Two antisense RNAs target the transcriptional regulator CsgD to inhibit curli synthesis

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Escherichia coli produces proteinaceous surface structures called curli that are involved in adhesion and biofilm formation. CsgD is the transcriptional activator of curli genes. We show here that *csgD* expression is, in part, controlled post-transcriptionally by two redundant small RNAs (sRNAs), OmrA and OmrB. Their overexpression results in curli deficiency, in accordance with the inhibition of chromosomally encoded, FLAG-tagged CsgD. Downregulation of *csgD* occurs by a direct antisense interaction within the *csgD* 5'-UTR, far upstream of the ribosome-binding site (RBS). OmrA/B downregulate plasmid-borne *csgD*-*gfp* fusions *in vivo*, and inhibit CsgD translation *in vitro*. The RNA chaperone Hfq is required for normal *csgD* mRNA and OmrA/B levels in the cell, and enhances sRNA-dependent inhibition of *csgD* translation *in vitro*. Translational inhibition involves two phylogenetically conserved secondary structure modules that are supported by chemical and enzymatic probing. The 5'-most element is necessary and sufficient for regulation, the one downstream comprises the RBS and affects translational efficiency. OmrA/B are two antisense RNAs that regulate a transcription factor to alter a morphotype and group behaviour.

The EMBO Journal (2010) 29, 1840–1850. doi:10.1038/emboj.2010.73; Published online 20 April 2010

Subject Categories: RNA; microbiology & pathogens

Keywords: antisense RNA; bacterial sRNA; curli regulation; Hfq; transcriptional activator

Introduction

Regulatory RNAs are ubiquitously present post-transcriptional regulators in bacteria and eukaryotes (Chapman and Carrington, 2007; Waters and Storz, 2009). Eukaryotic miRNAs, and most of the bacterial small RNAs (sRNAs), act by an antisense mechanism to target mRNAs. In bacteria such as *Escherichia coli*, >80 sRNAs have been experimentally validated. Most of these inhibit expression of target genes, and a few are activators (Waters and Storz, 2009).

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Received: 24 November 2009; accepted: 24 March 2010; published online: 20 April 2010

Initially, sRNAs appeared to target single genes, but multi-targeting of functionally related families of mRNAs has become a recurrent theme in recent years; this applies to, for example, *E. coli* and *Salmonella enterica* outer membrane protein (OMP) mRNAs targeted by RybB and OmrA/OmrB, and transporter mRNAs by GcvB (Guillier and Gottesman, 2006; Vogel and Papenfort, 2006; Sharma *et al.*, 2007). A second emergent pattern is the interplay of transcriptional and post-transcriptional regulation. Several transcription factor (TF)/Sigma factor mRNAs are targeted by sRNAs. Their gene products can in turn affect transcriptional induction of sRNAs, resulting in feedback or feedforward control, and creating sensitive regulatory networks that are implicated in rapid adaptive responses.

Most enterobacterial sRNAs are key players in reprogramming protein expression upon environmental change, in particular under stress conditions. Perhaps most striking are the many sRNAs that regulate outer membrane or surface proteins in *E. coli* and/or *S. enterica* (Mizuno *et al.*, 1984; Chen *et al.*, 2004; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005; Castillo-Keller *et al.*, 2006; Douchin *et al.*, 2006; Guillier and Gottesman, 2006; Papenfort *et al.*, 2006, 2008; Bossi and Figueroa-Bossi, 2007; Pfeiffer *et al.*, 2007). Examples of them are the sequence-related sRNAs RygA/SraE and RygB that had been identified in global screens in *E. coli* (Argaman *et al.*, 2001; Wassarman *et al.*, 2001; Vogel *et al.*, 2003) and were subsequently renamed OmrA and OmrB (OmpR-regulated sRNAs A and B; Guillier and Gottesman, 2006). Their genes reside next to each other between the *aas* and *galR* genes on the *E. coli* chromosome. Expression of OmrA and OmrB in exponentially growing cells is transcriptionally controlled by the EnvZ-OmpR two-component system. When induced, OmrA and OmrB decrease the steady-state levels of several OMP mRNAs including *cirA*, *fecA*, *fepA* and *ompT* (Guillier and Gottesman, 2006). More recent work showed that *cirA* and *ompT*, as well as *ompR*—the transcriptional activator of *omrA* and *omrB*—are direct antisense targets (Guillier and Gottesman, 2008).

