*), it seems possible that Crl binds only transiently to σ^S and either imposes a lasting modification or, by acting in a chaperone-like manner, confers a conformational change to σ^S; both mechanisms could increase the affinity of σ^S to core RNAP.

The regulation of Crl and its role in cellular physiology

Crl, σ^S, core RNAP and RssB are components of a complex protein–protein interaction network, whose proper functioning in the control of σ^S activity and degradation exquisitely depends on the relative affinities and actual cellular levels of all components involved. This requires complex fine-tuning of the regulation of at least Crl, σ^S and RssB, whose cellular levels have to be adjusted to each other in adequate ratios. For σ^S and RssB, this is achieved by σ^S control of the weak *rssB* transcription, which results in RssB being present at 10- to 20-fold lower levels than σ^S (Becker *et al*, 2000). Quantitative immunoblotting indicates that Crl in turn is present in a 5- to 10-fold excess over σ^S (our unpublished data), and similar to σ^S, exhibits increased expression during entry into stationary phase and/or at reduced temperature (Supplementary Figure S2). Crl expression, however, does not seem to be σ^S dependent, as it is not part of the σ^S regulon under various conditions tested (Weber *et al*, 2005). Moreover, like σ^S, Crl was found in the 'ClpXP-trap' and therefore may also be regulated by proteolysis (Flynn *et al*, 2003), although the N-terminally tagged protein used in this study for *in vitro* experiments was stable (Figure 7A and Supplementary Figure S8B).

Even our current limited knowledge about regulation of Crl raises interesting questions regarding its physiological role. Crl expression patterns (Supplementary Figure S2) and our microarray analysis (Table I) indicate that Crl plays a global role during entry into stationary phase. In addition, its effects are more pronounced when σ^S levels are relatively low (Supplementary Figure S4). This may result in σ^S becoming active earlier during entry into stationary phase in the presence of Crl. Does this also mean that Crl could support σ^S-dependent transcription during exponential phase? Slow but exponential growth on energy-poor carbon sources (e.g. alanine, acetate and proline) causes accumulation of σ^S and increased σ^S-dependent gene expression (Liu *et al*, 2005), suggesting that the role of Crl should be studied under such conditions. Moreover, there may be situations where intracellular σ^S levels do not significantly change, but increased expression of certain σ^S-dependent genes may take place owing to the induction of Crl. For example, the presence of external acetate in rich medium (at neutral pH; with low amounts of acetate that do not affect growth) significantly stimulates the σ^S regulon, but σ^S levels are not increased in parallel (Kirkpatrick *et al*, 2001; Polen *et al*, 2003). In addition, MqsR, a regulator that responds to autoinducer II, strongly and positively regulates *crl* expression as shown be genome-wide transcription analysis (Gonzalez Barrios *et al*, 2006), but the impact of this system on σ^S-dependent gene expression is unknown. Thus, a complete understanding of the physiological role of Crl will also require further studies of its regulation.

The major target process of Crl, that is, the competition between σ^S and σ^{70}, is the key regulatory process for the transition from exponential to stationary phase. During this transition, the cell reorganises its transcriptional machinery in a way that favours transcription by Eσ^S and other alternative RNAPs, with Eσ^S being the most prominent one. Apart from targeting its own broad regulon, Eσ^S also assumes control of housekeeping functions in stationary phase, for example, basal expression of ribosomal RNAs (Raffaelle *et al*, 2005). This holoenzyme switch is supported by the strong resemblance of the promoter consensus sequence for the two sigmas (Typas *et al*, 2007). In addition, numerous genes possess overlapping σ^S and σ^{70}-specific promoters, in order to secure continuous (but differential) expression during entry into stationary phase. Thus, Eσ^S induces the expression of a plethora of new genes and at the same time takes over the 'housekeeping' duties of the cell from Eσ^{70}. Of course, this does not mean that Eσ^{70} is dispensable or nonfunctional at this stage of growth, as it continues to express many genes important for the cell's nutritional competence. However, its significance is clearly reduced. Proteins like Crl ensure a balanced allocation of duties between the two sigmas in

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stationary phase and presumably also under other long-term stress conditions. Maintaining this balance is vital to the cell as it allows it to adjust its trade-off between self-preservation and nutritional competence according to the external milieu (King *et al*, 2004; Ferenci, 2005).

Materials and methods

Owing to space constraints and the multitude of methods used in this study, the detailed description of strains and experimental procedures has been moved to Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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