Targeting of csgD by the small regulatory RNA RprA links stationary phase, biofilm formation and cell envelope stress in Escherichia coli

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Summary

RprA is a small regulatory RNA known to weakly affect the translation of σ^S (RpoS) in Escherichia coli. Here we demonstrate that csgD, which encodes a stationary phase-induced biofilm regulator, as well as ydaM, which encodes a diguanylate cyclase involved in activating csgD transcription, are novel negatively controlled RprA targets. As shown by extensive mutational analysis, direct binding of RprA to the 5'-untranslated and translational initiation regions of csgD mRNA inhibits translation and reduces csgD mRNA levels. In the case of ydaM mRNA, RprA base-pairs directly downstream of the translational start codon. In a feedforward loop, RprA can thus downregulated > 30 YdaM/CsgD-activated genes including those for adhesive curli fimbriae. However, during early stationary phase, when csgD transcription is strongly activated, the synthesis of csgD mRNA exceeds that of RprA, which allows the accumulation of CsgD protein. This situation is reversed when csgD transcription is shut off – for instance, later in stationary phase or during biofilm formation – or by conditions that further activate RprA expression via the Rcs two-component system. Thus, antagonistic regulation of csgD and RprA at the mRNA level integrates cell envelope stress signals with global gene expression during stationary phase and biofilm formation.

Introduction

Post-transcriptional regulation by small RNAs has been found ubiquitously in bacteria and eukaryotes. Most bacterial small RNAs base-pair to target mRNAs and thereby affect translation and/or mRNA turnover. So far, more than 80 small RNAs have been experimentally demonstrated in Escherichia coli (Waters and Storz, 2009). For the present study the 105 nucleotide RNA RprA is of particular interest (Fig. S1). When overproduced, RprA can activate the synthesis of σ^S (RpoS) (Majdalani et al., 2001), the stationary phase and general stress sigma subunit of RNA polymerase (RNAP) (Hengge, 2011). RprA activates translation by an anti-antisense mechanism that relieves intramolecular base-pairing of rpoS mRNA in the translational initiation region (TIR) (Majdalani et al., 2002; McCullen et al., 2010). Although RprA is a major small RNA (Argaman et al., 2001; Wasarman et al., 2001; Majdalani et al., 2002), its physiological role has remained enigmatic. In contrast to RprA overproduction, a rprA knockout mutation hardly affected σ^S levels, and additional targets of RprA have not been identified.

Two lines of evidence suggested that RprA may have a function in the context of stationary phase and biofilm control: (i) the only known RprA target, rpoS mRNA, encodes the master regulator of stationary phase gene expression, which also activates the expression of biofilm components such as adhesive curli fimbriae (summarized in Hengge, 2011), and (ii) RprA expression is activated by the RcsC/RcsD/RcsB two-component signal transduction pathway, which is involved in biofilm maturation (Majdalani and Gottesman, 2005).

RcsC, RcsD and RcsB constitute a phosphorelay signalling pathway, which in enteric bacteria affects several stress responses, biofilm formation and capsule synthesis. The Rcs system is activated by numerous stimuli that include cell envelope perturbations, high osmolarity, desiccation, low temperature and growth on surfaces, but the underlying mechanisms are poorly understood. The membrane-bound histidine sensor kinase RcsC can activate the response regulator RcsB by phosphotransfer via the HPT protein RcsD. In the absence of appropriate stimuli, RcsC acts as a phosphatase for RcsB and...
thereby actively downregulates the output of the pathway (for a comprehensive review, see Majdalani and Gottesman, 2005). As a DNA-binding transcription factor, phosphorylated RcsB directly activates not only rprA (Fig. S1), but a number of genes involved in stress responses and biofilm formation, including genes involved in the production of the biofilm matrix polysaccharide colanic acid (Gottesman et al., 1985; Ferrières and Clarke, 2003; Hagiwara et al., 2003; Francez-Charlot et al., 2005; Castanie-Cornet et al., 2010; Krin et al., 2010). On the other hand, RcsB can directly repress flhDC which encodes the master regulator for flagella expression (Francez-Charlot et al., 2003).

Overall, the Rcs system can thus downregulate motility, maintain or increase cellular levels of σ^5 and modulate biofilm formation (Majdalani and Gottesman, 2005).

Global gene expression patterns are similar in stationary phase and in biofilms (Schembri et al., 2003; Beloin et al., 2004), and σ^5 is required for the expression of many genes involved in biofilm formation (Hengge, 2011). One of these genes is csgD, which encodes an important early biofilm regulator (Römling, 2005). Apart from σ^5-containing RNAP, csgD transcription also depends on the diguanylate cyclase YdaM and the transcription factor MlrA, which are expressed from σ^5-dependent genes as well (Römling et al., 1998; Brown et al., 2001; Weber et al., 2006; Pesavento et al., 2008). Among the CsgD-regulated target genes are the structural genes for adhesive curli fimbriae (csgBA) and yaiC which encodes a diguanylate cyclase involved in cellulose biosynthesis (Römling, 2005). For simplicity, this complex transcriptional cascade is referred to as the ‘curli control cascade’ in the following (summarized in Fig. S2).