A biocomputational target search algorithm (J Reimegård, unpublished data) suggested *csgD*, the first gene within the *csgDEFG* operon, as a putative direct target of both OmrA and OmrB. CsgD is a LuxR-type TF that is required for transcriptional activation of the divergent *csgBA* operon (Hammar *et al.*, 1995). CsgA and CsgB are the components of proteinaceous fibres on the cell surface called curli. Both curli and cellulose, another extracellular matrix component that is dependent on CsgD, are involved in adhesion and biofilm formation (Römling, 2005; Barnhart and Chapman, 2006). CsgD also activates transcription of *adrA* (Zogaj *et al.*, 2001); AdrA is a diguanylate cyclase that synthesizes cyclic di-GMP (c-di-GMP), a second messenger that stimulates cellulose production (Simm *et al.*, 2004). Expression of cellulose and curli give rise to the rdar morphotype, a red, dry and rough colony morphology on Congo red agar plates (Römling, 2005).

In this paper, we address the regulation of *csgD* by OmrA and OmrB. Overexpression of either of the two sRNAs decreases CsgD levels, which in turn abolishes curli formation and cellulose production. The mechanism by which inhibition is accomplished involves a phylogenetically conserved target structure far upstream of the *csgD* translation start site and appears to be distinct from translational control mechanisms reported so far. In terms of biological significance, the OmrA/B-dependent regulation of a key TF adds to the complexity of cell surface/outer membrane remodelling processes in adaptive responses and presents two redundant antisense RNAs that affect control of a morphotype and group behaviour.

Results

The *csgD* mRNA is a predicted target for OmrA and OmrB RNAs

A target prediction program developed in our group (Reimegård J, unpublished data), based on searches for sRNA-target RNA complementarity that incorporate structural accessibility and phylogenetic conservation of RNA pairs, suggested a region within the leader of the *csgD* mRNA as a high-scoring interaction site for the sequence-related OmrA and OmrB sRNAs (Supplementary Figure S1). The putative antisense interaction is 19 (OmrB) or 18 (OmrA) base-pairs long and involves the 5'-tails of the two sRNAs. Interestingly, the predicted target region is located in the middle of the ≈ 150 nucleotide long *csgD* leader, far upstream of the translation initiation region (see below). Thus, sRNA binding is unlikely to sterically interfere with initiating ribosomes.

Overexpression of *omrA* or *omrB* inhibits curli and cellulose production

CsgD is required for the formation of curli in *E. coli* (Hammar *et al*, 1995). If OmrA/B were to interact with *csgD* mRNA, this could result in either inhibition or activation, and thus we tested first whether ectopic overexpression of the sRNAs would downregulate curli production. A wild-type and a $\Delta\text{omrA/B}$ strain, carrying plasmids encoding either *omrA* or *omrB*, was plated on Congo red indicator plates; *E. coli* K-12 strains that express curli fibres form red colonies, whereas lack of curli gives white colonies. Figure 1A shows that the *E. coli* strain MC4100 *relA*⁺ harbouring the control plasmid was curli proficient (red), whereas constitutive overexpression of either of the two sRNAs inhibited curli formation (white). Moreover, as the observed effect was the same in the $\Delta\text{omrA/B}$ strain, either one of the two sRNAs alone was capable of inhibiting curli production (Figure 1B). This implies functional redundancy with respect to the sRNA-dependent repression of curli. CsgD also activates cellulose production, although indirectly. Cellulose proficiency can be monitored by growing bacteria on plates supplemented with the dye calcofluor (Zogaj *et al*, 2001). The *E. coli* strain MC4100 does not produce cellulose, and therefore we used the fecal strain TOB1, which expresses both curli and cellulose (Bokranz *et al*, 2005). TOB1 with plasmids that overexpressed *omrA* or *omrB* formed colonies with less intense fluorescence, indicating reduced cellulose production (Supplementary Figure S2). Thus, both OmrA and OmrB can inhibit the synthesis of the two extracellular matrix components curli and cellulose.

