A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of σ^S (RpoS) in *E. coli*

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The general stress σ factor σ^S (RpoS) in *Escherichia coli* is controlled at the levels of transcription, translation, and proteolysis. Here we demonstrate that the phosphorylated response regulator ArcA is a direct repressor of *rpoS* transcription that binds to two sites flanking the major *rpoS* promoter, with the upstream site overlapping an activating cAMP-CRP-binding site. The histidine sensor kinase ArcB not only phosphorylates ArcA, but also the σ^S proteolytic targeting factor RssB, and thereby stimulates σ^S proteolysis. Thus, ArcB/ArcA/RssB constitute a branched “three-component system”, which coordinates *rpoS* transcription and σ^S proteolysis and thereby maintains low σ^S levels in rapidly growing cells. We suggest that the redox state of the quinones, which controls autophosphorylation of ArcB, not only monitors oxygen but also energy supply, and we show that the ArcB/ArcA/RssB system is involved in σ^S induction during entry into starvation conditions. Moreover, this induction is enhanced by a positive feedback that involves σ^S -dependent induction of ArcA, which further reduces σ^S proteolysis, probably by competing with RssB for residual phosphorylation by ArcB.

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Controlled proteolysis of key regulatory factors in response to cellular and environmental signals plays a crucial role both in eukaryotic and prokaryotic cells. One of the most prominent prokaryotic examples is the degradation of the σ^S (RpoS) subunit of RNA polymerase (RNAP) in rapidly growing *Escherichia coli* cells, which is inhibited in response to several stress conditions: e.g., starvation, hyperosmotic shift, or pH downshift (for a summary of σ^S regulation, see Hengge-Aronis 2002). This results in a rapid increase of the cellular concentration of σ^S , which competes with the vegetative σ subunit σ^{70} , and thereby induces the general stress response (Hengge-Aronis 2000), which affects the expression of ~10% of the *E. coli* genes (Weber et al. 2005). In parallel, *rpoS* transcription can be enhanced in response to poor nutritional conditions and reduced growth rates (Hengge-Aronis 2002). In addition, the efficiency of *rpoS* mRNA translation is controlled by various stress-sensing mechanisms that involve small regulatory RNAs and the Hfq protein (Repoila et al. 2003). Whether these levels of control operate independently and additively or are somehow coordinated, is still an open question.

The present study started from the question of how σ^S proteolysis is regulated. σ^S degradation requires the complex ATP-dependent ClpXP protease as well as the response regulator RssB, which, in its phosphorylated form, binds to σ^S (thereby exposing a ClpX-binding site in σ^S), delivers σ^S to ClpXP, and is released from the complex (Becker et al. 1999; Klauck et al. 2001; Zhou et al. 2001). Thus, RssB acts as a proteolytic recognition or targeting factor for σ^S . This process is regulated by various mechanisms that affect specific substrate, i.e., σ^S recognition, and can integrate different stress signals (summarized by Jenal and Hengge-Aronis 2003). When bound to RNAP core enzyme, σ^S is protected against degradation (Zhou et al. 2001), and therefore, competition for RNAP core of various σ factors, whose cellular concentrations are highly variable, can affect the rate of σ^S degradation. In addition, RssB is present at cellular concentrations that are limiting or near-limiting for σ^S degradation, which allows control of σ^S proteolysis by titration of RssB under stress conditions that strongly and rapidly induce σ^S synthesis. The operating range of this titration control seems to be set by a homeostatic mechanism that is based on σ^S control of RssB expression (Pruteanu and Hengge-Aronis 2002). As RssB is a response regulator, it is obvious that control is also exerted by its state of phosphorylation. RssB is a typical

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“orphan” response regulator, as no cognate histidine sensor kinase is encoded in an operon with *rssB* or close to it. Evidence has been presented that acetyl phosphate can act as a physiologically relevant phosphodonator also in vivo, but as an acetyl phosphate-free *pta-ackA* mutant still degrades σ^S (albeit at a reduced rate), there must be additional phosphodonators for RssB (Bouché et al. 1998).

In this study, we present genetic and biochemical evidence that the ArcB protein is a physiologically important and directly acting histidine-sensor kinase for RssB. Moreover, we discovered that ArcA, i.e., the other response regulator served by ArcB, acts as a repressor for *rpoS* transcription. This branched phosphorelay system provides the first mechanism identified that coordinates *rpoS* transcription with σ^S proteolysis, and we propose that this occurs in response to signals that reflect the cellular energy supply.

Results

Mutations in arcB and arcA differentially affect the cellular σ^S level

Extensive genetic screens for defective σ^S proteolysis have always yielded mutations in *rssB*, *clpP*, or *clpX*, but never in a known histidine sensor kinase gene (F. Reindl, G. Kampmann, and R. Hengge, unpubl.). This suggested the existence of more than one “cross-talking” sensor kinase that phosphorylates RssB in a conditionally redundant way. Therefore, we decided to knock out potential candidate genes in a directed manner. We reasoned that complex phosphorelay systems that operate by a serial phosphotransfer which involves histidine-phospho-transfer (HPT) domains might be especially well suited for such cross-talk, as the HPT domain always interacts with more than one response regulator receiver domain. *E. coli* possesses five such phosphorelay systems [ArcB/ArcA, RcsC/YojN/RcsB, BarA/YecB(UvrY), EvgS/EvgA, and TorS/TorR]. We isolated knock-out mutants in all genes encoding these phosphorelay components and tested their effects on σ^S levels under various growth conditions (data not shown). Lesions that resulted in significantly elevated σ^S levels during log-phase growth, i.e., a phenotype consistent with the potential elimination of a RssB-activating factor, were mutations in *arcA* and *arcB* (Fig. 1).

Given the multilevel control of σ^S , the cellular σ^S level

Figure 1. During log-phase growth, σ^S levels are elevated in *arcA* and *arcB* mutants. Strain MC4100 and its *rpoS*, *rssB*, *arcA*, and *arcB* mutant derivatives were grown in LB medium. Using samples of 20 μ g total cellular protein taken at an OD_{578} of 0.15, σ^S levels were assayed by immunoblot analysis.