CsgD is also regulated by the Rcs phosphorelay system, but in contrast to the Rcs-mediated input into σ^5 control, CsgD is negatively controlled (Vianney et al., 2005). Downregulation of CsgD and curli expression can be triggered by the expression of YmgB, a small protein that acts via the Rcs pathway (Tschowri et al., 2009). The observation that this effect of YmgB could be suppressed by knocking out the rprA gene (Fig. S3) suggested that CsgD regulation by RcsB occurs indirectly via the RcsB-controlled small RNA RprA.

We therefore decided to study the molecular function of RprA in csgD and curli regulation in closer detail. Here we report that RprA directly interacts with the 5′-regions of both csgD mRNA and ydaM mRNA. Thereby, RprA can directly reduce csgD mRNA levels and translation and, indirectly via YdaM, reduce transcription of csgD. However, during entry into stationary phase, strong expression of excess csgD mRNA overcomes inhibition by RprA, which allows the synthesis of CsgD protein and the activation of more than 30 genes including those for curli fimbriae. Yet, activation of the Rcs two-component system resulting in only about fourfold induction of RprA is sufficient to completely shut off the expression of CsgD and its target genes.

Overall, csgD mRNA and the small RprA RNA cooperate to inversely control a large CsgD/RprA regulon that co-ordinates early stationary phase and biofilm gene expression with the Rcs cell envelope stress response.

Results

**Mutations in the Rcs phosphorelay system and RprA oppositely affect the expression of rpoS and genes in the curli control cascade**

In order to gain a first overview of the influence of the Rcs/RprA system in the curli control cascade, we systematically analysed the effects of single and double knockout mutations in the rcs genes and rprA on translational lacZ reporter fusions in various regulatory and target genes in this cascade (Fig. 1). Mutations in rcsC and rcsB actually have opposite effects, since in the absence of RcsC-dependent phosphatase activity, RcsB can be activated via ‘cross-talk’ from other unidentified phosphodonor (Majdalani and Gottesman, 2005). Consistent with activation of rpoS expression by RprA (Majdalani et al., 2001; 2002), rpoS expression was somewhat increased in the rcsC mutant, which could be suppressed by secondary mutations in either rcsB or rprA (Fig. 1A).

By contrast, two downstream target genes of the σ^5/CsgD control cascade, csgB and yaiC, showed the opposite pattern. These genes were strongly downregulated in the rcsC mutant, and this inactivation was completely relieved by knocking out rcsB or rprA (Fig. 1B and C). Among the regulatory genes in the curli control cascade, ydaM was similarly affected (but the effects were weaker than for csgB and yaiC; Fig. 1D) while mlrA expression was unaltered (data not shown). These data indicated that downstream of the positively RprA-regulated master regulator σ^5, there must be at least one negatively regulated RprA target in the curli control cascade. ydaM seemed one candidate, but the much stronger effects of the rcs/rprA mutations on the CsgD targets csgB and yaiC pointed to csgD expression as a possible major RprA target. Moreover, this was also in line with suppression of the effect of the YmgB/Rcs pathway on CsgD and curli expression by a mutation in rprA (Tschowri et al., 2009 and Fig. S3).

**RprA downregulates the expression of the biofilm regulator CsgD**

Therefore we analysed how the same rcs/rprA mutations affected the cellular levels of csgD mRNA, RprA and the CsgD and σ^5 (RpoS) proteins during entry into stationary phase (Fig. 2A). In the rcsC mutant, csgD mRNA as well as CsgD protein were strongly reduced, whereas RprA accumulated to higher levels than in the parental strain.
under these conditions, also a previously reported degradation product of RprA became apparent, i.e. RprA60–105; Argaman et al., 2001). The long ‘ladder’ of incomplete csgD mRNA fragments are 5′-end fragments since the Northern blot probe used here was complementary to nucleotides −148 to +90 of csgD mRNA. These data show that increased expression of RprA in the rcsC mutant correlated with strongly reduced expression of csgD. σS levels, however, were hardly affected (Fig. 2A), consistent with the relatively weak effects seen with the rpoS::lacZ translational fusion (Fig. 1A).

When assayed along the growth cycle (Fig. 2B), growth phase-dependent expression of CsgD was clearly apparent. csgD mRNA and traces of CsgD protein appeared around an OD578 of 3, and were maximal around an OD578 of 4, i.e. during early stationary phase. Notably, csgD mRNA as well as CsgD protein were no longer present later in stationary phase, i.e. in an overnight culture, whereas RprA as well as σS persisted (Fig. 2B). Disappearance of csgD mRNA and CsgD protein equally occurred in a rprA mutant background, and is therefore not caused by RprA but probably by a shutdown of csgD transcription and possibly CsgD proteolysis (Fig. 2B). With cells growing for several days as patches on agar plates, i.e. in surface biofilms, a similar pattern was observed. Both csgD mRNA and RprA were present after 6 and 24 h of growth, but csgD mRNA was no longer found after 72 h, whereas RprA continued to be present (Fig. 3).

