Modulating RssB activity: IraP, a novel regulator of σ^S stability in *Escherichia coli*

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The σ^S subunit of *Escherichia coli* RNA polymerase regulates the expression of stationary phase and stress response genes. σ^S is highly unstable in exponentially growing cells, whereas its stability increases dramatically upon starvation or under certain stress conditions. The degradation of σ^S is controlled by the phosphorylatable adaptor protein RssB and the ClpXP protease. RssB specifically directs σ^S to ClpXP. An unanswered question is how RssB-mediated degradation of σ^S is blocked by conditions such as glucose or phosphate starvation. We report here the identification and characterization of a new regulator of σ^S stability, IraP (inhibitor of RssB activity during phosphate starvation), that stabilizes σ^S both in vivo and in vitro. Deletion of *iraP* interferes with σ^S stabilization during phosphate starvation, but not during carbon starvation, and has a partial effect in stationary phase and nitrogen starvation. IraP interferes with RssB-dependent degradation of σ^S through a direct protein–protein interaction with RssB. A point mutant of IraP was isolated and found to be defective both for inhibition of σ^S degradation and interaction with RssB. Our results reveal a novel mechanism of regulation of σ^S stability through the regulation of RssB activity and identify IraP as a member of a new class of regulators, the anti-adaptor proteins.

[*Keywords*: RpoS; σ^{38}; YaiB; regulated proteolysis; starvation]

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Bacteria have evolved sophisticated stress response mechanisms that allow them to adapt to a broad range of stressful conditions. Gram-negative bacteria respond to different stresses by the synthesis and/or activation of alternative RNA polymerase σ factors that direct the transcription of regulons whose gene products will deal with the stress (Ishihama 2000). In *Escherichia coli*, the primary alternative σ factor, σ^S (also called σ^{38} or RpoS), is the master transcriptional regulator of stationary phase and the general stress response (Loewen and Hengge-Aronis 1994; Loewen et al. 1998). *E. coli* cells enter into stationary phase to cope with nutrient limitation and the different external environmental insults the bacteria may encounter during long periods of starvation. σ^S is required for the proper development of the stationary phase program. It promotes the expression of ~100 genes that help the cell to respond to starvation (Loewen et al. 1998; Lacour and Landini 2004), hyperosmotic stress (Hengge-Aronis 1996; Checroun and Gutierrez 2004), hypoosmotic stress (Stokes et al. 2003), acid resistance, alkaline resistance, heat shock, cold shock (Kandror et al. 2002), oxidative damage, and DNA damage (Almiron et al. 1992; Volkert et al. 1994; Serafini and Schellhorn 1999; Wolf et al. 1999; Frenkiel-Krispin et al. 2001; for review, see Hengge-Aronis 2002). Thus, σ^S is crucial to maintain cellular homeostasis under many conditions, reflected in the tight regulation of its cellular concentration and activity at all possible levels: transcription, translation, protein stability, and activity (Pratt and Silhavy 1998; Hengge-Aronis 2002; Bougdour et al. 2004).

σ^S protein stability is dependent on both an energy-dependent protease, ClpXP, and an adaptor protein, RssB. In exponentially growing cells, σ^S is maintained at a very low level due to active degradation by the ClpXP protease (Lange and Hengge-Aronis 1994; Schweder et al. 1996). σ^S stability increases (~10-fold) during entry into stationary phase or after exposure to certain stresses, allowing the accumulation of σ^S in the cells (for review, see Hengge-Aronis 2002). Among the known substrates for *E. coli* cytosolic proteases, σ^S is exceptional in that it is poorly recognized by the ClpXP protease alone; its degradation requires the adaptor protein RssB (also termed SprE, MviA in *Salmonella* and ExpM in *Erwinia*) (Bearson et al. 1996; Muffler et al. 1996; Pratt and Silhavy 1996; Zhou and Gottesman 1998; Andersson et al. 1999). RssB binds directly to σ^S and targets it to the ClpXP protease (Zhou et al. 2001). RssB belongs to the two-component response regulator family of proteins. The

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activity of proteins in this family is modulated by phosphorylation of the N-terminal receiver domain on a highly conserved residue, asp58 in the case of RssB (Bouche et al. 1998).

While RssB affinity for σ^S can be modulated by phosphorylation (Becker et al. 1999; Zhou et al. 2001), its ability to activate σ^S proteolysis is regulated by a mechanism that is independent of its ability to be phosphorylated. A nonphosphorylatable RssB protein is still active in vivo and, furthermore, can still respond to environmental signals (Peterson et al. 2004). In cells carrying an *rssB* mutation in the phosphorylation site, σ^S is stabilized in stationary phase and upon starvation. The intracellular amounts of ClpXP are constant during growth (Damerau and St John 1993; Schweder et al. 1996; Mandel and Silhavy 2005) but, paradoxically, RssB levels increase as the cells enter into stationary phase, at the same time as σ^S becomes stable (Becker et al. 2000; Gibson and Silhavy 2000; Ruiz et al. 2001; Pruteanu and Hengge-Aronis 2002). Therefore, RssB activity during stationary phase and starvation must be regulated by an undefined mechanism; unlike previously suggested models, this mechanism may not involve a kinase or a phosphatase.

The work described here was undertaken in order to identify new components involved in the regulation of σ^S proteolysis. An *E. coli* genomic DNA library was screened for clones that would affect the activity of an *rpoS-lacZ* translational fusion designed to be regulated solely at the level of protein stability. We report the isolation and the characterization of a new regulator of σ^S stability encoded by the previously uncharacterized *yaiB* gene. YaiB modulates the stability of σ^S in vivo and in vitro by counteracting the activity of RssB, resulting in the stabilization of σ^S. Because this protein is critically important for stabilization of σ^S after phosphate starvation, we have renamed the gene *iraP* for inhibitor of RssB activity during phosphate starvation.

Results

Identification of a novel regulator of σ^S stability

As discussed above, there are still unidentified components involved in the regulation of σ^S degradation. We reasoned that the use of a reporter system that would more specifically respond to changes in σ^S stability might allow the identification of novel cellular regulators of σ^S degradation. For this purpose, we used a translational fusion P_{BAD}-*rpoS990'-'lacZ* (C. Ranquet and S. Gottesman, in prep.) lacking the 5′ untranslated region of the *rpoS* mRNA containing the well-described translational control signals (for review, see Gottesman 2004). Expression of the reporter was driven by the P_{BAD} promoter to bypass the normal signals influencing *rpoS* transcription. The strain carrying this fusion, CRB316, gave slightly Lac$^+$ (red) colonies on MacConkey lactose plates without arabinose but became strongly Lac$^+$ in *clpP* (AB002) or *rssB* (AB003) mutant derivatives (data not shown).

To identify potential new regulators of σ^S stability, this strain was transformed with a pBR322-based *E. coli* genomic DNA library (Ulbrandt et al. 1997) with the expectation that overproduction of either positive or negative regulators of σ^S degradation from the plasmid would give white (Lac$^-$) or red (Lac$^+$) colonies, respectively, on MacConkey lactose indicator plates. After transformation with the plasmid library, six red and seven white colonies were isolated out of ~15,000 colonies. To eliminate plasmids affecting the transcription of the fusion rather than the stability of the σ^S-LacZ fusion protein, plasmids were isolated and introduced into a strain carrying the *rpoS750'-'lacZ* translational fusion, which encodes a hybrid protein that is subject to the same transcriptional, translational, and proteolytic regulation as σ^S itself. Only plasmids that affected the expression of both fusions were studied further. These plasmids were directly assayed for their effects on σ^S degradation, as described in Materials and Methods. Two plasmids survived these screens and showed an effect on σ^S degradation. One contained the complete coding sequence of *rssB* and produced white colonies, indicating increased degradation of the σ^S-LacZ reporter. This clone was not further studied. The second plasmid, which produced red colonies, diagnostic of decreased degradation of the σ^S-LacZ reporter, contained the complete coding sequence of *yaiB,* encoding a previously uncharacterized protein. For the reasons described below, we have renamed *yaiB* as *iraP*, and the protein product as IraP.

IraP inhibits intracellular degradation of σ^S

The insert carried by the clone containing *iraP* corresponds to the region located between 399,461 and 401,605 base pairs on the *E. coli* K12 chromosome. This region contains the coding sequence of *iraP* flanked by intergenic regions and part of the flanking genes *ddlA* and *phoA,* encoding, respectively, D-alanine-D-alanine ligase A and alkaline phosphatase. A series of deletions in the plasmid defined the *iraP* coding sequence as the active DNA sequence within this plasmid (data not shown). This assignment was confirmed by subcloning the ORF of *iraP* with its upstream region (387 nucleotides [nt] upstream of the *iraP* start codon) into the vector plasmid used for the library, pHDB3, yielding the pIraP plasmid, as described in Materials and Methods.