Figure 2. ArcA represses *rpoS* transcription. (A) Strain FS1 (which is MC4100 carrying the single-copy transcriptional *rpoS742::lacZ* fusion; circles) and its *arcA* (squares) and *arcB* (triangles) mutant derivatives were grown in LB medium, and OD_{578} (open symbols) and specific β -galactosidase activities (closed symbols) were determined along the growth curve. (B) The effect of an *arcA* mutation on σ^S protein levels were determined by immunoblot analysis in the *rssB* mutant background (in order to eliminate effects of the *arcA* mutation on σ^S proteolysis during entry into stationary phase; see Fig. 6). Cells were grown in LB medium, and samples were taken at OD_{578} as indicated (beyond an OD_{578} of 3.5, samples were taken at the times indicated, as the increase of cellular mass of the *arcA* mutant is slower than that of the parental strain).

is a highly integrative parameter; i.e., as a next step, it had to be tested whether *rpoS* transcription, translation, or σ^S proteolysis was affected. Moreover, the *arcB* mutant reproducibly showed higher σ^S levels than the *arcA* mutant (Fig. 1), which was a first indication that σ^S might not be a downstream target of the ArcB–ArcA phosphorelay, but that ArcB and ArcA may differentially affect σ^S control.

The response regulator ArcA is a direct repressor of rpoS transcription and can act independently of ArcB

Using a single-copy transcriptional *rpoS::lacZ* fusion [integrated at *att(λ)*], we tested whether *arcA* and *arcB* mutations affected *rpoS* transcription. The *arcA* mutant exhibited enhanced expression of the reporter fusion (three- to fourfold in log phase and twofold in stationary phase), whereas the *arcB* mutant was not significantly affected (Fig. 2A). Derepression of *rpoS* expression in the *arcA* mutant could also be seen by immunoblot analysis of σ^S itself (Fig. 2B). For this experiment, σ^S proteolytic control was eliminated by using an *rssB* mutant background, since during entry into stationary phase, ArcA also affects σ^S proteolysis (see below). These data indicate that ArcA directly or indirectly represses *rpoS* transcription and can do so even in an ArcB-independent manner. The latter finding suggests that either ArcA may exert this function also in the nonphosphorylated

state or that in the absence of ArcB, ArcA is phosphorylated by another histidine sensor kinase or small molecule phosphodonator.

In order to study whether ArcA-mediated repression of *rpoS* is direct or indirect, ArcA protein was purified and used for electrophoretic mobility shift and DNase I footprinting experiments with a 356-base-pair (bp) DNA fragment (see Material and Methods for details) carrying the major *rpoS* promoter (*rpoSp1*), which is responsible for regulation of *rpoS* transcription in response to nutritional conditions and growth rate (Lange et al. 1995). Both phosphorylated, as well as nonphosphorylated ArcA protein bound to this *rpoSp1* promoter fragment, but produced different band-shift patterns (Fig. 3A), consistent with previous similar observations at other ArcA-controlled promoters (Lynch and Lin 1996b) and the finding that nonphosphorylated ArcA forms a dimer, whereas ArcA phosphorylation results in a higher oligomeric state (Jeon et al. 2001).

DNase I footprinting analysis revealed that ArcA interacts with two sites in the *rpoSp1* region in a phosphorylation-dependent pattern (Fig. 3B). Site I is located upstream of the promoter and overlaps with a cAMP-CRP-binding site (Fig. 3C), from which cAMP-CRP acts as a class I activator for *rpoS* transcription during entry into stationary phase (F. Mika and R. Hengge, in prep.). Site II is located just downstream of the transcriptional start site. Both sites contain core regions highly similar to the ArcA consensus binding site (McGuire et al. 1999; Liu and de Wulf 2004; Salmon et al. 2005) (shown below the sequence of the promoter region in Fig. 3C). Site II is bound slightly better by phosphorylated ArcA and may act as a nucleation site (Fig. 3B). In contrast, nonphosphorylated ArcA produced only very minor alterations, but no clear protection pattern at both sites or elsewhere in the *rpoSp1* region (Fig. 3B).

Taken together, these data demonstrate that phosphorylated ArcA directly binds to two specific sites located upstream and immediately downstream of the *rpoSp1* region. The positions of these ArcA-binding sites suggest that phosphorylated ArcA acts as a direct repressor for *rpoS* transcription by a mechanism that includes an antiactivation for cAMP-CRP.

The histidine sensor kinase ArcB stimulates σ^S proteolysis by a mechanism that involves phosphorylation of RssB but does not require ArcA

As the *arcB* mutant is not affected in *rpoS* transcription, and in particular, ArcB is not required for ArcA-dependent repression of *rpoS* transcription in vivo (Fig. 2A), strongly increased σ^S levels in the *arcB* mutant (Fig. 1) indicate that the histidine sensor kinase ArcB also plays a role in the post-transcriptional control of σ^S . Expression of a translational *rpoS::lacZ* reporter fusion, which reflects *rpoS* transcriptional as well as translational control, exhibited similar effects of *arcA* and *arcB* mutations as the transcriptional fusion, indicating that ArcB does not affect *rpoS* translation (data not shown). Conse-

Figure 3. ArcA directly binds to two sites in the *rpoSp1* promoter region. (A) EMSA using a 356-bp fragment carrying the *rpoSp1* promoter region as well as ArcA-P (phosphorylated by acetyl phosphate) or ArcA (in amounts as indicated in the figure). (B) DNase I footprint of ArcA-P (phosphorylated by acetyl phosphate) or ArcA on a 291-bp fragment carrying the *rpoSp1* promoter region. ArcA-P-protected regions are marked. Numbering of base pairs is relative to the transcriptional start site. (C) Nucleotide sequence in the *rpoSp1* region with -35 and -10 regions, the transcriptional start site, binding sites for cAMP-CRP (centered at positions -61.5 and +56.5 with respect to the transcriptional start site; underlined), and binding sites for ArcA-P (boxed) indicated. The consensus site for ArcA-P binding is given in the boxes below the sequence of the *rpoSp1* region.

quently, we turned our attention to the control of σ^S proteolysis.