Figure 1 Overexpression of OmrA or OmrB inhibits curli production. *E. coli* strain MC4100 *relA*⁺ (A) and its ΔomrAB derivative (B) harbouring either of the plasmids pControl, pOmrA or pOmrB was streaked on Congo red agar plates and grown at 28°C for 48 h.

OmrA and OmrB downregulate chromosomally encoded *CsgD*

The experiments in Figure 1 monitored phenotypic effects that are consistent with *csgD* as the primary sRNA target. To test whether the two sRNAs indeed target the expression of *csgD*, we constructed a ΔomrAB strain with a C-terminal 3xFLAG tag sequence in the chromosomal *csgD* ORF. This tag does not compromise CsgD function indicated by formation of red colonies on Congo red plates (data not shown). Plasmids with *omrA*, *omrB*, or lacking inserts, were transformed into this strain, and CsgD-3xFLAG was monitored by western analysis using GroEL as loading control. Clearly, high levels of OmrA or OmrB severely decreased CsgD synthesis (Figure 2A, upper panel). Northern analysis showed, as expected, low endogenous levels of OmrA and OmrB in the wild-type strain, their absence in the ΔomrAB strain, and high levels in the presence of the overexpression plasmids (Figure 2A). Real-time RT-PCR showed that *csgD* mRNA levels were also severely decreased upon *omrA* or *omrB* overexpression (Figure 2B). As CsgD is the transcriptional activator of the *csgBA* operon (Hammar *et al*, 1995), we measured the effect of *omrA* or *omrB* overexpression on *csgBA* mRNA levels. OmrA/B-dependent downregulation of CsgD resulted in essentially undetectable levels of *csgBA* mRNA (Figure 2C). As CsgA and CsgB are the components of curli fibres, this accounts for the white colony phenotype (Figure 1).

OmrA and OmrB inhibit *csgD* expression directly by an antisense mechanism

Bioinformatics suggested *csgD* to be a direct target of OmrA/B. However, the above experiments cannot exclude that downregulation of *csgD* expression by OmrA/B occurred through inhibition of the expression of the transcriptional activator OmpR; OmpR is a known OmrA/B target (Guillier and Gottesman, 2008) and promotes *csgD* transcription. To experimentally assess the putative base-pairing interaction *in vivo* and to avoid complicating effects of OmpR, we constructed a plasmid-borne *csgD::gfp* translational fusion preceded by the constitutive $P_{\text{LtetO-1}}$ promoter (Urban and Vogel, 2007). When OmrA or OmrB was supplied from a second, compatible plasmid, inhibition of plasmid-encoded wild-type CsgD::GFP was observed (Figure 3B). Mutations of nucleotides within the predicted interaction sites, -67 to -64 (CTTC to GGAG) in the *csgD* 5'-UTR and nucleotides 5 to 8 (GAGG to CTTC) in *omrA* and *omrB*, were introduced to assess base-pairing (mutation M4; Figure 3A). The experi-

Figure 2 OmrA and OmrB overexpression downregulates *csgD* and *csgA*. *E. coli* strain MC4100 *relA*⁺ *csgD*::3xFLAG $\Delta omrAB$ harbouring plasmids as indicated were grown in LB at 28°C. Samples for protein and RNA analysis were withdrawn at an OD₆₀₀ value of 2.5. (A) Western blot analysis of chromosomally encoded CsgD-3xFLAG and GroEL, and Northern blot analysis of OmrA, OmrB and 5S rRNA levels. *csgD* mRNA (B) and *csgA* mRNA (C) levels determined by qRT-PCR.