The effect of multicopy *iraP* (pIraP) on σ^S stability was measured in cells grown at 37°C in Luria-Bertani broth (LB). As expected, in exponentially growing cells with the vector control (pHDB3), σ^S was rapidly degraded, whereas in cells carrying pIraP, the σ^S half-life was increased approximately threefold (Fig. 1A, exponential phase). In stationary phase, the half-life of σ^S was relatively short, ~3 min in the presence of the vector control (Fig. 1A). However, in stationary phase cells carrying *iraP* in multicopy, the σ^S half-life increased to ~21 min, a sevenfold increase over the vector control (Fig. 1A). The presence of a multicopy plasmid carrying *iraP* led to a modest (approximately twofold) increase in the intracellular amount of σ^S, presumably owing to decreased σ^S

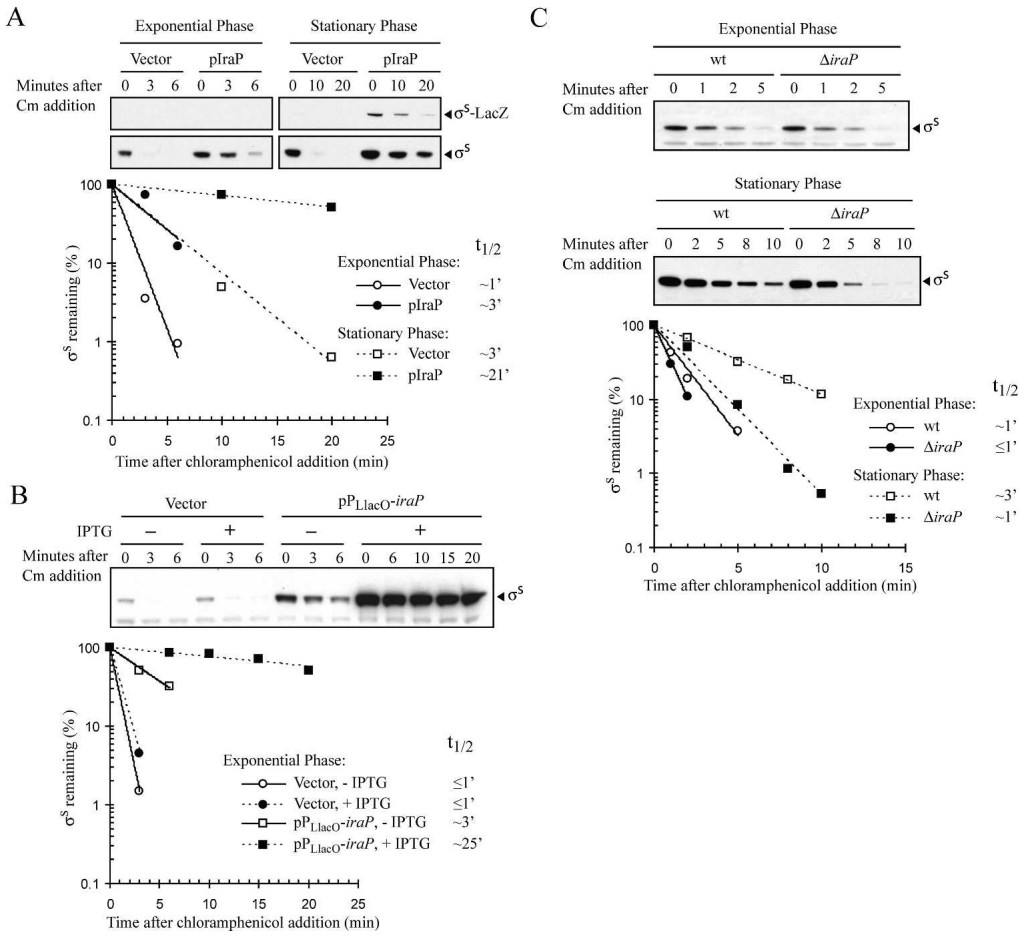

Figure 1. Effects of multicopy *iraP* and deletion of *iraP* on σ^S stability. The half-life of σ^S was measured in cells grown at 37°C in LB. Protein synthesis was inhibited with chloramphenicol at OD_{600} of ~0.3 and ~3 for the determination of σ^S half-life in exponential phase and stationary phase, respectively. Samples were removed at specific time points and analyzed by immunoblot with an anti-σ^S antiserum. (*A*) σ^S half-life was determined in the strain CRB316 (P_{BAD}-*rpoS990'-'lacZ* chromosomal fusion) containing either pHDB3, as vector control, or pIraP. The accumulation and the degradation of σ^S-LacZ are shown in the *top* panel and chromosomally-encoded σ^S in the *bottom* panel. Quantification of the σ^S signals is shown in the graph. The density for samples at time 0 was set to 100% for each of the chase experiments. Half-lives ($t_{1/2}$) were calculated by regression analysis of the exponential decay of σ^S. (*B*) Effect of *iraP* expression from a foreign inducible promoter on σ^S stability in the strain AB009 (Δ*iraP*::*kn*) carrying either the vector control pP_{LlacO} or pP_{LlacO}-*iraP*. The cells were grown until exponential phase in the presence or absence of 1 mM IPTG, as indicated. (*C*) Comparison of σ^S half-lives in the Δ*iraP* (AB006) and wild-type (MG1655) strains.

degradation (Fig. 1A, cf. vector and pIraP at 0 min). This decreased degradation permitted the accumulation and, consequently, the detection of σ^S-LacZ (Fig. 1A, stationary phase), and provides confirmation of the basis for the effect of IraP overproduction on the β-galactosidase activity of the P_{BAD}-rpoS990'-'lacZ fusion.

To confirm that the *iraP* ORF, rather than any sequences from the regulatory region, was responsible for the stabilization of σ^S, *iraP* was cloned under the control of the inducible promoter P_{LlacO} (described in Materials and Methods). The chromosomal copy of *iraP* was deleted (see Materials and Methods). σ^S half-life was monitored in exponential phase in a Δ*iraP* strain complemented by the pP_{LlacO}-*iraP* plasmid. σ^S was dramatically stabilized when *iraP* expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) in comparison to the uninduced cultures or cells with the vector control (Fig. 1B). σ^S was partially stabilized in strains carrying the uninduced pP_{LlacO}-*iraP* plasmid, probably reflecting leaky transcription from the repressed P_{LlacO} promoter. Furthermore, *iraP* induction also influenced the steady-state levels of σ^S (Fig. 1B, cf. the time 0 min for each set of samples), presumably due to the effect of IraP on σ^S stability. σ^S levels in cells containing the uninduced or induced P_{LlacO}-*iraP* plasmid were increased 10- and 40-fold, respectively, compared with the vector control.

Overexpression of IraP did not affect the activity of either a *rpoS'-lacZ* transcriptional fusion or a *rpoS477'-'lacZ* translational fusion, neither of which is degraded by ClpXP and RssB (data not shown). Thus, IraP does not affect transcription from the *rpoS* promoter or expression and folding of stable σ^S fusion proteins. Together with our observations of the effect of multicopy *iraP* on the P_{BAD}-*rpoS990'-'lacZ* fusion, containing a foreign pro-

moter and no upstream sites for translational control, these results strongly suggest that IraP acts at the level of σ^S protein stability.

IraP is required for σ^S stabilization during some but not all starvation conditions

To determine whether the *iraP* gene is required for the stabilization of σ^S in stationary phase, the half-life of σ^S was measured in both the wild-type strain and a strain deleted for *iraP*. In exponentially growing cells, the deletion of *iraP* had only a modest effect on σ^S stability. However, in stationary phase cells growing in rich broth, σ^S half-life was approximately threefold reduced in the Δ*iraP* strain (Fig. 1C). In cells lacking *iraP*, σ^S is almost as unstable in stationary phase as in exponential phase in the wild-type strain; therefore, IraP contributes significantly to stationary phase stabilization of σ^S.

Expression of a σ^S-dependent fusion was also examined; as expected from the modest effect on accumulation and stability in LB, there was a slight decrease (twofold or less) in reporter gene expression in the *iraP* mutant and a slight increase (twofold or less) with *iraP* on a multicopy plasmid (pIraP) (data not shown).