When RprA was constitutively overproduced from a plasmid, csgD mRNA as well as CsgD protein were absent (Fig. 2C). When overproduced, also the truncated form of RprA (RprA60–105), which corresponds to the highly conserved region of RprA (Fig. S1), was sufficient to downregulate csgD mRNA and CsgD protein (Fig. 2C).

Overall, these data demonstrate that RprA can downregulate csgD expression at the mRNA level, even when only moderately overproduced (such as in the rcsC mutant).

RprA reduces expression of CsgD and YdaM also when expressed from ectopic promoters

In order to study a putative direct interaction of RprA with its target(s) in the curli control pathway, we used a con-
A convenient test system in which both the small RNA of choice and its putative target gene are constitutively expressed from ectopic promoters on compatible plasmids. The target gene is fused to gfp, which allows to use the level and/or activity of the Gfp hybrid protein as a simple readout of the system (Urban and Vogel, 2007). Using an ectopic promoter for expression of these gfp fusion constructs (with the same 5′-ends as present in the wild-type mRNAs) uncouples target gene expression from putative complex indirect effects of RprA, such as RprA acting on rpoS and ydaM (Fig. 1A and D), with σ5 activating ydaM transcription, and both activating csgD transcription (Fig. S2). Direct effects on target mRNAs, however, are maintained. As shown for csgD and ydaM (Fig. 4B), the gfp fusions reproduced the effects of RprA overproduction seen with translational single copy lacZ fusions in ydaM and the CsgD target genes csgB and yaiC (Fig. 4A), whereas the other regulatory gene in the curli control pathway, mlrA, was not affected. These data suggested that RprA may directly act on the mRNAs of csgD and ydaM.

RprA interferes with translation and reduces csgD mRNA levels by direct interaction

The wild-type csgD transcript as well as the csgD::gfp mRNA used here both contain the 148 nucleotide 5′-untranslated leader region (5′-UTR). Predictions of interaction between RprA and the csgD leader revealed extensive complementarity in two regions termed sites I and II (Fig. 5A). Region I, which extends from positions −119 to −84 of csgD mRNA and is interrupted into two half-sites (Ia and Ib) by a small loop region (−107 to −99), is complementary to a region between nucleotides 60 and 97 in RprA (termed anti-sites Ia and Ib). csgD mRNA region II (from −14 to +7) overlaps with the TIR and is

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complementary to nucleotides 28 to 45 of RprA (anti-site II). Regions I and II on csgD mRNA are separated by a stretch of 70 nucleotides that was shown to form a long stem-loop structure (Holmqvist et al., 2010) (Fig. 5A).

In order to sort out how these regions of complementarity might contribute to RprA-mediated regulation of csgD, we isolated a series of 5′- and internal deletions in the 5′-UTR as well as point mutations in regions I and II on the csgD::gfp constructs (Fig. 5B). With these constructs, we assayed both mRNA and protein levels in the absence and presence of the RprA-overproducing plasmid (Fig. 6). The 5′- as well as precise internal deletions (Fig. 6A, left and right panel respectively) demonstrated that deleting site Ia alone (construct −103/+90, left panel lanes 7 and 8) or Ib alone (construct −148/+90 Δ−87 to −96, right panel, lanes 17 and 18) did not relieve strong downregulation of csgD::gfp mRNA and CsgD::Gfp protein by RprA. However, eliminating the entire site Ia/Ib was sufficient to generate increased amounts of CsgD::Gfp protein (constructs −83/+90 and −36/+90, left panel lanes 9, 10 and 11, respectively, and −148/+90 Δ−87 to −119, right panel lanes 19, 20). csgD::gfp mRNA levels were also increased, although not to the level seen in the absence of the RprA plasmid, suggesting that translation is reduced only when transcript levels fall below a certain threshold. Deleting the long stem-loop region between −29 and −82 of csgD mRNA, which leaves intact sites Ia/Ib and II, did not affect the ability of RprA to downregulate csgD::gfp mRNA and CsgD::Gfp protein (last two lanes in Fig. 6A).

As site II contains the translation initiation site, its role in a putative interaction with RprA could not be studied by simple deletion. We therefore isolated precise exchanges in nucleotides that are predicted to interact with RprA but that are not part of either the Shine-Dalgarno (SD) site or the initiation codon [CC(−1,4)GG, see Fig. 5A]. On its own, this exchange in site II did not affect RprA-mediated downregulation of CsgD::Gfp (Fig. 6B). However, when combined to a 4 bp exchange in site Ib [CAGC(−87to−90)GTCG] or a deletion of the entire site Ib (Δ−87 to −96), which by themselves also had no effects, translational inhibition was relieved (Fig. 6B). This indicated that regions I and II both contribute to, but to some extent are also functionally redundant in RprA-mediated regulation.