ments were conducted in a $\Delta omrA/B$ strain background. As seen in Figure 3B, all mismatched combinations of OmrA, or OmrB, and target sequences (e.g. *omrA*-wt/*csgD*-M4, *omrA*-M4/*csgD*-wt etc.) failed to significantly decrease CsgD-GFP levels. Importantly, efficient inhibition was obtained when plasmid pCsgD-M4::GFP was challenged with either pOmrA-M4 or pOmrB-M4. As the combination of both mutations

Figure 3 Regulation requires an antisense interaction between OmrA/B and *csgD* mRNA *in vivo*. (A) Base-pair mutations introduced in OmrA, OmrB and *csgD*. (B) MC4100 *relA*⁺ $\Delta omrAB$ harbouring plasmids as indicated was grown in LB at 37°C and samples were withdrawn at an OD₆₀₀ value of 0.5. Western blot analysis of CsgD-GFP and GroEL (loading control). (C) *In vitro* translation of *csgD*-3xFLAG or *csgD*-M4-3xFLAG mRNAs in the absence or presence of OmrB, OmrB-M4 or MicA RNAs (see Materials and methods for details). Western blot analysis shows CsgD-3xFLAG protein levels. The asterisk indicates an unspecific cross-hybridization with a protein in the extract and serves as loading control.

restored regulation, we conclude that OmrA and OmrB inhibit expression of *csgD* by an antisense mechanism that requires direct sRNA-mRNA base-pairing.

To address the question of whether additional factors may be required, or whether the antisense interaction alone is sufficient for regulation, we used an *in vitro* assay that supports translation of added mRNAs and permits assessment of inhibition by including the sRNAs (Maki *et al*, 2008). Thus, transcription does not occur in the reaction mixture, and all added RNAs are stable throughout the incubation (Sharma *et al*, 2007 and data not shown). The *csgD*-3xFLAG mRNA was provided as translation template. Figure 3C shows that sRNA-dependent inhibition of CsgD-3xFLAG translation is faithfully recapitulated, as is the sequence specificity of interaction: upon addition of OmrB RNA to a concentration of 1 μ M ($\approx$ 12-fold molar excess over mRNA), the CsgD translation rate was strongly reduced. In line with the results obtained *in vivo*, base-pair mutations abolished regulation whereas the combination of *csgD*-M4 mRNA and OmrB-M4 restored inhibition (Figure 3C). An unrelated sRNA, MicA (Udekku *et al*, 2005), had no effect.

We also conducted a toeprint analysis. *In vitro* transcribed *csgD* mRNA, incubated in the presence or absence of OmrA, OmrB, MicA or MicF, was assayed for formation of

Figure 4 Specific inhibition of initiation complex formation by OmrA/B. Toeprinting was carried out as described (see Materials and methods). An autoradiogram of this assay is shown, with additions of components indicated. The position of the *csgD*-specific toeprint is indicated, as is the position of the RNA duplex that results in a reverse transcription block. Sequencing ladders were generated with the 5'-end-labelled primer used in toeprints.

30S translation initiation complexes by primer extension. The autoradiogram in Figure 4 shows a 30S/tRNA_{fMet}-dependent toeprint 15/16 nt downstream of the *csgD* start codon, which was inhibited by OmrA and OmrB, but not by two control sRNAs, MicA and MicF. In the assays where OmrA/B were present, base-pairing to their target region far upstream is indicated by a strong reverse transcription stop (Figure 4).

The *in vivo* and *vitro* results thus confirm that binding of the sRNA to its target mRNA is direct and entails inhibition of CsgD synthesis. The *in vitro* experiments further suggest that translational inhibition suffices to account for the effect, and mRNA degradation is not required. Finally, auxiliary factors appear not to be needed, although sRNA efficiency might be modulated by proteins absent from this assay (see below).