Stationary phase growth in complex media such as LB involves multiple changes in the available nutrients and the environment. Many specific stress responses lead to stabilization of σ^S, including two well-defined conditions, carbon starvation (Lange and Hengge-Aronis 1994; Zgurskaya et al. 1997) and phosphate starvation (Ruiz and Silhavy 2003; Peterson et al. 2004). To evaluate the role of *iraP* under these conditions, we assayed σ^S stability following glucose or phosphate limitation in both wild-type and Δ*iraP* strains. Exponentially growing cells in minimal medium were harvested and resuspended in fresh medium lacking either phosphate or the carbon source. After 1 h of starvation, chloramphenicol was added and σ^S half-life was monitored. Prior to nutrient starvation, σ^S was very unstable, with a half-life of ~2 min (Fig. 2A). Only a small effect of the Δ*iraP* mutation on σ^S degradation was noticed in exponentially growing cells in minimal media, as was seen in LB (Fig. 1C). After 1 h of glucose starvation, σ^S was dramatically stabilized, as previously reported; σ^S stabilization during carbon starvation was not significantly affected by the absence of IraP (Fig. 2B).

Very different results were obtained after phosphate starvation. As previously observed (Peterson et al. 2004), σ^S became stable when the cells were starved for phosphate. However, in the Δ*iraP* strain, σ^S remained unstable in phosphate-starved cells (Fig. 2C). Complementation of Δ*iraP* with pIraP restored stabilization of σ^S during phosphate starvation (Fig. 2D), confirming that IraP is required for full σ^S stabilization during phosphate limitation, although there is also an IraP-independent effect of phosphate starvation (4-min half-life, cf. the 1-min half-life without starvation). These data show that requirements for σ^S stabilization are different during phosphate starvation and glucose starvation; IraP is required for the former but not the latter.

To test whether IraP affects σ^S stabilization only in phosphate starvation, we measured the σ^S half-life in the wild-type and Δ*iraP* strains during nitrogen starvation, a condition in which σ^S is also stabilized (Mandel and Silhavy 2005). σ^S levels increased only modestly and the protein was only partially stabilized (half-life of ~15 min) after 1 h of nitrogen starvation with the wild-type strain (data not shown), consistent with the data of Mandel and Silhavy (2005). In the Δ*iraP* strain, the σ^S half-life was reduced from 15 to 4 min, indicating that IraP also participates in σ^S stabilization during nitrogen starvation (data not shown).

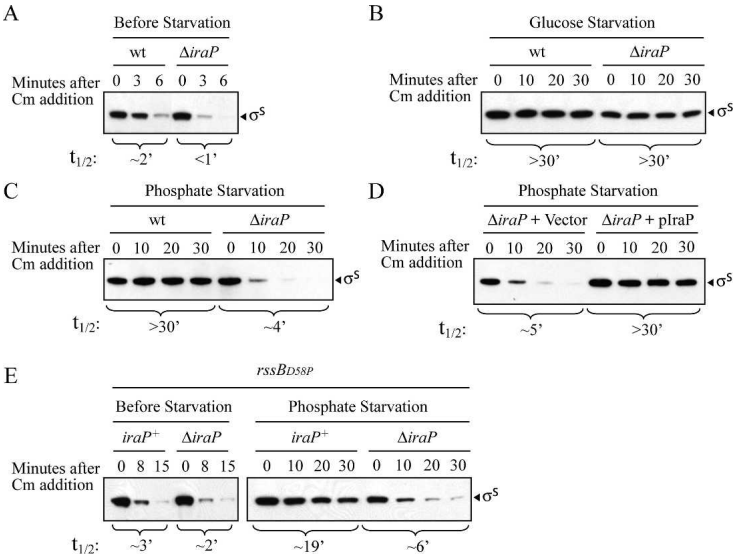

Figure 2. Effect of Δ*iraP* on σ^S stability during carbon and phosphate starvation. The half-life of σ^S was measured in cells grown at 37°C in MOPS medium as described in Materials and Methods. σ^S half-life was determined in exponentially growing cells and after 1 h of glucose or phosphate starvation as indicated. Protein synthesis was inhibited with chloramphenicol at an OD_{600} ~0.3. Samples were removed at specific time points and analyzed by immunoblot with an anti-σ^S antiserum. To obtain the same amount of σ^S at time 0 of each of the chase experiments, the amount of cell extract used for exponential phase cultures was twice the volume used for starved cells. σ^S half-lives ($t_{1/2}$) were determined from plots of intensity versus time. (*A–C*) σ^S degradation was monitored in the wild-type (MG1655) and Δ*iraP*::*kn* (AB006) strains before (*A*, exponential phase) and after glucose (*B*) or phosphate (*C*) starvation. (*D*) Complementation of Δ*iraP* by pIraP. σ^S half-life was determined in phosphate-starved cells with the strain AB006 (Δ*iraP*::*kn*) containing either the vector control pHDB3 or pIraP. (*E*) The half-life of σ^S was measured in strains YN868 (*rssBD58P*) and AB010 (*rssBD58P*, Δ*iraP*::*kn*) in both exponential phase and after 1 h of phosphate starvation.

Our results highlight the differences between different starvation conditions in affecting σ^S stability. IraP is critically important in phosphate starvation and plays a significant role in stationary phase and nitrogen starvation, but has no measurable effect in glucose starvation.

IraP regulates RssB activity by a phosphorylation-independent mechanism

While σ^S stability is controlled by RssB and ClpXP (Muffler et al. 1996; Pratt and Silhavy 1996; Zhou and Gottesman 1998), it was theoretically possible that another system was responsible for σ^S degradation in the absence of *iraP*. If so, σ^S might be unstable in an *iraP* mutant host, even in the absence of *rssB*. This was tested by comparing the σ^S half-life in an *rssB*-null strain (AB012) with that in a *rssB iraP* double mutant (AB013). The σ^S half-life was longer than 30 min in both strains (data not shown), consistent with IraP acting within the RssB pathway.

If IraP acts directly within the RssB pathway, does it function to change RssB phosphorylation status? To determine this, we measured σ^S half-life in cells containing a chromosomal *rssB* allele unable to be phosphorylated. In this strain, the *rssB* codon for asp58, the location of phosphorylation (Bouche et al. 1998), was mutated to encode proline (*rssBD58P*, Y. Zhou and S. Gottesman, in prep.), a change previously shown to be partially active for σ^S degradation (Becker et al. 2000). Peterson et al. (2004) previously observed that cells carrying a chromosomal copy of *rssB* in which asp58 was mutated to ala retained the ability to promote σ^S degradation in exponentially growing cells, and that σ^S was stabilized after phosphate starvation. Our results agree; σ^S was degraded in exponential phase, albeit at a slightly slower rate than with the wild-type *rssB*, in both *iraP*$^+$ and Δ*iraP* cells carrying *rssBD58P* (Fig. 2, cf. A and E, before starvation). After 1 h of phosphate starvation, σ^S was stabilized in the *iraP*$^+$ *rssBD58P* strain (Fig. 2E). Thus, the *rssBD58P* mutation did not abolish the ability of the cell to downregulate σ^S degradation in response to environmental signals, although the stabilization was significantly less than in the *rssB*$^+$ host.

As in the *rssB*$^+$ strain, phosphate starvation did not lead to σ^S stabilization in the Δ*iraP* derivative of *rssBD58P* strain (Fig. 2E, phosphate starvation). The effect of IraP overproduction in stabilizing σ^S (Fig. 1A) and of the loss of IraP (Δ*iraP* strain) in destabilizing σ^S (Fig. 1C) in LB cultures could also be detected in the *rssBD58P* background (data not shown). Thus, IraP still affects σ^S stability in a strain in which RssB cannot be phosphorylated. These data, taken together, indicate that IraP inhibits σ^S proteolysis by regulating RssB activity in a manner that does not require the potential to phosphorylate or dephosphorylate the asp58 residue.

Identification and characterization of a mutant of IraP

RssB is the limiting protein governing σ^S stability; it is generally made at low levels, and increasing the level of RssB increases σ^S degradation (Pruteanu and Hengge-Aronis 2002). We used a strain, referred to here as *rssB*$^{+++}$, carrying the P_{BAD}-*rpoS990'-'lacZ* fusion as well as a transposon insertion upstream of *rssB* (*rssA2*::cm) that increases levels of RssB and therefore decreases σ^S levels below our detection limit (Fig. 3A, vector; Ruiz et al. 2001; Mandel and Silhavy 2005) to develop a simple screen for point mutants in *iraP* that could be used both in vivo and in vitro as controls to further examine IraP mechanism of action.