With the hypothesis in mind that ArcB could be a sensor kinase that directly phosphorylates and thereby activates RssB, we tested the effects of the *arcA* and *arcB* mutations on σ^S proteolysis in strains that expressed either wild-type RssB (Fig. 4A,B) or RssB^{D58P} (Fig. 4C,D). As D58 is the phosphorylated aspartyl residue in the

Figure 4. A mutation in *arcB* reduces σ^S proteolysis in the presence of wild-type RssB, but not with RssB^{D58P}. (A–D) A *rssB::Tn10* derivative of strain MC4100 (circles in B,D) as well as its *arcA* (diamonds) and *arcB* (squares) mutant derivatives, which expressed either wild-type RssB (A,B) or RssB^{D58P} (C,D), were grown in M9 medium containing 0.4% glycerol (no inducer, i.e., arabinose, was added, in order to obtain RssB levels similar to those in strains expressing RssB from the chromosome). During log-phase growth (at an OD₅₇₈ of 0.6), σ^S degradation was assayed by pulse-chase labeling of cells, followed by immunoprecipitation and SDS-PAGE. PhosphorImager data are shown in A and C, and the corresponding quantitation is given in B and D, respectively. (E–H) MC4100 (circles in F,H) as well as its *arcB* mutant derivative (squares in F,H) were grown in LB medium. At an OD₅₇₈ of 1.0 (E,F) and 2.0 (G,H), σ^S proteolysis was assayed by the addition of 200 μ g/mL chloramphenicol and immunoblot analysis. Immunoblots are shown in E and G, the corresponding quantification is given in F and H, respectively.

RssB receiver domain, RssB^{D58P} cannot be phosphorylated. It nevertheless displays residual activity that supports σ^S degradation at a reduced rate (Fig. 4D; Klauck et al. 2001). In the strains used in Figure 4A and B, these *rssB* alleles were plasmid expressed under the control of the *araBAD* promoter (in a *rssB::Tn10* genetic background, in the absence of inducer, basal RssB expression from these constructs produces cellular RssB levels comparable to those in wild-type strains [Klauck et al. 2001]). In the presence of wild-type RssB, the *arcB* mutation reduced the in vivo rate of σ^S degradation from a half-life of ~3.2 min to 6.5 min (Fig. 4A,B). The *arcA* mutation, however, did not alter σ^S proteolysis, indicating that ArcB does not affect σ^S proteolysis via the ArcB–ArcA phosphorelay. In the strain that expresses RssB^{D58P}, the *arcB* mutation did not alter σ^S degradation (Fig. 4C,D), suggesting that ArcB acts via phosphorylation of D58 in RssB.

The effect of the *arcB* mutation on σ^S proteolysis was also observed with strains that express wild-type RssB from the chromosome as well as under different growth conditions. For instance, we measured σ^S half-lives of 2.5 and 5 min in wild-type and *arcB* mutant strains, respectively, during log-phase growth in rich LB medium (at an

OD of 1.0) (Fig. 4E,F). During gradual transition into stationary phase, the effect of the *arcB* mutation on σ^S proteolysis became more pronounced. At an OD of 2.0, σ^S half-lives were 5 min and >15 min in wild-type and *arcB* mutant strains, respectively (Fig. 4G,H) (also, under these conditions, the *arcA* mutation had no effects; data not shown). These data indicate that the degree to which ArcB contributes to σ^S proteolysis is variable. Under certain conditions—e.g., during rapid log-phase growth—the ArcB function in σ^S degradation seems to be partially substituted for by other systems, whereas ArcB becomes a major determinant for σ^S proteolysis when cells gradually enter stationary phase.

ArcB phosphorylates and thereby activates the σ^S proteolysis targeting-factor RssB

The in vivo data described above suggest that the ArcB histidine sensor kinase acts as a direct phosphodonor for the “orphan” response regulator RssB. We therefore tested whether ArcB can phosphorylate RssB in vitro and whether it does so in similar or different ways as it phosphorylates ArcA. ArcB was purified as a soluble variant

lacking the first 77 amino acids, which contain the membrane-spanning domains of wild-type ArcB (this N-terminally truncated form of ArcB was previously shown to be active in vitro) (Georgellis et al. 1997). Purified ArcB⁷⁸⁻⁷⁷⁸, RssB, ArcA, and [γ^{32} P]ATP were coincubated in various combinations and phosphorylated proteins were visualized by SDS-PAGE and PhosphorImager analysis. ArcB⁷⁸⁻⁷⁷⁸ transphosphorylated both ArcA and RssB, no matter whether present alone or together in approximately equimolar concentrations (Fig. 5A). ArcA efficiently competes with RssB for transphosphorylation from ArcB⁷⁸⁻⁷⁷⁸, as shown with increasing concentrations of ArcA (Fig. 5B,C). Increasing concentrations of RssB, however, do not significantly interfere with ArcA phosphorylation (Fig. 5D,E), which is explained by the strongly different kinetics of phosphorylation of RssB and ArcA (Fig. 5F,G). Since ArcA phosphorylation from ArcB⁷⁸⁻⁷⁷⁸ is ~10 times more rapid than RssB phosphorylation, ArcA phosphorylation is not significantly reduced even in the presence of increased concentrations of RssB. This also means that in the presence of a limited ArcB-P supply, RssB phosphorylation is exquisitely sensitive against even small changes in the concentration of competing ArcA (but not vice versa), a feature that the cell may use to control RssB phosphorylation and thereby σ^S proteolysis (see below; Fig. 6).

We also tested whether transphosphorylation from ArcB to RssB produces active RssB able to bind to σ^S in

vitro (Fig. 5H). In an in vitro RssB- σ^S interaction assay (based on affinity chromatography and coelution of the binding partners) (Becker et al. 1999), we observed that the previously demonstrated acetyl-phosphate-mediated phosphorylation and activation of RssB could be fully replaced by ArcB⁷⁸⁻⁷⁷⁸ and [γ^{32} P]ATP, and that the presence of ArcA in the assay also interfered with RssB binding to σ^S , as expected for ArcA competing with RssB for ArcB-dependent phosphorylation (Fig. 5H).

As phosphorylated RssB does not exhibit intrinsic autophosphatase activity and is stable over at least 2 h (Klauck et al. 2001), we could also test whether ArcB can act as a phosphatase for prephosphorylated RssB and whether reverse phosphate flow from prephosphorylated RssB via ArcB⁷⁸⁻⁷⁷⁸ to ArcA can occur. Using the direct radioactive transphosphorylation assay as well as the functional RssB- σ^S interaction assay, we found that this was not the case (data not shown). This indicates that ArcB-dependent phosphorylation of RssB is a one-way reaction and that RssB is dephosphorylated by other factors, perhaps as part of its proteolytic targeting cycle (Klauck et al. 2001). This is in contrast to ArcA, for which ArcB can act as a phosphatase (Georgellis et al. 1998). The latter can actually be seen in the kinetic experiment (Fig. 5F,G), where initially rapidly accumulating phosphorylated ArcA is reduced again over time as was also observed previously (Tsuzuki et al. 1995; Georgellis et al. 1997).