In order to genetically demonstrate a direct interaction between RprA and csgD::gfp mRNA, we isolated changes in RprA that are complementary to the point mutations in site Ib and site II, i.e. to CAGC(−87to−90)GTCG and CC(−1,4)GG (mutations 1 and 3 in Fig. 5A). Combining these mutant constructs (as well as their wild-type counterparts) with all mutant/wild-type versions of RprA revealed a clear suppression pattern (Fig. 6C). Consistent with the data described above, any single mutation alone (either in the csgD construct or in RprA), i.e. a single mismatch in putative base-pairing, did not significantly relieve RprA-mediated downregulation of csgD::gfp expression. Combining any two mutations (either both in csgD or in RprA or ‘crosswise’ combinations of mutations in both) that would result in two putative mismatches relieved downregulation of csgD::gfp. However, combinations of two mutations in csgD and RprA that would restore the putative base-pairing also restored downregulation of csgD::gfp. Moreover, the effects of both csgD mutations together could be successively suppressed by combining them with either one or both complementary mutations in RprA (Fig. 6C). These data are a clear indication of direct base-pairing between csgD::gfp mRNA and RprA both in sites I and II.

This result was further corroborated by suppression obtained with another mutation in site II (TTT(−5to−7)AAA; mutation 2 in Fig. 5A) and the complementary exchange in RprA (Fig. S4). In addition, we observed that this TTT/AAA exchange resulted in elevated CsgD::Gfp protein levels (in the absence of the RprA plasmid; Fig. S4). This mutation in
site II actually disrupts a small stem-loop structure which overlaps with the TIR (blue nucleotides in Fig. 5A, structure shown in Fig. S4) and reduces translation as recently shown (Holmqvist et al., 2010).

In conclusion, these data indicate that: (i) an extensive region in the 5′-UTR of csgD mRNA (sites Ia/Ib and II) is involved in a partially redundant manner in RprA-mediated downregulation of csgD expression; (ii) RprA interacts directly with csgD mRNA at these sites; (iii) this interaction does not only reduce translation but also csgD mRNA levels; and (iv) the long stem-loop region between -29 and -83 of csgD mRNA is not involved in the action of RprA and does not seem to generally affect csgD expression under the conditions tested here.

RNase III, RNase E and Hfq are not essential for RprA-mediated downregulation of csgD mRNA

Small RNAs that reduce target mRNA levels usually do so by stimulating endonucleolytic attack, either by allowing double strand specific RNase III to cleave in the duplex region which allows further rapid decay of the resulting fragments (Arraiano et al., 2010), by specifically recruiting the target mRNA to a Hfq-RNase E complex (Morita et al., 2005; Pfeiffer et al., 2009; Caron et al., 2010) or by allowing RNase E to access RNA that is not protected by ribosomes when translation initiation is inhibited by the sRNA (Massé et al., 2003; Chen et al., 2004; Afonyushkin et al., 2005; Wagner, 2009). In order to test whether RNase III, RNase E or Hfq are involved, we assayed the effect of RprA on csgD mRNA in the corresponding rnc, rne and hfq mutants. While a specific small csgD mRNA fragment was degraded in the RprA-overexpressing strains in an RNase III-dependent manner, RprA-mediated downregulation of full size and all other fragments of csgD mRNA was independent of RNase III (Fig. S5). Since RNase E is essential, we used a temperature-sensitive rne mutant (Goldblum and Apririon, 1981). At the non-permissive temperature, the RprA effect on csgD mRNA was slightly less pronounced but still

Fig. 5. The 5′-region of csgD mRNA and RprA, their putative interaction sites and relevant genetic constructs.
A. The part of csgD mRNA showing complementarity to RprA (sites Ia, Ib and II) and including the putative stem-loop between nucleotides -83 and -28 [interaction was predicted with the RNAhybrid2.2 program (Rehmsmeier et al., 2004); numbering refers to the translational start site on csgD mRNA] as well as full-size RprA in its putative folded structure are shown [secondary structures predicted by Mfold (Zuker, 2003); numbering is from the 5′ to the 3′ end]. Regions in RprA likely to interact with csgD mRNA are highlighted in red (anti-sites Ia, Ib and II). Blue nucleotides indicate a region in csgD mRNA that can form a small stem-loop structure that includes the TIR. Numbers in circles refer to mutated nucleotides as indicated.
B. Positions of deletions and point mutations that were isolated on pSB25, which carries the -148 to +90 region of csgD inserted precisely behind the P_Lud promoter and fused to gfp. Numbers in the names of the fusion constructs refer to the nucleotide sequence in (A).
csgD and ydaM are novel RprA targets

[Diagram A: Diagram showing mRNA and anti-sites for RprA binding sites]

[Diagram B: Table listing RprA binding sites and related constructs]

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clearly visible (Fig. S6), suggesting that RNase E may somewhat contribute to, but is not essential for RprA-mediated downregulation of \textit{csgD} mRNA. Also a mutation in \textit{hfq} relieved downregulation of \textit{csgD} by RprA only slightly (Fig. S7), suggesting that Hfq plays an auxiliary role in the formation of the RprA/\textit{csgD} mRNA complex, but is not essential for downregulating \textit{csgD} mRNA.