Deletion of *hfq* results in low *csgD* mRNA, OmrA and OmrB levels

Many chromosomally encoded antisense RNAs require the Sm-like RNA-binding protein Hfq for regulation *in vivo* (Brennan and Link, 2007). As OmrA and OmrB can be co-immunoprecipitated with Hfq (Wassarman *et al*, 2001; Zhang

Figure 5 Hfq effects on antisense regulation of *csgD-gfp* mRNA *in vivo* and *csgD*-3xFLAG *in vitro*. MC4100 *relA*⁺ $\Delta omrAB$ and MC4100 *relA*⁺ $\Delta omrBA \Delta hfq$ harbouring plasmids as indicated was grown and sampled as in Figure 3. (A) Western blot analysis of CsgD-GFP and GroEL, and Northern blot analysis of OmrA, OmrB and 5S rRNA levels. (B) *csgD-gfp* mRNA levels determined by qRT-PCR. Columns follow the strain order as in (A). (C) *In vitro* translation of *csgD*-3xFLAG and *ompA*-3xFLAG mRNAs in the absence or presence of OmrB and Hfq (see Materials and methods for details). Concentrations of OmrB and Hfq (hexamer) are indicated. Translation of OmpA product is unaffected by OmrB and is used as an internal control for the observed CsgD translation rate effects. The two frames are from the same gel. Relative CsgD levels are given: values in lanes 2–4 and 6–8, are relative to the values in lanes 1 and 5 (both set to unity), respectively.

et al, 2003) and *in vitro* affinity is very high (K_d -value of ≈ 1 nM; Supplementary Figure S3), we tested whether this protein is required for regulatory efficiency. The effect of *omrA/B* overexpression on *csgD::gfp* expression was measured in a pair of isogenic strains, one of which was deleted for the *hfq* gene. Figure 5A shows, as before, that OmrA and OmrB inhibit CsgD::GFP synthesis in the wild-type strain. By

contrast, no such effect is seen in the Δhfq strain. Two points are worth making. First, CsgD-GFP protein and *csgD::gfp* mRNA levels are much lower in a Δhfq background, irrespective of the presence or absence of OmrA/B (Figure 5A and B). As transcription of the *csgD::gfp* mRNA is driven by a promoter that is not affected by Hfq (Urban and Vogel, 2007), this suggests that Hfq is required to stabilize this mRNA, aid its translation which in turn may stabilize the mRNA, or cause the observed effect indirectly via a different sRNA. Second, unlike in the inhibition of chromosomally encoded CsgD-3xFLAG (Figure 2), sRNA-dependent repression of plasmid-encoded CsgD in the wt strain is not accompanied by a severe decrease in mRNA level (Figure 5B). Hence, the direct effect of OmrA/B seen here is on translational inhibition. This is in line with the *in vitro* translation results in Figure 3C and the toeprinting assay in Figure 4. Why the sRNAs fail to regulate in a Δhfq strain cannot be conclusively explained. However, it is apparent that Hfq, probably by binding (Wassarman *et al*, 2001), protects both sRNAs from degradation; OmrA and OmrB signals are strongly decreased in the Δhfq strain (Figure 5A, cf. OmrA/OmrB wt and OmrA/OmrB Δhfq), in good agreement with an earlier report (Guillier and Gottesman, 2008). Low intracellular concentration of the sRNAs alone might account for poor inhibitory efficiency, but additional Hfq-dependent effects on binding rate are not excluded.

The effect of Hfq on sRNA-dependent regulation of CsgD expression was also tested in the *in vitro* translation system. Increasing concentrations of OmrB, either alone or prebound

to Hfq, were added to *csgD*-3xFLAG mRNA and preincubated for 5 min, followed by translation for 30 min. All reactions contained equal concentrations of *ompA*-3xFLAG mRNA so that OmpA protein synthesis could serve as a loading control for quantification. In the presence of Hfq, OmrB efficiently inhibited CsgD synthesis even at the lowest concentration; in absence of Hfq, inhibition was quite ineffective (Figure 5C). At higher OmrB concentrations, Hfq was not required for inhibition of CsgD synthesis (Figures 3C and 5C). The results thus indicate that Hfq increases the regulatory efficiency of OmrB on the *csgD* target *in vitro*.