Figure 5. In vitro phosphotransfer between ArcB, RssB, and/or ArcA, and σ^S binding of ArcB-phosphorylated RssB. (A) Purified ArcB (0.067 μ M), RssB (0.85 μ M), and/or ArcA (1.0 μ M) were coincubated for 30 min in the presence of [γ^{32} P]ATP in the combinations as indicated in the figure (for details of protein purification and the transphosphorylation conditions, see Materials and Methods). (B) In order to test competition between ArcA and RssB for phosphorylation by ArcB, the assay was done as in A, but increasing concentrations of ArcA were used as indicated in the figure. A quantification of this competition experiment is shown in C, with RssB phosphorylation given as the percentage of maximal phosphorylation in the absence of ArcA, and ArcA phosphorylation given in arbitrary (PhosphorImager) units. (D) Phosphorylation by ArcB of ArcA and RssB was assayed as in A, but increasing concentrations of RssB were used as indicated. The quantification is shown in E. (F) Kinetics of phosphorylation by ArcB were compared for ArcA (1 μ M) and RssB (0.85 μ M) using ArcB preincubated with [γ^{32} P]ATP and by starting the reaction with the addition of the respective response regulator. Samples were withdrawn for SDS-PAGE at time points as indicated in F and quantitative data are shown in G. (H) Phosphorylation of RssB was assayed by its binding to σ^S using an affinity chromatography and coelution ("pull-down") assay. The constituents of the assay mixture were used in combinations as indicated in the figure (for concentrations and details of the procedure, see Materials and Methods).

*A positive feedback in stationary-phase accumulation of σ^S involving σ^S -dependent *arcA* transcription and an inhibitory role of ArcA in σ^S proteolysis*

All of our data presented above can be summarized in a model (Fig. 7) in which the complex sensor kinase ArcB acts as a phosphodonor for two response regulators, ArcA and RssB, which repress *rpoS* transcription and stimulate σ^S proteolysis, respectively. Due to inactivation of ArcB by oxidized quinones (Georgellis et al. 2001a; for a detailed discussion, see below), entering into poor nutritional—i.e., energy-starvation—condition (in the presence of oxygen) should reduce ArcB-mediated phosphorylation of ArcA and RssB, and as a consequence, σ^S should be induced. Additional data suggest an even more intricate fine-regulation under such stress conditions: In the course of genome-wide microarray analyses comparing wild-type and *rpoS* mutant strains (Weber et al. 2005), we found *arcA* to be under significant σ^S control under two of the tested σ^S -inducing stress conditions (entry into stationary phase, and osmotic upshift) (Fig. 6A). We verified this by constructing a single-copy chromosomal transcriptional *arcA::lacZ* fusion, which was found to be stationary phase-induced in a partly σ^S -dependent manner (Fig. 6B). These data indicate that σ^S , which accumulates during entry into stationary phase, stimulates the expression of ArcA.

As ArcA efficiently competes with RssB for phosphorylation by ArcB (especially under conditions where autophosphorylation of ArcB is reduced as mentioned above), increased ArcA levels may result in further reduced levels of phosphorylated RssB, and consequently, further reduced σ^S proteolysis. We therefore tested whether an *arcA* mutation has an effect on σ^S proteolysis in starved cells. After entry into stationary phase, wild-type cells exhibit a σ^S half-life of ~20 min (in contrast to fully supplemented growing cells, where σ^S half-life is between 1.5 and 4 min, depending on the medium com-

Figure 6. In stationary phase, *arcA* expression is partly σ^S dependent and ArcA contributes to σ^S stabilization. (A) Expression ratios in *rpoS*⁺ versus *rpoS*::Tn10 strains are given for the *arcA* gene under conditions where σ^S is highly expressed (an OD₅₇₈ of 4.0 in LB medium corresponds to transition into stationary phase; “pH 5” indicates 40 min after downshift from pH 7 to pH 5 at an OD₅₇₈ of 0.4 in LB medium; “NaCl” indicates 20 min after the addition of 0.3 M NaCl at an OD₅₇₈ of 0.3 in M9/0.4% glycerol medium; sample treatment and statistical analysis of data were described in detail by Weber et al. [2005]; a ratio >2 indicates significant control by σ^S). (B) MC4100 carrying the single copy *arcA159::lacZ* fusion (circles) and its *rpoS::kan* derivative (triangles) were grown in LB medium, and OD₅₇₈ (open symbols) and specific β -galactosidase activities (closed symbols) were determined along the growth curve. (C) Strain MC4100 and its *arcA* derivative were grown in M9/0.1% glucose, and 45 min after entry into stationary phase, σ^S degradation was assayed by pulse-chase labeling of cells, followed by immunoprecipitation and SDS-PAGE (only PhosphorImager-quantified data are shown).

position) (Fig. 4; Lange and Hengge-Aronis 1994). We found that the *arcA* mutant indeed does not stabilize σ^S to this extent, but exhibits a σ^S half-life of 11 min in stationary phase (Fig. 6C). These data were obtained with glucose-starved cells. In glycerol-starved cells, σ^S half-lives were 25 and 14 min for *arcA*⁺ and *arcA* mutant strains, respectively (data not shown).

We conclude that while ArcA does not play a role in the control of σ^S degradation in log-phase cells (Fig. 4A,B), ArcA contributes to stabilizing σ^S in stationary phase (Fig. 6B).

Discussion

*The ArcB/ArcA/RssB phosphotransfer network maintains low σ^S levels in nonstressed cells by inhibiting *rpoS* transcription and promoting σ^S proteolysis*

In this study, we have demonstrated that the ArcB histidine sensor kinase and the ArcA response regulator down-regulate σ^S in log-phase cells. However, they do not exert this control as a cognate two-component system. Rather, ArcA acts as direct repressor at the *rpoS*P1 promoter even in the absence of ArcB, and ArcB acts as a sensor kinase that directly phosphorylates and thereby activates the response regulator RssB, which targets σ^S for proteolysis. Thus, under conditions where ArcB autophosphorylates, this ArcB/ArcA/RssB “three-component system” operates in a branched phosphorelay pathway or network (Fig. 7A). This network probably contains additional components, as RssB can also be activated by acetyl phosphate in a physiologically relevant way (Bouché et al. 1998; see also below). Phosphorylation of ArcA by acetyl phosphate can also occur, and in the absence of ArcB, is likely to become relevant for ArcA activity (Pena-Sandoval et al. 2005).