\textbf{Fig. 6.} Genetic identification of regions in the 5'-UTR/TIR of \textit{csgD::gfp} mRNA relevant for RprA-mediated regulation and evidence for direct interaction of the two RNAs. In an \textit{rprA::kan} derivative of MC4100, effects of RprA overproduction were assayed in the presence of 5'- and internal deletions (A) and point mutations (B) in the 5'-UTR/TIR of \textit{csgD::gfp} mRNA. In (C) all possible combinations of point mutations in either site I or site II (or both) of \textit{csgD::gfp} mRNA with RprA carrying either one or both complementary exchanges in anti-site I or II are shown. Allelic changes and their locations are symbolized by pairs of black (wild-type sequence) or white circles (mutant sequences), with the left and right circles indicating the status of site I/anti-site I and site II/anti-site II respectively. 'co' stands for the control plasmid (pJV300, which expresses a small nonsense RNA). Cells were grown in LB at 37°C to an OD\textsubscript{578} of 4.0. mRNA and protein levels of the \textit{csgD::gfp} reporter fusion were determined by Northern and immunoblot analyses respectively.
Moreover, this implies that RprA can affect CsgD expression not only directly, but also indirectly via YdaM.

**CsgD and RprA tightly cooperate in controlling global gene expression**

Our identification of the csgD and ydaM mRNAs as novel direct targets for RprA also raised the question whether RprA has even more targets. In addition, since CsgD is itself a transcriptional regulator, CsgD-controlled genes may constitute a significant or even major subset of the RprA regulon.

Using microarray-based transcriptome analysis, we identified the regulons controlled by RprA and CsgD and the extent to which these overlap. During entry into stationary phase (in LB at 28°C), when csgD mRNA physiologically accumulates to high levels, comparing csgD+ and csgD strains revealed a large regulon of predominantly positively CsgD-regulated genes including the curli operon csgBA (Fig. 8, Table S1). While RprA is also expressed under these conditions (Fig. 2), knocking out rprA did not reveal any genes with significant differential regulation on the microarrays (data not shown), consistent with the very minor effects also on the expression of rpoS and known CsgD target genes (see Figs 1 and 2). These results indicate that when wild-type cells just enter into stationary phase and are not subject to any other stress, the ongoing RprA synthesis is not sufficient to significantly reduce mRNA levels of csgD or affect any other putative target genes.

However, what would be the consequences for global gene expression if RprA was further induced in response to some conditions that may further activate the Rcs system? In order to simulate this situation we again used the rcsC mutant, in which RcsB is activated and RprA shows approximately fourfold higher expression (Fig. 2A), and compared this strain to the rcsC rprA double mutant. This allowed us not only to identify RprA-regulated genes (with a majority being under negative RprA control), but revealed an almost complete and inverse overlap between the RprA and CsgD regulons, i.e. negatively RprA-regulated genes were found to be under positive control of CsgD and vice versa (Fig. 8, Table S1). This pattern was even apparent for genes that were just weakly regulated by one regulator and below the threshold of significance (set to ratios > 0.5 or < 0.2) for the other (Table S1). The only clear exception to this pattern seemed to be gadE and the gadBC operon, which exhibited positive regulation by RprA and only very weak negative effects of CsgD. This suggested that RprA may be able to positively target gadE, which encodes a regulator that activates gadBC expression. rpoS and ydaM did not show up as RprA target genes in the microarray data, probably because higher levels of RprA in the rcsC mutant do not affect these mRNAs strongly enough (Fig. 1).

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Taken together, these data allow several conclusions: (i) RprA-regulated genes expressed during entry into stationary phase are largely identical to CsgD targets and thus constitute a common ‘CsgD/RprA regulon’ inversely controlled by these two regulators; (ii) when wild-type cells enter into stationary phase, RprA is expressed but functionally silent and the large set of genes under positive control of csgD is expressed; (iii) this set of genes as well as csgD itself are negatively regulated by RprA, when RprA is expressed at only moderately higher levels (as in the rcsC mutant); (iv) while most effects on global gene expression of RprA are thus linked to altered levels of csgD mRNA and/or CsgD protein, gadE seems to be RprA-regulated in a csgD-independent manner and therefore may be an additional direct target for RprA besides csgD, ydaM and rpoS.

**Discussion**

A regulator of regulators – the small regulatory RNA RprA modulates the expression of several globally acting transcription factors

With this study we have identified csgD and ydaM as novel direct targets for the small regulatory RNA RprA (Figs 3–6). In addition, our data provide preliminary evidence that also gadE (Table S1) is controlled by RprA in a csgD-independent and possibly direct manner. RprA regulation of ydaM sets up a feedforward loop (FFL), which combines direct downregulation of csgD by RprA at the mRNA level with indirect downregulation via YdaM, which encodes a diguanylate cyclase producing c-di-GMP that is essential for csgD transcription initiation (Weber et al., 2006). This FFL arrangement may enhance and speed up csgD downregulation under sudden stress conditions incompatible with proper assembly of the amyloid curli fibres.