OmrA and OmrB bind to the *csgD* mRNA leader *in vitro*

Binding between 5'-end-labelled OmrA/B and a molar excess of unlabelled *csgD* mRNA was assayed by gel shift analysis. Both sRNAs form complexes with the target RNA. Binding as a function of concentration or of incubation time is shown in Supplementary Figure S3. As several other chromosomally encoded antisense RNAs (Udekwi *et al*, 2005), OmrA and OmrB exhibit low association rate constants of $\approx 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Supplementary Figure S3 and data not shown). To examine the interaction in detail and to map the sRNA-binding site, we used footprinting. 5'-end-labelled *csgD* mRNA (Figure 6A) or OmrB (Figure 6B) was incubated with or without an excess of the unlabelled complementary RNA partner, followed by partial digestion with RNase T1 or lead(II) acetate as indicated. T1 cleaves after G residues in single-stranded RNA regions, and lead(II) cleaves the RNA backbone in unstructured regions (Lindell *et al*, 2002). On the *csgD* leader RNA,

Figure 6 Footprinting of *csgD* mRNA–OmrB interaction *in vitro*. **(A)** Partial enzymatic and chemical cleavage of 5'-labelled *csgD* mRNA (nts 1–130 relative to the transcription start site) in the presence or absence of unlabelled OmrB. The predicted interaction region is highlighted by yellow squares. Blue squares indicate increased accessibility to probes on OmrB binding. Numbers: G residues relative to the *csgD* AUG start codon; C: mock-treated control; OH: alkaline ladder; T1L: RNase T1 digest under denaturing conditions; T1: RNase T1 digest under native conditions; Pb: lead(II) acetate. **(B)** Partial lead(II) cleavage of 5'-labelled OmrB with or without unlabelled *csgD* mRNA. Yellow square: predicted interaction region. Numbers: G residues relative to the 5'-end of OmrB. Triangle: increasing *csgD* mRNA concentrations according to Materials and methods. **(C)** Schematic drawing of structure changes in the *csgD* 5'-UTR on OmrA or OmrB binding. Nucleotides in yellow show the interaction sequences in *csgD* mRNA and OmrB. Blue nucleotides highlight sequences in *csgD*, which on OmrB interaction are rendered single stranded. The *csgD* SD sequence and the AUG start codon are indicated by red boxes.

OmrB-dependent protection was observed across the region predicted to participate in base-pairing (yellow box, Figure 6A); protection from T1 cleavage was seen at G₋₆₂ and G₋₆₁, and protection from lead(II) ranges from nucleotides -69 to -63. The remaining nucleotides that are predicted to take part in the interaction (nt -80 to -69) were already protected in the absence of OmrB. They appear to be involved in intramolecular base-pairing with nucleotides -41 to -30, as this sequence becomes accessible to cleavage upon OmrB binding (blue box, Figure 6A). This indicates that OmrB-*csgD* mRNA pairing forces the opening of a local stem-loop structure indicated schematically in Figure 6C. Addition of increasing concentrations of *csgD* mRNA to end-labelled OmrB mapped the interaction site to the predicted region within the 5'-tail (yellow box, Figure 6B). Protection from lead(II) cleavage was seen from nt 9 to 19. Altogether, the protections observed on both RNAs suggest 19 base-pairs to be formed.