A plasmid carrying *iraP-his*$_6$ under control of the Lac repressor (pQE-IraP-His$_6$) was introduced into the *rssB*$^{+++}$ strain and induced with IPTG for 15 min. The stability of σ^S was then monitored by addition of tetracycline followed by sampling at various times. Figure 3A shows that after 15 min of induction, σ^S was highly expressed and was stable for >30 min, demonstrating both that the IraP-His$_6$ protein is active and that it can overcome the somewhat higher RssB levels in the *rssB*$^{+++}$ strain. In addition, the somewhat higher RssB levels allowed us to monitor the RssB protein by immunoblot. No change in intracellular levels of RssB was observed in cells overexpressing IraP, and RssB levels remained stable during the tetracycline chase (Fig. 3A). Therefore, IraP does not appear to affect RssB levels or stability.

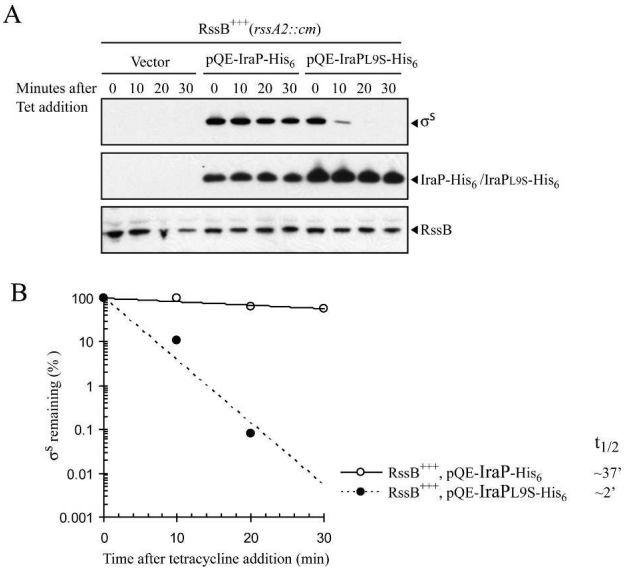

Figure 3. IraP, but not IraPL9S, overcomes RssB overexpression. (*A*) The half-life of σ^S was measured in exponentially growing cells at 37°C in LB after 15 min of induction with 1 mM IPTG. Protein synthesis was inhibited with tetracycline at an OD_{600} of ~0.3, and samples were removed at specific time points and analyzed by immunoblot with an anti-σ^S antiserum. The strain AB011 (*rssA2*::*cm* background) containing the vector control pQE-80L, pQE-IraP-His$_6$, or pQE-IraPL9S-His$_6$ was used. In the absence of IPTG, σ^S was not detected with any of the plasmids listed above (data not shown). The levels of His-tagged IraP protein expressed were analyzed by immunoblot using an anti-RGS-His$_4$ antibody. RssB levels were determined by immunoblot with anti-RssB antiserum. (*B*) Quantification of the levels of σ^S in panel *A*. σ^S half-lives ($t_{1/2}$) were determined from plots of the log of intensity versus time.

The effect of IraP-His$_6$ overexpression on σ^S levels in the *rssB*$^{+++}$ strain can easily be monitored on indicator plates with the P$_{BAD}$-*rpoS990'-'lacZ* fusion. A strain carrying this fusion and the *rssB*$^{+++}$ mutation is white on MacConkey lactose plates (fusion protein degraded); colonies are dark red on McConkey lactose plates (fusion protein stabilized) when IraP is induced. This change in phenotype allowed us to screen for loss-of-function mutants of *iraP* using random mutagenesis, as described under Materials and Methods. Several mutants of *iraP-his$_6$*, each containing a single amino acid substitution and each able to synthesize significant levels of the protein, were obtained; one, *iraPL9S-his$_6$*, has been studied. IraPL9S-His$_6$ was much less effective at stabilizing σ^S than was IraP-His$_6$ (Fig. 3).

The levels of the His$_6$-tagged mutant and wild-type proteins were analyzed by immunoblot. Unexpectedly, the IraPL9S-His$_6$ mutant protein was more abundant than was IraP-His$_6$ (Fig. 3A); we do not currently have an explanation for this difference. The levels of IraP (either mutant or wild type) did not change during an antibiotic chase, suggesting that IraP is not itself a substrate for degradation.

IraP interferes directly and specifically with the RssB-mediated degradation of σ^S

To investigate the mechanism of action of IraP and whether it acts directly to stabilize σ^S, we assayed σ^S degradation in vitro with purified proteins. As previously reported (Zhou et al. 2001), σ^S was degraded in the presence of ClpXP, RssB, and ATP, as measured by SDS-PAGE analysis (Fig. 4A). Acetyl phosphate, which is a known phosphate donor for the phosphorylation of RssB (Bouche et al. 1998), also stimulated σ^S degradation, as previously described (Zhou et al. 2001). To test whether IraP acts directly to inhibit σ^S degradation, IraP-His$_6$ was purified under native conditions (see Materials and Methods) and included in the σ^S degradation reaction mixtures. In the complete reaction mixture, in the absence of IraP, ~80% of σ^S was degraded after 30 min of incubation (Fig. 4A). When IraP was present in the reactions, σ^S was not detectably degraded. Thus, IraP is a direct inhibitor of σ^S degradation.

We then asked whether the L9S mutation in IraP affects its in vitro activity. Figure 4B shows that IraPL9S-His$_6$ did not inhibit σ^S degradation. Thus, both in vivo and in vitro the L9S mutant protein is inactive. Figure 4B also shows that IraP-His$_6$ or IraPL9S-His$_6$ is not detectably degraded in vitro by ClpXP, confirming that IraP is not itself a substrate of ClpXP.

Given that IraP directly affects σ^S degradation, IraP might act by interfering with the activity of either the ClpXP protease or the adaptor protein RssB, or by protecting σ^S from interacting with either of these factors. To test the first possibility, we examined the effect of IraP on the degradation of another ClpXP substrate, GFP-SsrA (Kim et al. 2000; Singh et al. 2000; Farrell et al. 2005). In the absence of IraP-His$_6$, when σ^S and GFP-SsrA were incubated together with ClpXP and RssB, both

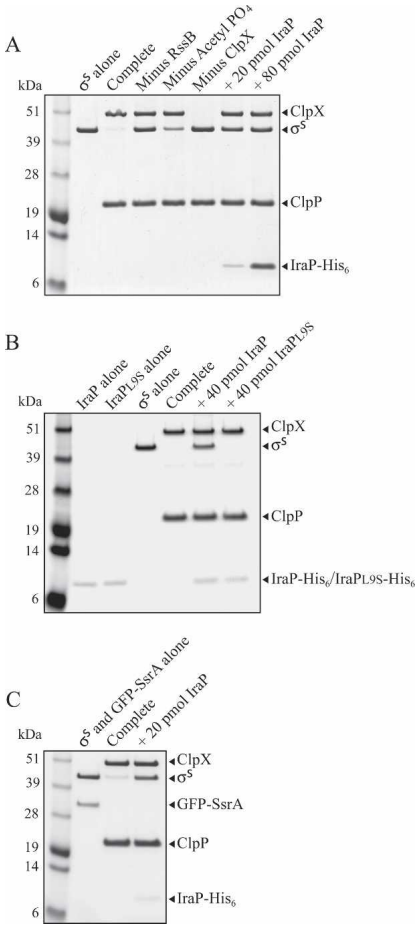

Figure 4. IraP inhibits σ^S degradation by ClpXP and RssB in vitro. The degradation reactions were carried out as described in Materials and Methods with purified components. Reaction products were separated by SDS-PAGE and stained with Coomassie blue. (*A*) The reactions were performed using 20 pmol of σ^S in the presence or absence of IraP-His$_6$. RssB, acetyl phosphate, and ClpX were omitted where indicated. (*B*) The reactions were performed as above, in the presence or absence of either IraP-His$_6$ or IraPL9S-His$_6$. (*C*) Ten picomoles of σ^S was mixed with 10 pmol of GFP-SsrA, in the presence or absence of IraP-His$_6$, and degradation reactions were performed as above.

were degraded (Fig. 4C). In the presence of IraP-His$_6$, σ^S was protected from degradation but GFP-SsrA was not (Fig. 4C, last lane). Similarly, degradation of RepAΔ25-SsrA, another known ClpXP substrate (Sharma et al. 2005), was not inhibited by IraP-His$_6$ (data not shown). These results indicate that IraP acts specifically on the σ^S degradation pathway and, importantly, that IraP does not inactivate ClpXP.