The other RprA targets, i.e. rpoS, csgD and possibly gadE, all encode globally acting transcription factors. With σ2 (the product of the rpoS gene) activating the transcription of csgD as well as of gadE in complex feedforward cascades, these regulators are the key components of a regulatory network that co-ordinates global gene expression during stationary phase, biofilm formation and stress conditions (summarized in Hengge, 2011). Within this transcriptional network, RprA introduces differential control at the mRNA level by maintaining or further stimulating the expression of σ2 and GadE while downregulating that of YdaM/CsgD when required.

The small regulatory RNA RprA directly binds to csgD mRNA, interferes with translation and reduces the cellular level of csgD mRNA

Our finding that RprA also downregulates csgD when expressed from an ectopic promoter (Figs 4, 6 and S4) as Fig. 8. The RprA and CsgD regulons during entry into stationary phase. E. coli K-12 strain W3110 and its derivatives were grown in LB at 28°C to an OD578 of 4 and genome-wide transcriptome analysis was performed as detailed in Experimental procedures. The following strains were compared: for the RprA regulon, rcsC::cat versus rcsC::cat rprA::kan; for the CsgD regulon: csgD+ versus DcsgD. Ratios of differential gene expression (listed in Table S1) were transformed into a ‘heat map’ as described in Experimental procedures, with (A) showing genes under negative control (green) by RprA and positive control (red) by CsgD, and (B) showing oppositely regulated genes; genes were ordered by the magnitude of RprA dependence.

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well as phenotypic compensation by complementary nucleotide exchanges in the two RNAs (Figs 6C and S4) indicate that RprA directly binds csgD mRNA. This interaction involves separate regions of extensive RprA complementarity on csgD mRNA (Fig. 5A): (i) site I (−119 to −84) consists of two parts, la and lb, separated by a small loop region; (ii) site II (−14 to +7) overlaps with the TIR and is clearly required for translational inhibition by RprA (Figs 5 and S4). These segments act in a partially redundant manner, as introducing deletions or point mutations in either site la, lb or site II alone did not reduce the effects of RprA on csgD mRNA or CsgD protein. However, introducing the same lesions in two of the three regions (no matter in which combinations) alleviated translational inhibition, indicating that RprA binding was no longer strong enough to efficiently interfere with ribosome entry (Fig. 5). RprA interacts directly with the SD region and also reduces its cellular level so efficiently that csgD mRNA can hardly be detected upon even moderate overproduction of RprA (Fig. 2). This downregulation is directly linked to the interaction of these two RNAs and reduced csgD mRNA translation, since it occurs also with csgD::gfp mRNA expressed from an ectopic promoter, and lesions in csgD mRNA and/or RprA that relieve translational inhibition do also relieve transcript downregulation (Figs 6 and S4). Even in the absence of RprA, csgD mRNA levels are actually sensitive to a variation in the rate of translation, as can be seen with the −83/+90 construct (Fig. S4). Here, the TTT(−5t0−7)AAA mutation, which eliminates a small translation-inhibitory stem-loop structure (Holmqvist et al., 2010) that overlaps with the csgD TIR region (blue nucleotides in Fig. 5A, structure shown in Fig. S4C), results in an about twofold increase in CsgD::Gfp protein levels and several fold higher csgD::gfp mRNA level (Fig. S4A).

In principle, at least two molecular mechanisms could account for RprA-mediated regulation of csgD mRNA levels: RprA binding to the 5′-part of csgD mRNA and inhibition of csgD translation initiation may (i) stimulate mRNA turnover and/or (ii) induce premature termination of transcription. Since site I of the 5′-UTR of csgD mRNA, which features a particularly long stretch of complementarity to RprA, emerges from RNAP right after transcription initiation, RprA may bind to the nascent csgD transcript already and stimulate its co-transcriptional endonucleolytic cleavage and decay. However, although small RNA-dependent modulation of transcript stability is relatively common (see reviews by Arraiano et al., 2010; Caron et al., 2010), neither the double-strand specific RNase III nor RNase E is essential for RprA to downregulate csgD mRNA, although the RprA effect was somewhat less pronounced in the absence of active RNase E (Figs S5 and S6). This suggests that RNase E contributes to some extent to the effect of RprA on csgD mRNA as it does in the effects of the small RNAs RyhB or MicC on their respective target mRNAs (Massé et al., 2003; Chen et al., 2004; Afonyshev et al., 2005; Wagner, 2009). However, stimulating RNase E-dependent degradation does not seem to be the only mechanism by which RprA acts on csgD mRNA.