Both branches of this network cooperate to maintain

Figure 7. A model for the function of the ArcB/ArcA/RssB “three-component system” in σ^S control under different environmental conditions. (A) Under conditions of high energy supply and/or low oxygen tension, components of the respiratory chain including quinones are reduced and ArcB autophosphorylates (Georgellis et al. 2001a). ArcA and RssB are phosphorylated and cooperate to keep σ^S levels low by repressing *rpoS* transcription and stimulating σ^S proteolysis, respectively. Accessory components are acetyl phosphate, which contributes to RssB and perhaps also to ArcA phosphorylation (Bouché et al. 1998; Pena-Sandoval et al. 2005), as well as cAMP-CRP, which in log-phase cells has an indirect inhibitory effect on *rpoS* transcription (Lange and Hengge-Aronis 1994; F. Mika and R. Hengge, in prep.). (B) Upon energy starvation and/or high oxygen tension, oxidized quinones interfere with ArcB autophosphorylation (Malpica et al. 2004) and RssB and ArcA get less phosphorylated. σ^S levels increase as *rpoS* transcriptional repression by ArcA is relieved and σ^S proteolysis is slowed down (since the cellular level of phosphorylated RssB is limiting for the overall rate of σ^S degradation) (Pruteanu and Hengge-Aronis 2002). Accessory factors are a decrease of acetyl phosphate levels under energy limitation conditions (McCleary and Stock 1994) and increased levels of cAMP-CRP, which now acts as a class I activator for transcription from *rpoS*p1 (F. Mika and R. Hengge, in prep.). In addition, high σ^S levels stimulate the expression of ArcA, which contributes to σ^S stabilization (probably by competing with RssB for residual phosphorylation from ArcB).

low σ^S levels in rapidly growing cells. This is not only the first demonstration of a coordination between two different levels of σ^S control, i.e., *rpoS* transcription and σ^S proteolysis, but our data also provide the mechanistic basis of this process. The *rpoS*p1 promoter region contains two ArcA-binding sites, which are occupied by phosphorylated ArcA (Fig. 3). Site I overlaps with a

cAMP-CRP-binding site, from which cAMP-CRP acts as a class I activator for *rpoS* expression during entry into stationary phase (F. Mika and R. Hengge, in prep.). Site II is located immediately downstream of the transcriptional start site. Thus, it is conceivable that an ArcA oligomer (which is known to form upon phosphorylation [Jeon et al. 2001]) binds to both sites and thereby may induce the formation of a DNA loop that includes the *rpoS*p1 promoter sequence. Due to the overlap with the activating cAMP-CRP-binding site, phosphorylated ArcA would not only act as a “classical” repressor, but also as an antiactivator for cAMP-CRP. A similar arrangement, in which cAMP-CRP and ArcA compete, has also been observed in the *ptsG* promoter (Jeong et al. 2004). With nonphosphorylated ArcA, the binding pattern in the *rpoS*p1 region is different. As shown by gel retardation, ArcA binds, but a smaller complex is formed (Fig. 3A), consistent with nonphosphorylated ArcA forming a dimer (Jeon et al. 2001) and previous observations of similar ArcA band-shift patterns at other promoters (Lynch and Lin 1996b; Jeon et al. 2001; Jeong et al. 2004). In the footprinting assay, nonphosphorylated ArcA produced some very minor alterations but no clear protection in the *rpoS*p1 region (Fig. 3B). These data suggest that in vivo repression by ArcA in the absence of ArcB is not exerted by nonphosphorylated ArcA, but that under these conditions, ArcA is still phosphorylated, most likely from acetyl phosphate.

In the second branch of this phosphorelay network, ArcB directly phosphorylates RssB (Fig. 5). This is consistent with a recently published study of in vitro transphosphorylation tested for all possible combinations of *E. coli* histidine-sensor kinases and response regulators, which reported that phosphotransfer could occur between ArcB and RssB (Yamamoto et al. 2005). However, RssB could also be phosphorylated by the chemotaxis-sensor kinase CheA, which seems of questionable physiological relevance in view of the highly dynamic CheA phosphorylation and dephosphorylation operating on a millisecond-to-second time scale (Sourjik 2005). Moreover, RssB seemed to interfere with transphosphorylation within many “cognate” two-component pathways, without being itself phosphorylated by the corresponding sensor kinases (Yamamoto et al. 2005). Our data shown here not only provide evidence that phosphorylation of RssB by ArcB is physiologically relevant and results in active RssB able to bind σ^S , but also demonstrate intricate differences in ArcA and RssB phosphorylation by ArcB that can contribute to fine-tuning of the physiological roles of phosphorylated ArcA and RssB.

While ArcA phosphorylation by ArcB is rapid (Fig. 5F,G) and reversible (Georgellis et al. 1998), RssB phosphorylation is 10-fold slower and irreversible (Fig. 5F,G). The “kinetic” advantage of ArcA means that ArcA can efficiently interfere with RssB phosphorylation, but not vice versa. Thus, even moderate up-regulation of ArcA (as observed in stationary-phase cells, see Fig. 6B) should result in down-regulation of RssB activity and, therefore, σ^S stabilization (Fig. 6C; for further discussion, see below). Another interesting observation is that small con-

centrations of RssB (up to equimolar with ArcA) reproducibly stimulated ArcA phosphorylation to some extent (Fig. 5D,E). This raises the question of whether one response regulator can allosterically affect phosphotransfer from a sensor kinase dimer to another response regulator, even though its own phosphorylation is less efficient (or even absent, as suggested by the interference of RssB with *in vitro* phosphotransfer in several other two-component systems, as reported by Yamamoto et al. 2005). Such biochemical details and physiological implications of protein interactions and phosphotransfer reactions in two-component networks, in particular if they consist of phosphorelay systems, will have to be clarified in future studies.