IraP interacts directly with the adaptor protein RssB

The above results showed that IraP controls the half-life of σ^S and acts within the RssB/ClpXP pathway to protect σ^S from degradation. One simple way to accomplish this would be a direct IraP interaction with RssB, blocking

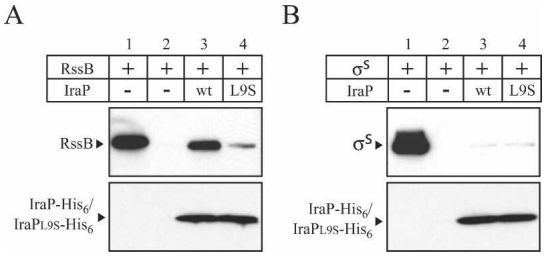

Figure 5. Interaction of IraP and RssB. Purified RssB (*A*) or σ^S (*B*) was incubated with an excess of either IraP-His_6-magnetic beads (lane *3*) or IraPL9S-His_6-magnetic beads (lane *4*) or the magnetic beads alone (lane *2*), as described in Materials and Methods. The proteins adsorbed to the matrix were washed and eluted with imidazole. RssB, σ^S, and IraP were separated by SDS-PAGE and visualized by immunoblot. Lane *1* shows the input amount of RssB (*A*) or σ^S (*B*) used per reaction.

the RssB interaction with σ^S. To test whether IraP interacts directly with RssB, purified IraP-His_6 was bound to Ni^{2+}-NTA-magnetic beads and incubated with purified RssB. Following elution with imidazole buffer and analysis by immunoblot, ~25% of the RssB introduced to the IraP-His_6-Ni^{2+}-NTA-magnetic beads matrix was retained and coeluted with IraP-His_6 (Fig. 5A, cf. lanes 1 [input] and 3 [protein eluted]). RssB did not bind to the Ni^{2+}-NTA-magnetic beads in the absence of IraP-His_6 (Fig. 5A, lane 2).

We then asked whether the L9S mutant of IraP, which in vivo and in vitro had dramatically diminished activity, had any effect on the interaction between IraP and RssB. With use of the assay described above, significantly less RssB coeluted with the mutant IraP compared with the wild-type IraP protein (Fig. 5A, cf. lanes 3 and 4). Similar results were obtained by using crude extracts as a source of RssB (data not shown).

A similar experiment was performed to probe for association of σ^S and IraP-His_6. We found that σ^S was not retained and therefore was not coeluted with either IraP-His_6 or IraPL9S-His_6 attached to the magnetic beads (Fig. 5B).

These results demonstrate a direct and specific interaction between IraP and RssB, and suggest that the L9 residue of IraP is important for this interaction with RssB. We conclude that IraP-dependent stabilization of σ^S relies on a direct protein–protein interaction between IraP and RssB.

Discussion

IraP belongs to a novel class of regulator: the anti-adaptor proteins

We report here the identification and the characterization of a novel regulator of σ^S stability, IraP. IraP provides a critical link to the previously unexplained environmental regulation of σ^S stability. IraP acts in a direct manner to inhibit σ^S degradation. Deletion of *iraP* reverses the stabilization of σ^S after phosphate starvation (Fig. 2C) and partially blocks stabilization in stationary phase (Fig. 1C) and after nitrogen starvation (data not shown). In vivo and in a purified in vitro system, IraP inhibits σ^S degradation by interfering specifically with RssB-dependent degradation by ClpXP (Figs. 1, 3, 4; data not shown). IraP is not itself subject to degradation by ClpXP or other cellular proteases (Figs. 3A, 4B). Consistent with these observations, in vivo analysis of two classes of ClpXP substrates, represented by Dps and SsrA-tagged proteins, do not accumulate when *iraP* is overexpressed (data not shown), and in vitro, two SsrA-tagged substrates of ClpXP are not affected by IraP (Fig. 4C; data not shown).

Our model for IraP action is summarized in Figure 6. Under specific stress signals (phosphate starvation, for instance), IraP becomes available, probably because synthesis increases, although this has not yet been demonstrated. IraP directly binds RssB; we suggest that this

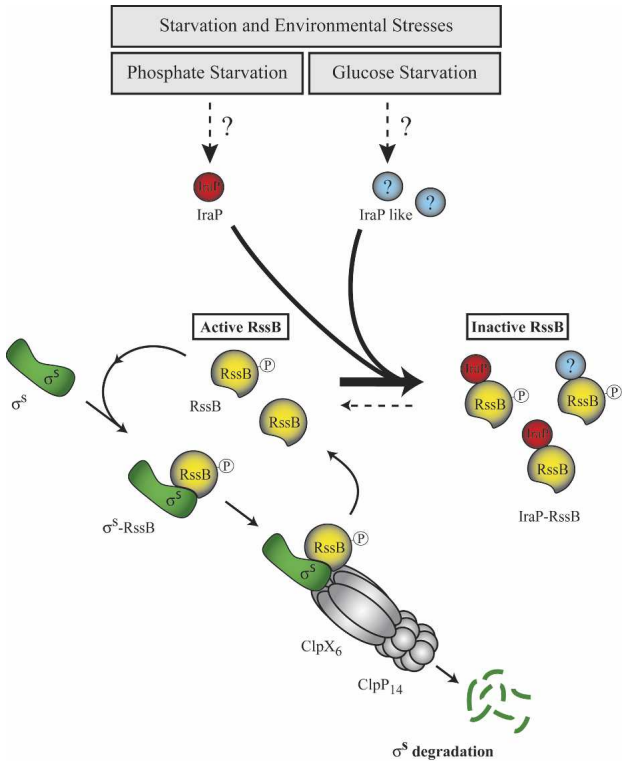

Figure 6. Model of regulation of σ^S degradation by IraP. In exponentially growing cells, RssB delivers σ^S to ClpXP for degradation. During the reaction, RssB itself is not consumed; RssB acts catalytically and is able to carry out multiple cycles of σ^S binding and delivery to ClpXP (Zhou et al. 2001). Phosphorylation of RssB stimulates σ^S degradation. During phosphate deprivation and under other conditions (i.e., nitrogen starvation), IraP stabilizes σ^S through a direct interaction with RssB; the model suggests that the RssB–IraP complex is unable to bind and target σ^S for degradation, although failure to bind σ^S remains to be shown. The pathway for *iraP* induction is not known but is independent of the PhoR/PhoB phosphate regulatory pathway. σ^S is also stabilized under glucose starvation independently of IraP; we suggest that another IraP-like protein acts in that case. Other mechanisms regulate σ^S synthesis (not shown).

binding sequesters RssB, thereby preventing it from interacting with σ^S (Fig. 6). Direct interaction is supported by the association of the wild-type IraP and RssB shown in Figure 5 and the partial loss of that association with the impaired L9S mutant form of IraP. As indicated in the model, we propose that other stress conditions that lead to σ^S stabilization (e.g., glucose starvation) may do so via other IraP-like proteins made under those specific stress conditions. The availability of a variety of different RssB inhibitors combined with the complex regulation of σ^S synthesis (Hengge-Aronis 2002) would allow the cell to modulate σ^S accumulation and activity in response to a large number of different conditions.

Sequestering RssB with specific anti-adaptors provides an elegant and novel way to block degradation under a variety of environmental conditions. We assume that σ^S is stabilized because it is needed to activate promoters in response to the stress. Therefore, any modification or interaction with σ^S itself might compromise its activity. The intracellular levels of RssB are very low (estimated to be only a few molecules per cell) (Becker et al. 2000). If IraP binds RssB stoichiometrically and with high affinity, a low amount of IraP should be sufficient to inactivate RssB. Consistent with this model, increased expression of RssB leads to more rapid degradation of σ^S but can be overcome by overexpression of IraP (Fig. 3). Because RssB amounts are normally low, even a modest increase in the levels of a protein such as IraP may be sufficient to titrate RssB and totally block σ^S turnover. Thus, in this model, rapid degradation of σ^S is the default situation, and only under appropriate environmental conditions is degradation blocked by IraP and/or similar anti-adaptors.

RssB can also act as an anti-σ factor for σ^S (Zhou and Gottesman 1998; Becker et al. 2000); therefore, even in the absence of degradation, inactivating RssB will free σ^S to activate its promoters. The sites on RssB that interact with IraP and σ^S have not yet been identified, but we expect these interactions to be mutually exclusive. Preliminary experiments to examine whether this is so are consistent with our model. RssB attached to beads was able to bind RpoS; RpoS binding was reduced when RssB was preincubated with IraP (data not shown).