In addition, rapid binding of RprA to nascent csgD transcripts may also affect transcript elongation. RNAP and the immediately following, i.e. ‘leading’ ribosome can directly interact via NusG protein which results in a mechanistic coupling of transcription and translation (Burmann et al., 2010; Proshkin et al., 2010; Roberts, 2010). In the absence of such a physically linked ribosome, the elongated RNAP is prone to ‘backtracking’ and premature termination of transcription by termination factor Rho, an effect long known as polarity (Adhya et al., 1974; Franklin, 1974; Richardson et al., 1975). Thus, How does RprA RNA reduce the cellular level of csgD mRNA?

RprA can impose kind of a ‘death kiss’ on csgD mRNA, as it does not only interfere with its translation but also...
excess RprA, by disfavouring ribosome binding to nascent csgD mRNA emerging from RNAP, may increase the frequency of premature termination of nascent csgD transcripts. By contrast, when csgD is expressed in excess over RprA, the majority of nascent csgD transcripts could pick up a ‘leading’ ribosome and these transcripts would be elongated and translated.

RprA-induced degradation as well as premature transcriptional termination of nascent csgD transcripts could equally produce the trail of incomplete csgD 5′-fragments visible on the Northern blots (Fig. 2). Moreover, the two processes are not necessarily mutually exclusive. Finally, since both processes would result from an inhibition of translational initiation, our data also suggest another mechanistic detail. Downregulation of csgD mRNA was also observed with overproduction of the RprA(60–105) fragment, which can base-pair to region Ia/ib only, but not directly to the TIR (Figs 2 and S5). This is consistent with the proposal by Holmqvist et al. (2010) that a region upstream of the csgD TIR contributes to translational initiation as a transient ribosome loading site (Unoson and Wagner, 2007). This proposal was based on the finding that OmrA/B, which inhibits translation initiation of csgD mRNA, binds to the long stem-loop region right next to site Ia/ib and therefore also upstream of the TIR (Holmqvist et al., 2010).

RprA and CsgD mRNA set up an RNA network that underlies the co-ordination of stationary phase, biofilm formation and the cell envelope stress response

When E. coli cells enter into stationary phase or during initial biofilm formation (Figs 2 and 3), csgD mRNA is strongly activated, which allows csgD mRNA as well as CsgD protein to accumulate. While RprA is synthesized in parallel, its knock-out has hardly any effect on the expression of csgD mRNA, indicating that most of these fragments can contribute to trapping RprA. Overall, early stationary phase is thus a ‘CsgD-ON’ state characterized by high levels of csgD mRNA and the synthesis of CsgD protein and a ‘silencing’ of RprA activity even though RprA is actually synthesized.

Our microarray data (Fig. 8, Table S1) show that in this ‘CsgD-ON’ state, a CsgD/RprA regulon is activated that consists of more than 30 genes which are not expressed in a csgD mutant or when csgD expression is downregulated by enhanced RprA synthesis (in the rcsC mutant). The molecular mechanisms for activation of these genes can be rather different. In some cases, which include the csgBA operon (Römling et al., 2000; Ogasawara et al., 2011), CsgD protein can directly control transcription. The mRNAs of some other genes, however, could be direct targets for RprA, which ‘escape’ this control as long as RprA remains trapped by highly expressed csgD mRNA. Some genes of the CsgD/RprA regulon may even be targets for additional csgD mRNA-binding small RNA(s) (such as OmrA/B) that could also be sequestered by highly expressed csgD mRNA. In this scenario, the csgD gene actually has a dual role in global gene expression – its mRNA acts in a RNA network that prevents RprA and perhaps other small regulatory RNAs from affecting their other target mRNAs, while its protein product acts as a transcription factor.

In this RNA network, the status of expression of the entire CsgD/RprA regulon must be highly sensitive to changes in the ratio of expression rates for csgD and rprA. Either upregulation of RprA expression or downregulation of csgD expression (or both at the same time) should result in switching from a ‘CsgD-ON/RprA-OFF’ to a ‘CsgD-OFF/RprA-ON’ state. What is the physiological context in which this may occur? The Rcs system, which activates RprA expression, responds to a variety of cell envelope perturbations (Majdalani and Gottesman, 2005). It is easy to imagine that the massive assembly of curli fimbriae, which is typical for early stationary phase cells, becomes detrimental and has to be shut down when cells have to cope with envelope stress. Another condition, where the ratio of expression for csgD mRNA and RprA shifts in favour of RprA is later in stationary phase (Figs 2 and 3). Here, both csgD mRNA as well as CsgD protein disappear, while RprA expression continues and also ωs protein remains present. That RprA is not required for csgD mRNA and CsgD to disappear (Fig. 2) suggests that csgD transcription is inactivated and CsgD protein is degraded. Upon shutdown of csgD mRNA synthesis, RprA would now be free to bind to other potential targets, i.e. certain mRNAs synthesized late in stationary phase. The same may apply to other small RNAs bound by csgD mRNA such as OmrA/B (see above). Overall, what seems to be emerging here, is a fine-tuned network of directly
interacting mRNAs and small RNAs, in which every player can be a regulator as well as a target. The architecture and physiological impact of this non-hierarchical regulatory RNA network and its multiple connections with the hierarchical transcriptional network of *E. coli* will have to be elucidated in future studies.