*Physiological signals integrated
by the ArcB/ArcA/RssB phosphotransfer network*

rpoS transcriptional repression by ArcA and ArcB-mediated phosphorylation and activation of RssB and, therefore, σ^S proteolysis, cooperate to maintain low cellular σ^S levels in rapidly growing cells. From this, the question arises as to which stress signals can induce σ^S levels by specifically acting upon the ArcB/ArcA/RssB network. Previous work has demonstrated that the redox state of the quinones and, therefore, the respiratory chain control autophosphorylation of ArcB (Georgellis et al. 2001a). Oxidized quinones induce intermolecular disulfide bond formation between two ArcB molecules, which results in a loss of ArcB autophosphorylation activity (Malpica et al. 2004). As the redox state of the respiratory chain depends on the concentration of the terminal electron acceptor, the activity of the ArcB–ArcA system is controlled by oxygen supply and is therefore crucially involved in the control of gene expression during transition to anaerobic conditions (Lynch and Lin 1996a). Therefore, the function of the Arc system is usually studied using anaerobic cultures (Liu and de Wulf 2004; Salmon et al. 2005). Yet, several studies have observed ArcA-dependent gene regulation also under aerobic or microaerobic conditions (Alexeeva et al. 2003; Levanon et al. 2005) (also, our *in vivo* data presented here were obtained with aerated cultures). In particular, if cells are grown in a chemostat, i.e., with a certain defined carbon and energy supply, NADH/NAD⁺ ratios remain high up to a certain relatively high-threshold oxygen concentration, and then drop sharply (Alexeeva et al. 2003).

All of these data are consistent with the redox state of the respiratory chain being the result of a balance between energy (i.e., electron) supply and the concentration of oxygen (i.e., terminal consumption of electrons). Thus, the activity of the Arc system seems to integrate information about oxygen supply as well as energy supply, and under the standard laboratory moderately aerobic growth conditions, transition from excess carbon and energy supply to starvation is probably decisive for ArcB activity. In conclusion, σ^S accumulation in cells entering into starvation is likely to be controlled by the interference of oxidized quinones with ArcB autophosphorylation and, therefore, reduced phosphorylation of RssB and

ArcA, which results in reduced σ^S proteolysis and a relief of *rpoS* transcriptional repression (Fig. 7B). This energy supply signaling in the control of σ^S may be further accentuated by acetyl phosphate, which can phosphorylate both RssB and ArcA, and whose cellular concentration also varies with the energy supply (McCleary and Stock 1994).

While being an antagonist of σ^S expression in log phase, ArcA cooperates with σ^S in a positive feedback loop in stationary phase

By acting as a repressor for *rpoS* transcription, ArcA plays an antagonistic role for σ^S in exponentially growing cells. During entry into stationary phase, transcriptional repression by ArcA becomes less pronounced (Fig. 2A,B). This effect is probably enhanced by competition with cAMP–CRP, which binds to a site overlapping with the ArcA-binding site I (Fig. 3C) and acts as an activator for *rpoS* transcription under these conditions (our unpublished data). In parallel, the role of ArcA changes to that of an agonist of σ^S , as it becomes part of a positive feedback that further stabilizes σ^S ; *arcA* expression increases in a partially σ^S -dependent manner (Fig. 6A,B), and ArcA plays a positive role in σ^S stabilization in starved cells (Fig. 6C), as even small increases in ArcA concentration efficiently interfere with RssB phosphorylation by ArcB (especially when phosphorylated ArcB is in reduced supply as in cells that enter into an energy starvation situation).

This positive role of ArcA in the accumulation of σ^S during entry into stationary phase correlates with an overlap in the physiological functions of ArcA and σ^S . In genomic microarray studies (Weber et al. 2005), it was observed that σ^S has an impact on many genes in central energy metabolism. During entry into stationary phase, σ^S positively affects the expression of many fermentative genes and down-regulates genes of the TCA cycle and aerobic respiration. This pattern is strikingly similar to the physiological control exerted by ArcA (Lynch and Lin 1996a; Liu and de Wulf 2004; Salmon et al. 2005) and it is possible that σ^S affects many of these genes indirectly by stimulating the expression of ArcA. In summary, ArcA and σ^S are an example where an antagonistic relationship between two global regulators under one condition (i.e., in log phase) can change into cooperation under other conditions (i.e., in stationary phase), which underlines the highly dynamic structure of global regulatory networks.

Conclusions and perspectives

A major conclusion of our study is that the complex histidine-sensor kinase ArcB serves the two response regulators ArcA and RssB in a branched pathway. This suggests that the distinction between “cognate” and “noncognate”, i.e., “cross-talking” two-component systems, is questionable and may reflect our incomplete knowledge rather than physiological reality. It may be

more reasonable to distinguish between linearly operating two-component pathways and two-component phosphotransfer networks with multiple inputs and outputs. In the future, methods will have to be worked out to make this distinction in a physiologically relevant manner.

In addition, ArcB is obviously not the only sensor kinase that activates RssB, as the severity of the effects of the *arcB* mutation on σ^S proteolysis is conditional, suggesting the presence of other relevant sensor kinases that control RssB activity and, therefore, σ^S degradation under certain conditions. This indicates that not only the activity of the signaling components but also the architecture of a complex signal integration network is variable and regulated. Which of all possible signal-transducing and regulatory circuits actually operate, may strongly depend on the actual conditions. This also means that large-scale studies—e.g., of putative interactions between the proteins of an organism (Butland et al. 2005) or between all members of a certain protein family such as the two-component system proteins (Yamamoto et al. 2005) under a single standard condition or in vitro—reveal an overall regulatory potential, but not the relevant regulatory circuitry under certain physiological conditions.

Materials and methods

Bacterial strains and growth conditions

All strains used in this study are derivatives of strain MC4100 (Casadaban 1976). The following mutant alleles were introduced by P1 transduction (Miller 1972) into either MC4100, FS1 (MC4100 carrying the *rpoS742::lacZ* operon fusion; see below) or FS110 (MC4100 carrying the *arcA159::lacZ* operon fusion, see below): *arcA::kan*, *arcB::kan*, *arcA::tet*, *arcB::tet* (Kwon et al. 2000; Georgellis et al. 2001b; Liu and de Wulf 2004), *rpoS::kan* (Bohannon et al. 1991), and *rssB::Tn10* (Muffler et al. 1996). To provide RssB in *trans*, the plasmids pBadRssB or pBadRssB^{D58P} (Becker et al. 2000) were introduced into *rssB::Tn10* strains.