RssB shares homology with the response regulator family of proteins, and its affinity for σ^S is modulated by phosphorylation of the conserved residue asp58 located in the N-terminal domain (Bouche et al. 1998; Zhou et al. 2001). We observed that IraP regulated the stability of σ^S in cells carrying either wild-type *rssB* or a mutant allele of *rssB* that cannot be phosphorylated (D58P) (Fig. 2; data not shown). This is consistent with and provides an explanation for recent work showing that a nonphosphorylable RssB protein retains partial activity and that σ^S turnover is regulated upon starvation even with the mutant RssB (Peterson et al. 2004). However, some differences in the effectiveness of IraP and phosphate starvation were observed in the *rssBD58P* mutant. In the *rssBD58P* background during phosphate starvation, the σ^S half-life is ~19 min, which is less than the >30 min observed with the wild-type *rssB* allele (Fig. 2C,E). Possibly, the D58P mutation somewhat compromises the ability of IraP to interact with RssB and counteract its activity.

These results do not rule out a role for phosphorylation in modulating σ^S stability; a role for phosphorylation would be consistent with the differences we observed in σ^S turnover in the *rssBD58P* strain compared with wild-type *rssB*. Acetyl-phosphate is known to phosphorylate RssB in vitro, and changing its level of synthesis affects σ^S turnover in vivo (Bouche et al. 1998). Recently, Mika and Hengge (2005) have shown that the sensor kinase ArcB can also phosphorylate RssB and modulate, to some extent, its activity.

The physiological function of IraP during phosphate starvation

IraP is a small protein (86 amino acids) predicted to be highly helical. The only homologs are found in other Enterobacteria that also contain *rpoS* and *rssB* (*Escherichia* species, *Shigella, Salmonella* species, and *Erwinia* species); the N-terminal region where point mutants were identified is particularly well conserved. In *E. coli, S. flexneri*, and *Salmonella*, *iraP* is located upstream of *phoA* or *psiF*, genes induced under phosphate starvation. It was this linkage to phosphate-regulated genes that led us to test the effect of *iraP* mutations after phosphate starvation; our results show that IraP is particularly important under this stress condition (Fig. 2C).

However, while the expression of *phoA* and other members of the Pho regulon are regulated by the PhoR/PhoB two-component regulatory system in response to phosphate deprivation (Wanner 1996), the IraP-dependent stabilization of σ^S was not affected by a *phoB* mutation (data not shown). Therefore, the phosphate starvation must affect IraP expression and/or activity by another mechanism. Our data are consistent with the previous observation that RssB is inactivated upon phosphate starvation, in a PhoBR-independent manner (Ruiz and Silhavy 2003). We do not currently have an explanation for how the phosphate starvation signal is sensed through *iraP*. Northern blots show a clear but transient induction of *iraP* transcript accumulation (~25-fold) upon phosphate starvation, suggesting transcriptional regulation of *iraP* expression as at least one component of the regulation (data not shown). Both Northern blots and transcriptional fusions show an increase in *iraP* expression upon entry to stationary phase (data not shown). If increased transcription of *iraP* is the primary event leading to stabilization upon starvation, it still remains to be determined what happens when cells exit from phosphate starvation, and whether there is an active process to overcome IraP-dependent stabilization.

Regulated degradation of σ^S is only one of the levels of control of this important protein. It is known, for instance, that σ^S is also up-regulated at the level of synthesis upon phosphate starvation, most probably by a currently undefined small RNA (Ruiz and Silhavy 2003). Possibly because of these complex additional levels of regulation, the changes in the steady-state level of σ^S in

iraP mutants are modest. This provides a simple explanation for why mutant searches using screens that reflect σ^S degradation have not previously been successful in identifying targets other that *rssB*. It is possible, as well, that mutations that impair the stabilization pathway are compensated by an increase in *rpoS* expression. This increase in synthesis of σ^S may itself also partially titrate RssB (Zhou and Gottesman 1998; Zhou et al. 2001; Pruteanu and Hengge-Aronis 2002). Such an increase in σ^S synthesis might be the explanation for the IraP-independent stabilization of σ^S after phosphate starvation (Fig. 2A,C, cf. exponential phase and phosphate starvation in the *iraP* mutant), although possibly another anti-adaptor could play a role here as well.

The increase in synthesis and decrease in degradation of σ^S suggest that its accumulation during phosphate starvation is important, but the function of increased σ^S under these conditions is not known. Increased σ^S is found after many stress treatments, including those that induce other, dedicated repair and recovery pathways (such as oxidative stress). σ^S induction may provide a general stress response, before committing the cell to the full-fledged specific response. In some agreement with this, a recent report suggests that high levels of σ^S negatively affect most of the phosphate starvation genes, possibly by competition with the primary σ factor, σ^{70}, for core RNA polymerase (Taschner et al. 2004); this paradoxical effect might reflect a need to delay the PhoB-dependent response. σ^S positively controls the expression of *pstS*, which encodes the high-affinity inorganic phosphate transporter Pst; transport of phosphate through this system may contribute to the block in induction of the Pho system (Taschner et al. 2004).

IraP-independent control of σ^S stability

IraP regulates σ^S turnover during phosphate starvation and participates in the stabilization of σ^S during nitrogen starvation and stationary phase (Figs. 1C, 2C; data not shown). However, IraP is not needed for σ^S stabilization during glucose starvation (Fig. 2B). These results indicate that other mechanisms or pathways, independent of IraP, participate in the control of σ^S turnover.

It is unlikely that RssB activity is titrated by a high amount of σ^S in glucose-starved cells because the rate of σ^S synthesis is very low in these conditions (McCann et al. 1993; Lange and Hengge-Aronis 1994; Zgurskaya et al. 1997; Mandel and Silhavy 2005). The simplest explanation would be the existence of another anti-RssB regulator, which would be induced or become active specifically during glucose starvation and would play a similar role to IraP (Fig. 6). Additional evidence for other regulators is suggested by the stabilization of σ^S in cells carrying an *hns* mutation (Yamashino et al. 1995; Y. Zhou and S. Gottesman, in prep.), encoding the histone-like protein H-NS, which represses expression of many genes. The stabilization of σ^S in the *hns* mutant is not affected by Δ*iraP* (data not shown), suggesting that at least one other anti-RssB regulator is repressed by H-NS.

The regulation of σ^S degradation results from the integration of many input signal pathways (Hengge-Aronis 2002). IraP clearly controls σ^S stabilization, and its overexpression leads to a significant accumulation of σ^S. Mandel and Silhavy (2005) pointed out that starvations for different nutrients do not trigger identical responses. We believe that starvation for different nutrients and different stress conditions elicit specific signal transduction pathways converging to regulate σ^S stability. These pathways may overlap to some extent because we observed that IraP stabilizes σ^S in more than one condition, although the most pronounced effect was upon phosphate starvation.

Regulating proteolysis in bacteria

Intracellular proteases play a critical role in maintaining cell homeostasis by clearing damaged proteins and controlling the levels of many proteins, including global regulators (Gottesman 2003). Regulated proteolysis allows the cell to adjust to changing conditions by changing the rate of turnover of some of these key regulators. In eukaryotes, the complex ubiquitin ligase machinery provides a target for regulating proteolysis of specific proteins under specific conditions. In bacteria, no ubiquitin tagging exists, and a small number of ATP-dependent proteases are responsible for degradation of all unstable proteins, frequently by directly interacting with the substrate protein. In some cases, adaptors such as RssB provide the link to the protease and the presumed target for environmental regulation, but how RssB was able to respond to many different signals was not understood. Anti-adaptors, of which IraP is a defining example, provide the missing link in understanding this important regulatory circuitry.

Regulated proteolysis of important transcriptional regulators is found in other bacteria, including *Bacillus subtilis* and *Caulobacter crescentus* (for review, see Jenal and Hengge-Aronis 2003). A parallel to our findings with IraP and RssB is found in the regulated degradation of ComK, a central regulator of competence genes in the naturally competent bacterium *B. subtilis* (for review, see Hamoen et al. 2003). ComK is degraded in exponentially growing cells but not in stationary phase; degradation requires the ClpCP protease, related to ClpXP, and the adaptor protein MecA (Turgay et al. 1998; Persuh et al. 1999). A small protein, ComS, is made in stationary phase and required for the development of competence (D'Souza et al. 1994; Hamoen et al. 1995). In vitro, ComS disrupts the ternary complex of MecA–ClpC–ComK through a direct interaction with MecA (Turgay et al. 1997, 1998; Ogura et al. 1999). Presumably disruption is required in vivo to save ComK from degradation, thus allowing the development of competence. Thus, ComS, like IraP, functions as an anti-adaptor, regulating proteolysis under specific environmental conditions.