**Experimental procedures**

**Bacterial strains and growth conditions**

The strains used in this study are derivatives of the *E. coli* K-12 strains W3110 (Hayashi et al., 2006) and MC4100 (Casadaban, 1976). The otherwise isogenic strains BL322 and BL321 carry the mc wild-type allele encoding endoribonuclease RNase III and a non-functional mc mutant allele respectively (Studier, 1975). The isogenic strains N3433 and N3431 carry the me wild-type allele and an me⁹ allele (rne-3071) respectively; the latter expresses a thermolabile endoribonuclease RNase E that is rapidly inactivated upon shift to the non-permissive temperature of 43°C (Goldblum and Apirion, 1981). The hfg·omega knockout allele was previously described (Tsui et al., 1994). The construction of mutant alleles, plasmids and single copy lacZ and plasmid-encoded gfp reporter fusions is described in detail in Supporting information (including primers shown in Table S2).

Cells were grown in LB medium (Miller, 1972) under aeration at 28°C (strains with wild-type control of csgD) or 37°C (strains in which csgD is under ectopic promoter control). Antibiotics were added as detailed in Supporting information. Growth was monitored by measuring the optical density at 578 nm (OD₅₇₈).

**SDS-PAGE and immunoblot analysis**

Sample preparation for SDS-PAGE and immunoblot analysis were performed as described previously (Lange and Hengge-Aronis, 1994). Three, five or ten micrograms of cellular protein was applied per lane. Polyclonal sera against csgD and CsgD (custom-made by Pineda-Antikörper-Service, Berlin) or a monoclonal antibody against Gfp (Roche), goat anti-rabbit and anti-mouse IgG alkaline phosphatase conjugate (Sigma) and a chromogenic substrate (BCIP/NBT; Boehringer Mannheim) were used.

**Northern blot analysis**

For RNA preparation and Northern blot analysis, cells were grown in LB medium and harvested at an OD₅₇₈ as indicated in the figure legends. TRIZOL reagent (Invitrogen) was used to isolate total RNA according to the manufacturer’s protocol. Northern blot analysis was performed as described previously (Papenfort et al., 2006) with some changes. Five micrograms of total RNA denatured in Ambion loading dye II (Ambion) was separated on 4.5% polyacrylamide gels containing 7 M Urea and transferred to positively charged Nylon Membranes (Roche) by electro-blotting in a tank blotter (Peplab). Northern probes were random Dig-labelled PCR fragments generated with primer pairs listed in Table S2 and Dig labelling mix (Roche) according to the manufacturers protocol. The csgD probe was complementary to the 5’-end of csgD mRNA (nucleotides −44 to +70, and the rprA probe was complementary to full-size RprA. Prehybridization and hybridization of membranes with DNA probes was carried out in Dig Easy Hyb (Roche) at 47°C overnight. Membranes were washed twice at 42°C for 5 min in 2× SSC/0.1% SDS and twice in 0.1× SSC/0.1% SDS for 30 min at room temperature. Detection of Dig-labelled DNA probes was performed after blocking in blocking solution (Roche) with Dig anti-Fab fragments (Roche) and CDP Star (Roche) as described (Mika and Hengge, 2005). Signals were visualized on Lumi films (Roche) with an Optimax Typ TR developing machine.

**Microarray analysis**

For RNA preparation for microarray analysis, cells were grown in LB at 28°C to an OD₅₇₈ of 4.0. Cell lysis, RNA isolation, DNasel treatment and phenol/chloroform extraction were as previously described (Webber et al., 2005). *E. coli* K-12 microarrays containing 4288 gene-specific 50mer oligo-nucleotide probes representing the whole *E. coli* genome (MWG, Ebersberg, Germany) were used. Hybridization with Cy3/5-dCTP-labelled cDNA, fluorescence detection and image analysis were as described in the Supplement to Pesavento et al. (2008). Each microarray experiment was done at least twice (biological replicates; with a dye swap in cDNA labelling). Genes were considered differentially regulated when signal-to-noise ratios exceeded a factor of three, the sum of median intensity counts was above 200, and relative RNA level differences (ratios) were at least twofold in both of the two independent experiments. The original datasets have been deposited in the Array Express database (accession numbers: E-MEXP-2620 for the RprA regulon, E-MEXP-2797 for the CsgD regulon).

Significantly altered gene expression signals (average of the ratios obtained in independent experiments; Table S1) were ordered by their magnitude, and these clustered data were transformed into a ‘heat map’ by the program Mayday 2.9, which provides a platform for visualization, analysis and storage of microarray data (Dietzsch et al., 2006). For visualization (Fig. 8), log₂ values of the signals were used, with a colour range corresponding to values between −4.5 and +4.5.

**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 μmol of o-nitrophenol per min per mg of cellular protein (Miller, 1972). Experiments showing the expression of lacZ fusions along the entire growth cycle were done at least twice, and a representative experiment is shown.

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