Cells were grown at 37°C under aeration in Luria-Bertani (LB) or minimal medium M9 supplemented with 0.1% glucose or 0.1% or 0.4% glycerol (Miller 1972). Antibiotics were added as recommended (Miller 1972). Growth was monitored by measuring the optical density at 578 nm (OD₅₇₈).

Construction of chromosomal *lacZ* fusions

pFS1 containing the *rpoS742::lacZ* operon fusion is a derivative of pRL45 (Lange and Hengge-Aronis 1994). A 573-bp fragment from pBR322 containing 309 bp of the *bla* gene and the adjacent noncoding region was amplified using primers 5'-AACATGACCGGTCTTGAAGACGAAAGGGCCTCG-3' (AgeI) and 5'-CTCTTACTGTCTATGCCATCCG-3', which binds upstream of the ScaI site in the *bla* gene. This fragment was used to replace the corresponding 837-bp ScaI-AgeI fragment of pRL45, which also contains the upstream promoters *nlpDp1+2*. In the resulting plasmid pLH45 (L. Heidingsfelder and R. Hengge, unpubl.) the SD sequence (bold) was improved by introducing point mutations (lowercase characters) with the primers 5'-CTCTAGAAGCTTCTAGTTAGGAGGATTAAGAAATGAAAGGG-3' (HindIII) and 5'-GGACCATTTTCGGCACAGCCGGGAAGGGCTGG-3', which binds downstream of the BssHII site in *lacZ*. A

1586-bp fragment was amplified and cloned into the HindIII/BssHII-treated pLH45 plasmid, resulting in pFS1.

To obtain the *arcA159::lacZ* operon fusion, a 788-bp fragment of the *arcA* promoter region including 629 bp of the upstream region as well as 159 bp of the *arcA* gene, was amplified from the chromosome. Primers used were 5'-CCAACCGGTGCATGCGGTGATCACTGTCAACT-3' (AgeI) and 5'-CACTAAGCTTCGCATGATCACCAGGTTGATGT-3' (HindIII) (Compan and Touati 1994). This fragment was cloned into the AgeI/HindIII-treated vector pFS1, replacing the complete *rpoS* part of the *rpoS742::lacZ* region, resulting in plasmid pFS23.

These transcriptional *rpoS742::lacZ* and *arcA159::lacZ* fusions were transferred to the *att(λ)* location of the chromosome of MC4100 via phage λRS45 as described (Simons et al. 1987), resulting in strains FS1 and FS110, respectively. Single lysogeny was tested by a PCR approach (Powell et al. 1994).

Determination of β-galactosidase activity

β-Galactosidase activity was assayed by use of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate and is reported as micromol of *o*-nitrophenol per minute per milligram of cellular protein (Miller 1972). Experiments in which the activity of *lacZ* reporter fusions were determined were done at least three times, and representative experiments are shown in the figures.

Overexpression and purification of RssB, σ^S , ArcA, and ArcB

S-tag-TRX-His6-RssB (Bouché et al. 1998) and 6His- σ^S were purified as reported previously (Becker et al. 1999).

To obtain the overexpression plasmid pQE30ArcA, the entire coding region of *arcA* was amplified using the primers 5'-TATGGATCCCAGACCCCGCACATT-3' (BamHI) and 5'-GTCAAGCTTTTAATCTTCCAGATC-3' (HindIII). The resulting fragment was digested with BamHI and HindIII and subsequently cloned into the equivalently treated vector pQE30 (QIAGEN).

To obtain the overexpression plasmid pQE30ArcB⁷⁸⁻⁷⁷⁸, the *arcB* gene, excluding the membrane spanning domains (located in amino acids 1–77), was amplified with the primers 5'-CTGGATCCCATATGGAGCAACTGGAGGAGT-3' (BamHI) and 5'-CATAGTCGACCATATCGCGCACCCCGGTCT-3' (SalI) (Georgellis et al. 1997), digested with BamHI and SalI, and cloned between the corresponding restriction sites of pQE30. Plasmids were verified by DNA sequencing.

Purification of 6His-ArcB and 6His-ArcA was done as recommended by the QIAGEN protocols 8, 9, and 12 (QIAGEN) for the native purification of cytoplasmic proteins. The buffers were used as recommended, with the exception that the sodium phosphate buffer was replaced by 50 mM Tris (pH 8). Cells were disrupted in a French press cell at 1000 psi. In the disruption buffer, 20 mM β-mercaptoethanol (Roth) was included. The washing procedure was extended until the OD₂₈₀ was <0.01. After the elution of ArcB⁷⁸⁻⁷⁷⁸, imidazol was removed by dialysis against storage buffer containing 10% glycerol. ArcA was further purified by gel filtration on a Sephacryl S200HR column (Amersham Biosciences). Proteins were concentrated in Centricon 10 U (Amicon) or Vivaspin Concentrators (Vivaspin) and dialyzed in storage buffer (10 mM Tris at pH 8.0, 0.1 mM EDTA, 10 mM MgCl₂, 200 mM KCl, 50% glycerol). To determine protein concentrations, the Coomassie protein reagent (Pierce) was used with BSA as a standard.

SDS-PAGE and immunoblot analysis

Sample preparation for SDS-PAGE (Laemmli 1970) and immunoblot analysis were performed as described previously (Lange and Hengge-Aronis 1994). A polyclonal serum against σ^S , a goat

anti-rabbit IgG alkaline phosphatase conjugate (Sigma), and a chromogenic substrate (BCIP/NBT; Boehringer Mannheim) were used for visualization of σ^S bands.

In vivo protein degradation assays

The procedure used for pulse labeling of cells with L-[35 S]methionine and immunoprecipitation of σ^S was described previously (Lange and Hengge-Aronis 1994). Exponentially growing cells were harvested at an OD₅₇₈ of 0.6 and pulse labeled for 60 sec, followed by chase times between 20 sec and 20 min. For immunoprecipitation, a polyclonal serum against σ^S was used. Immunoprecipitated proteins were quantified directly from dried gels by using a Fluorescent Image Analyzer FLA-2000G (Fuji Photo Film Co.), the Image Reader for Macintosh version 1.5E, and Image Gauge version 3.45.