The examples of ComS and IraP suggest that environmental regulation of proteolysis in bacteria may be mediated in many cases by anti-adaptor proteins. If the small size of these proteins (46 amino acids for ComS, 86 amino acids for IraP) is typical for the anti-adaptors, they

may be poor targets for mutagenesis and may, in some cases, not be annotated on genome maps. *comS* is encoded within the ORF of another gene, making it even harder to predict computationally (Hamoen et al. 1995). Multiple anti-adaptors made under somewhat different conditions may also obscure the detection of strong phenotypes unless precisely the right environmental conditions are tested. The approach taken here of looking for overproduction phenotypes has allowed us to identify IraP and suggest that IraP and ComS are the founding members of a large family of important regulatory molecules.

Materials and methods

Bacterial strains and plasmids

The genotypes of *E. coli* K12 strains and plasmids used in this work are described in Table 1. All the strains used are derivatives of MG1655, made by P1 transduction as indicated, selecting for the appropriate antibiotic-resistant marker. CRB316, a derivative of MG1655 containing a P_{BAD}-*rpoS-lacZ* translational fusion integrated into the *lac* site, was a gift from C. Ranquet (National Institutes of Health, Bethesda, MD) (C. Ranquet and S. Gottesman, in prep.).

To construct the deletion/insertion mutation of *iraP*, the λ Red recombination system was used as described (Yu et al. 2000). Briefly, a PCR fragment was obtained by amplifying either the kanamycin or chloramphenicol resistance cassettes of the strain MC4100, *ybeW*::GBKn cassette, obtained from J.M. Ghigo (Institut Pasteur, Paris, France), and SG30024 (*clpP*::*cm*) respectively. The antibiotic resistance cassettes were amplified by PCR using primers yaiBHRL-catF (5′-aatgttaacttctccatactttggataaggaaataca gacTACCTGTGACGGAAGATCAC-3′) and yaiBHRR-catR (5′-ccgtgacaactttatgacagctgttgaaaagattaactttGGGCACCAATAACT GCCTTA-3′) for amplification of the *cat* cassette and with the primers yaiBHRL-KnF (5′-aatgttaacttctccatactttggataaggaaatac agac AAAGCCACGTTGTGTCTCAA-3′) and yaiBHRL-KnR (5′-ccgtgacaactttatgacagctgttgaaaagattaactttTTAGAAAAACTCATC GAGCA-3′) for amplification of the kn cassette. The capital letters of the primers above are identical to the 5′ and the 3′ ends of the respective antibiotic resistance cassettes. The rest of the primers are homologous to the regions flanking the coding sequence of *iraP*. The resulting PCR products were recombined into the chromosome of strain NM1100 (N. Majdalani, National Institutes of Health, Bethesda, MD), a Δ*lac* derivative of MG1655 carrying the mini-λ prophage which encodes the λ Red functions (Court et al. 2003). The transformed cells were selected on LB plates containing either 25 µg/mL Cm or 50 µg/mL Kn at 32°C. Recombinant products were verified by sequencing, and the mutations were transferred into MG1655 by P1 transduction.

Plasmid pIraP was constructed by amplifying the *iraP* region by PCR using the primers yaiBregionF (5′-GATTGTTG CAATCTTCTGCTG-3′) and yaiBregionR (5′-GCTGTTG AAAAGATTAACTT-3′). The resulting PCR fragment, encompassing the *iraP* chromosomal region with 387 nt upstream of the *iraP* start codon and 21 nt downstream of the stop codon, was cloned into PCRII-TOPO (Invitrogen). The resulting plasmid was digested with SacI and XbaI, and the resulting linear fragment containing *iraP* was cloned into pHDB3, cut with the same restriction enzymes. The resulting plasmid was called pIraP.

Plasmid pP_{LlacO}-*iraP* was constructed by amplifying the coding sequence of *iraP* by PCR using the primers AatII+1-RBS-ATG-yaiBF (5′-TAAGACGTC*A*GCGGATAACAATTTCACAC ATAATTCATT**AAAGAGGAG**AAATTAACTATGAAAAATC TCATTGCTGAGTT-3′) and EcoRI-yaiBregionR (5′-TGGGAA TTCGCTGTTGAAAAGATTAACTT-3′). These primers amplify the entire ORF of *iraP*, adding the optimal ribosome-bind-

Table 1. *Strains and plasmids*

Strain or plasmid	Genotype	Reference or source
Strains		
MG1655	Wild-type *E. coli*	
DJ480	MG1655 Δ*lac* X74	D. Jin (National Cancer Institute, Frederick, MD)
DJ624	DJ480 *mal*::$lacI^q$	D. Jin (National Cancer Institute, Frederick, MD)
MJM282	MC4100 *rssA2*::*cm*	(Mandel and Silhavy 2005)
CRB316	MG1655 Δ*lacI-lacZ*::P_{BAD}-*rpoS990'-'lacZ*	C. Ranquet and S. Gottesman, in prep.
AB002	CRB316 *clpP*::*cm*	This study[a]
AB003	CRB316 *rssB*::*tet*	This study[a]
AB006	MG1655 Δ*iraP*::*kn*	This study[a]
AB007	CRB316 Δ*iraP*::*cm*	This study[a]
AB009	DJ624 Δ*iraP*::*kn*	This study[a]
YN868	DJ480 $rssB_{D58P}$	Y. Zhou and S. Gottesman, in prep.
AB010	YN868 Δ*iraP*::*kn*	This study[a]
AB011	CRB316 *rssA2*::*cm*	This study[a]
AB012	MG1655 *rssB*::*tet*	This study [a]
AB013	AB006 *rssB*::*tet*	This study[a]
Plasmids		
pQE-80L	Amp^R	Qiagen
pQE-IraP-His_6	pQE-80L/*iraP-his*$_6$	This study
pQE-IraPL9S-His_6	pQE-80L/*iraPL9S-his*$_6$	This study
pHDB3	Amp^R	(Ulbrandt et al. 1997)
pIraP	pHDB3/*iraP*	This study
pP_{LlacO}	pBR322/P_{LlacO}	(Guillier and Gottesman 2006)
pP_{LlacO}-*iraP*	pBR322/P_{LlacO}-*iraP*	This study

[a]Made by P1 transduction.

ing site (bold) of the expression vector pQE80 (Qiagen) and two restriction sites, AatII and EcoRI. The start codon of IraP is underlined and the expected +1 of transcription is in italics. After digestion with AatII and EcoRI, the PCR product was introduced into the AatII and EcoRI sites of pBR-plac (referred to here as pP_{LlacO}) (Guillier and Gottesman 2006). The resulting plasmid, called pP_{LlacO}-*iraP*, contains an artificial promoter P_{LlacO} (Lutz and Bujard 1997) driving the transcription of *iraP*.

To construct the plasmid pQE-IraP-His_6, the coding sequence of the *iraP* gene was cloned in a His_6 tag pQE-80L vector (Qiagen), as follows. We PCR-amplified the coding region of *iraP* using the chromosomal DNA from the MG1655 strain as template and the primers EcoRI-rbs-yaiBF (5′-ATCGGAATTCATTAAAGAGGAGAAATTAACTatgaaaaatctcattgctgagttgttat-3′) and PstI-3*-6hisSGRG-yaiBR (5′-ATAACTGCAGTCAGCTAATTAAGCTTAGTGATGGTGATGGTGATGCGATCCTCTTCCctgacgaggatgcttcaataact-3′). The sequences corresponding to *iraP* are indicated in lowercase letters and the sequence coding for GRGSHis_6 directly at the C-terminal end of IraP is underlined. The PCR fragment was digested by EcoRI and PstI and cloned into pQE-80L cut by the same enzymes, yielding the pQE-IraP-His_6 plasmid.

All plasmid sequences were checked by sequencing.

Media and growth conditions

Cells were grown in LB or morpholinepropanesulfonic acid (MOPS) minimal medium (Teknova) supplemented with 0.4% glucose, 0.2% $(NH_4)_2SO_4$, 1.32 mM K_2HPO_4, and 1 µg/mL thiamine. MacConkey agar plates with 1% lactose, containing ampicillin when used with plasmids, were used in analyses of strains carrying the P_{BAD}-*rpoS990′-′lacZ* chromosomal fusion. All liquid cultures were grown under aerobic conditions at 37°C, and growth was monitored by measuring the optical density at 600 nm (OD_{600}). Exponential phase and stationary phase correspond to an OD_{600} of ~0.3 and ~3, respectively. Stationary phase cells were obtained 2 h after the break in the exponential growth curve.

For starvation experiments, MOPS was made without K_2HPO_4 (phosphate starvation medium), without $(NH_4)_2SO_4$ (nitrogen starvation medium), or without glucose (carbon starvation medium). Cells were first grown overnight in complete MOPS minimal medium, then subcultured into fresh minimal glucose medium at an OD_{600} of 0.01 (~1:400 dilution) and grown to mid-logarithmic phase (OD_{600} of ~0.3) for the following treatments. Cells were washed twice by filtration with prewarmed (37°C) starvation medium, resuspended in the same volume of starvation medium, and incubation at 37°C was continued for 1 h.

Assay for σ^S degradation in vivo

Cells were grown in LB or in MOPS media as described above. Exponential phase refers to an OD_{600} of ~0.3 and stationary phase to an OD_{600} of ~3. Cells were treated with chloramphenicol (200 µg/mL) or tetracycline (100 µg/mL), and 1-mL samples were removed at the indicated time points and treated as described below.

Electrophoresis and immunoblot analysis of proteins

Whole-cell extracts were prepared as follows: Cells grown in LB or MOPS media were assayed for OD_{600}, and 1-mL samples were removed and precipitated with 5% ice-cold tricarboxylic acid (TCA). Precipitated pellets were washed with 500 µL of 80% cold acetone and then resuspended in a volume of sodium dodecyl sulfate (SDS) sample buffer normalized to the OD_{600}. Samples were analyzed using NuPAGE 12% Bis-Tris gels (Invitrogen), transferred, and probed with a 1:4000 dilution of anti-σ^S antiserum, a 1:1200 dilution of anti-RssB antiserum, or a 1:1000 dilution of anti-RGS-His_4 antiserum (Qiagen). The blots were developed with the ECL system (Amersham) and quantified with ImageJ Software (National Institutes of Health).

Library screening

The multicopy plasmid DNA library screen was performed as described previously (Majdalani et al. 2001). Briefly, transformations were performed by electroporation of a pBR322-based library (Ulbrandt et al. 1997) into CRB316, plated on MacConkey lactose plates containing 50 µg/mL ampicillin to give ~500 colonies per plate, and incubated at 37°C. We screened ~15,000 colonies and isolated six red colonies and seven white colonies. Plasmid DNA was purified using Qiagen's Minipreps kit, and insert junctions were sequenced using primers pBRlib.for (5′-CCTGACGTCTAAGAAACCATTATTATC-3′) and pBRlib.rev (5′-AACGACAGGAGCACGATCATGCG-3′).

Isolation of point mutants in iraP by random mutagenesis

Random mutagenesis of *iraP* was performed at a low mutation rate (three to 16 mutations per kilobase) using the GeneMorph II EZClone Domain Mutagenesis Kit (Stratagene) according to the manufacturer's specifications. A mutant plasmid library of *iraP* was generated using pQE-IraP-His_6 as template and the primers RMiraP-HisF (5′-CAGAATTCATTAAAGAGGAGAAATTAACTATG-3′) and RMiraP-HisR (5′-GTGATGCGATCCTCTTCC-3′). XL10-Gold ultracompetent cells were transformed with 1.5 µL of EZclone reaction mixture. The transformed cells were cultured in a total volume of 150 mL of LB supplemented with 100 µg/mL ampicillin for 10 h at 37°C. Plasmid library DNA was isolated using HiSpeed Plasmid Maxi Kit (Qiagen) and eluted with 600 µL of 1× TE buffer.

The screen for nonfunctional mutants of *iraP* was done by electroporation of the *iraP* mutant plasmid library (see above) into AB011, a strain carrying the chromosomal P_{BAD}-*rpoS990′-′lacZ* fusion and a *rssA2::cm* mutation, which leads to overexpression of *rssB* from the chromosome (Ruiz et al. 2001; Mandel and Silhavy 2005). When transformed with pQE-80L, AB011 gave white colonies (Lac^-), whereas transformation with pQE-IraP-His_6 gave red colonies (Lac^+) on MacConkey plates supplemented with 1% lactose and 50 µg/mL ampicillin. We used this phenotype to isolate mutations within the *iraP* coding sequence. Among ~10,000 red colonies, 14 white clones were identified. The plasmids isolated were transformed again into AB011, and all gave the expected Lac^- phenotype. To discriminate between full-length nonfunctional mutants of IraP and truncated proteins due to the introduction of stop codons, colony-immunoblot against the C-terminal RGS-His_6 tag was performed using an anti-RGS-His_4 antibody (Qiagen) according to the manufacturer's instructions. The RGS-His_6 tag was detected for five clones out of the 14 white colonies isolated. Sequence analysis showed that four plasmids each contained a single substitution; all were in the 5′ region of the coding sequence of *iraP* (the most conserved region); three of these, L4P, L9S, and A13D, were in fully conserved residues, and one, A6T, was in a partially conserved residue. Only the L9S substitution, which seemed to be the tightest mutant, was further studied; this mutation changes a TTA codon to TCA.

Proteins

Overproduction and purification of IraP-His_6 and IraPL9S-His_6 were performed as follows: The DH5α strain (Invitrogen), trans-

formed with pQE-IraP-His_6 or pQE-IraPL9S-His_6, was grown in LB medium containing 100 µg/mL ampicillin to an OD_{600} of 0.6. IPTG was added to 1 mM. After 5–6 h of induction, cells were harvested and stored at –80°C until use. Cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole at pH 8.0) and sonicated. The lysates were centrifuged at 10,000 × g for 30 min at 4°C, and the soluble fractions were applied to a Ni^{2+}-NTA column (Invitrogen) equilibrated with lysis buffer. The column was washed with 10 column volumes of buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole at pH 8.0), followed by elution with 250 mM imidazole, in buffer A. The eluates were desalted using an Amersham Biosciences PD-10 column equilibrated with 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 100 mM KCl, and 0.1 mM DTT. Glycerol was added to a final concentration of 10% (v/v), and the samples were stored at –80°C. Final preparations of IraP-His_6 and IraPL9S-His_6 were >90% pure as judged by SDS-PAGE.

ClpX (Grimaud et al. 1998), ClpP (Maurizi et al. 1994), and RepA(Δ25)-SsrA (Sharma et al. 2005) were isolated as described. σ^S was isolated from cells carrying pLHN14 and expressing σ^S (Nguyen et al. 1993) using a described procedure (Tanaka et al. 1993) with some modifications. RssB was purified as described, with minor modifications (Zhou et al. 2001). His_6-GFP-SsrA was isolated by Talon resin (Clontech) column chromatography as described by the manufacturer. All proteins were>90% pure as determined by Coomassie staining following SDS-PAGE. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin as a standard. Throughout, proteins are expressed as moles of RssB monomers, ClpX hexamers, ClpP tetradecamers, σ^S monomers, His_6-GFP-SsrA monomers, IraP-His_6 monomers, and IraPL9S-His_6 monomers.

Assay for σ^S degradation in vitro

Reaction mixtures were assembled in a 20-µL final volume of buffer (20 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 140 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5% glycerol [v/v], 0.005% Triton X-100 [v/v]) containing 5 mM ATP, 10 mM acetyl phosphate, σ^S (20 pmol), IraP (20–80 pmol), RssB (1 pmol), $ClpX_6$ (2 pmol), and $ClpP_{14}$ (2 pmol), unless otherwise indicated. All of the proteins were diluted into the reaction buffer containing 0.05% Triton X-100 before use. The mixtures were incubated at room temperature for 30 min, and the reactions were stopped by the addition of 20 µL of SDS sample buffer. Protein samples were analyzed by SDS-PAGE. SeeBlue Plus2 (Invitrogen) prestained protein standards were used for molecular weight estimation.

Protein–protein interaction assay

Fifty microliters of Ni^{2+}-NTA magnetic agarose beads (Qiagen) was added to 500 µL of interaction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM $MgCl_2$, 0.005% Tween 20, 20 mM imidazole at pH 7.5) containing 900 pmol of IraP-His_6 or IraPL9S-His_6. After 1 h at 4°C with agitation, the beads were incubated with bovine serum albumin (30 mg/mL) for 15 min, washed with 600 µL of interaction buffer, and resuspended with 200 µL of the same buffer. RssB (10 pmol) or σ^S (10 pmol) was added and, after incubation for 1 h at room temperature, the supernatant was removed and the beads were washed with 600 µL of interaction buffer. The proteins were eluted with 50 µL of interaction buffer containing 0.05% Tween 20 and 250 mM imidazole. Fifty microliters of SDS sample buffer was added to the eluted protein, and 20 µL of the mixture was analyzed by immunoblot. RssB, σ^S, IraP-His_6, and IraPL9S-His_6 were detected by immunoblot analysis using antiserum against RssB, σ^S, and RGS-His_4, respectively.

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Modulating RssB activity: IraP, a novel regulator of σ^S stability in *Escherichia coli*

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