Alternatively, σ^S degradation was determined after inhibition of de novo synthesis of proteins by adding 200 μ g/mL chloramphenicol to cells grown as described in the respective figure legends. Samples were taken as indicated in the figure legends, and σ^S was visualized by SDS-PAGE and immunoblotting.

Protein-DNA interaction assays

Electrophoretic mobility shift assays (EMSA) were performed in 20- μ L reaction mixtures, including increasing amounts of purified ArcA protein (previously phosphorylated) and 25 ng of DNA. The reaction buffer contained 100 mM Tris-HCl (pH 7), 100 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, and 10% glycerol (Lynch and Lin 1996b). Reaction mixtures were incubated for 20 min at room temperature and subsequently loaded onto 4% polyacrylamide gels. Gels were run in 0.5 $\times$ TBE (Sambrook et al. 1989) and stained with ethidium bromide. As a DNA fragment, a 356-bp PCR product was used that contains the *rpoS* promoter region from +163 to -193, which was obtained with the primers 5'-ACGTTGGTCAGACCTTGCAG-3' and 5'-CCTCAGAAGCGCCAAAGGTT-3'.

For DNase I footprint experiments, the primers 5'-DIG-AAC GTTGGTCAGACCTTGCAGGT-3' and 5'-TACTGGTTGAT GTACTGCTGACA-3' were used to isolate a 291-bp fragment containing the *rpoSp1* promoter region from +164 to -127. PCR fragments were purified and complex formation between DIG-labeled DNA (6.5 nM final concentration) and purified ArcA (0.9–7.4 μ M final concentration corresponding to 0.5–4 μ g) was allowed in 20 μ L for 20 min at room temperature in binding buffer as described above for the EMSA. Then, 5 μ L of DNase I (Roche) of an appropriate dilution was added to the reaction and incubated at room temperature for 20 sec. The reaction was stopped by the addition of 50 μ L of stop solution (15 mM EDTA, 10 mg/mL yeast carrier tRNA). The samples were extracted with chloroform-phenol, precipitated with ethanol, and separated on a 6% polyacrylamide gel. DNA fragments were transferred to a nylon membrane (Schleicher and Schuell) and cross-linked. The Nylon membrane was blocked for 30 min in blocking buffer (0.1 M maleic acid at pH 7.5, 0.15 M NaCl) with 1% blocking reagent (Roche) and incubated with 1:10000 diluted anti-DIG antibody (Roche) in blocking buffer for 1 h. Digested DNA fragments were visualized using CDP-Star (Roche) in detection buffer (0.1 M Tris-HCl at pH 9.5, 0.15 M NaCl). Protected regions were identified by comparison with a DNA sequence ladder generated with the same DIG-labeled primer as used for amplification of the DNA fragment by PCR (Heroven et al. 2004).

Phosphorylation and transphosphorylation assays

Phosphorylation of ArcA for EMSA was carried out in EMSA reaction buffer described above, including 50 mM acetylphos-

phate for 1 h at 30°C. Phosphorylation of ArcB and RssB, used in the RssB- σ^S interaction assay, was done with 250 μ M ATP for 10 min or 50 mM acetylphosphate for 20 min, respectively, at 30°C in transphosphorylation buffer (TP) (33 mM Hepes-KOH at pH 7.4, 70 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol, 10% glycerol) (Jeon et al. 2001). ArcB phosphorylation for transphosphorylation experiments was done under the same conditions with 1.66 mM [γ^{32} P]ATP (6000 Ci/mmol) for 5 min.

To demonstrate transphosphorylation from ArcB to RssB and/or ArcA, the response regulators (1 μ M) were incubated alone or in combination in 10- μ L reaction mixtures containing ArcB (molar ratio SK:RR 1:15). Transphosphorylation was started by the addition of 1.66 mM [γ^{32} P]ATP and stopped after 30 min by the addition of 4 μ L of 4 $\times$ protein sample buffer (60 mM Tris at pH 6.8, 2% SDS, 10% glycerol, 3% β -mercaptoethanol, 0.005% bromophenol blue). Determination of the time course of phosphorylation of ArcA and RssB was started by the addition of the response regulators to previously phosphorylated ArcB. Ten-microliter samples were withdrawn at the timepoints indicated, inactivated by the addition of 4 $\times$ protein sample buffer, and kept on ice until the last sample was taken.

In competition experiments involving ArcB and both response regulators ArcA and RssB, one response regulator was used at a constant concentration (1 μ M for ArcA, 0.85 μ M for RssB) and increasing amounts of the other response regulator (0.5–5 μ M) were included in the reaction. Transphosphorylation was allowed for 10 min and stopped as indicated above. Samples were immediately subjected to gel electrophoresis on 15% polyacrylamide gels. Labeling of proteins was quantified directly from dried gels using a Fluorescent Image Analyzer FLA-2000G (Fuji Photo Film Co.), the Image Reader for Macintosh version 1.5E, and Image Gauge version 3.45.

Protein-protein interaction assay

In vitro interaction assays of σ^S and RssB were performed as described previously (Becker et al. 1999). Equimolar amounts (3.7 μ M) of S-TRX-His6-RssB, ArcA with or without 50 mM acetylphosphate, or ArcB⁷⁸⁻⁷⁷⁸ (molar ratio SK:RR 1:15) and 100 μ M ATP, were incubated for 10 min at room temperature in 20- μ L reaction mixtures in TP buffer. σ^S (3.7 μ M) was added and incubation was extended for 20 min. Proteins were bound to 50 μ L S-protein agarose (Novagen) via S-TRX-His6-RssB, washed twice with 500 μ L of binding buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 5 mM MgCl₂), and eluted with 50 μ L of 0.2 M sodium citrate (pH 2). Twenty microliters of the supernatant were run on a 12% SDS gel and subjected to immunoblot analysis by using polyclonal sera against σ^S and RssB. To test for potential dephosphorylation of RssB by ArcB, phosphorylated RssB was bound to S-protein agarose for 20 min and washed twice with TP buffer to remove excess acetylphosphate. Increasing amounts of ArcB were added and incubated for 1 h, followed by a 20-min incubation period with equimolar amounts of σ^S to allow RssB- σ^S complex formation. Samples were treated as described above.

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