Expression and regulation of *csgD* involves distinct modules

Secondary structure prediction (CONTRAFold; Do *et al*, 2006) of the *csgD* leader sequence reveals two structure elements that are compatible with *in vitro* probing results (Figure 6; Holmqvist E, unpublished data), and moreover are supported by phylogenetic comparisons. An analysis of the most divergent enterobacterial sequences by Alifold (Hofacker *et al*, 2002) shows conservation of these elements despite significant nucleotide replacements, deletions and insertions, relative to *E. coli* K12 (Supplementary Figure S4). The upstream structure (stem-loop 1/SL1, nt -80 to -30 in *E. coli*) has two stems interrupted by a large bulge (Figure 6C; Supplementary Figure S4). The OmrA/B target sequence stretches from the bottom of the lower stem and extends throughout the bulge. The G₋₆₂G₋₆₁ dinucleotide, complementary to the sRNA 5'-end at which binding likely initiates, is accessible in this bulge (Figure 6A), and progression then frees the complementary 3'-side of the stem. The downstream stem-loop (SL2, nt -10 to +16) sequesters the Shine-Dalgarno (SD) sequence. Its predicted ΔG° -value of ≈ -8.2 kcal/mol at 37°C (RNAeval; Hofacker, 2003) suggests that initiation of translation should be ineffective. Indeed, destabilization of the lower stem of SL2 (mutation SL2M1; Figure 7A) resulted in higher synthesis of CsgD::GFP fusion protein *in vivo* (Figure 7C) but sRNA-mediated regulation was unaffected. Removal of the 5'-most 66 nucleotides (pCsgD₋₈₃::GFP; Figure 7B) from the *E. coli* sequence did not significantly change *csgD*::*gfp* expression or control. To investigate the effect of the upstream structure on CsgD synthesis, the 5' part of the *csgD* mRNA was systematically truncated in the context of the *csgD*-*gfp* fusion. 5'-truncations up to 20 nucleotides from the AUG supported significant *csgD*::*gfp* expression; in several cases increased expression was observed (Figure 7B). This suggests that sequences upstream of -20 are not required for translational activity, even though they may affect translation levels. Truncated fusions that lacked the target sequences (-60 through -20; Figure 7B) failed to be regulated by OmrA. To test whether the upstream element carries all determinants needed for regulation, we replaced the authentic ribosome-binding site (RBS) and the entire SL2 by the *luc* SD-start codon sequence fused to *gfp*. Plasmid pCsgD-lucRBS::GFP

Figure 7 Effect of the stem-loop elements SL1 and SL2 on *csgD* expression. (A) Secondary structure representation of the *csgD* 5'-UTR based on structure probing and phylogenetic conservation (S4). Nucleotides in yellow indicates the site of OmrA/B base-pairing. Blue boxes: SD, start and stop codons of the putative leader peptide. The fusion point between the leader peptide and GFP (in LP-GFP) is shown. Red boxes: SD and start codon of the *csgD* and *luc* reading frames, respectively. Numbers refer to 5'-truncation end points in *csgD*::*gfp*-encoding plasmids. The nucleotide substitution in mutation SL2M1 is shown in red. (B, C) Western blot showing expression and regulation from cells carrying fusion plasmids as indicated; GroEL is used as loading control.

shows both expression and regulation (five-fold downregulated compared with 25-fold for pCsgD::GFP; Figure 7C).

We observed that a short, four codon leader ORF is present upstream of *csgD*. Its putative SD sequence (GAGG, -47 to -44), is located within the stable rightward stem of SL1 (Figure 7A), and a putative GUG start codon in the lower stem (-33 to -31). In several control systems, for example control of *fur* by the sRNA RyhB (Vecerek *et al*, 2007) or *repA* by CopA in plasmid R1 (Blomberg *et al*, 1992), an antisense RNA inhibits translation of an ORF, which is translationally coupled to the downstream reading frame. Translation of the leader ORF was tested by fusing nucleotide -22 to *gfp*

in-frame (plasmid pLP::GFP) and showed no expression in the presence or absence of OmrA/B (Figure 7C).

Thus, it appears that the *csgD* leader is comprised of two modules, the target structure SL1, including the unpaired bulge, which is required for regulation, followed by SL2 whose stability affects translational efficiency.

Discussion

In this report, we show that two *E. coli* sRNAs (OmrA and OmrB) control expression of the key TF CsgD that is required for production of curli and, indirectly, cellulose. Some sRNAs that regulate TFs have been reported earlier. Three sRNAs (DsrA, OxyS and RprA) regulate the expression of the stationary phase/stress Sigma factor RpoS in response to distinct environmental signals. In addition, DsrA and OxyS also regulate two other TFs, HNS and FhlA, respectively (for a review see Repoila *et al*, 2003). Regulatory proteins can also be direct targets of sRNAs. For instance, the sRNAs CsrB and CsrC in *E. coli* (and many related sRNAs in other bacteria) sequester the CsrA protein, a translational repressor, thereby causing complex effects on morphogenetic traits such as biofilm formation and group behaviour (Babitzke and Romeo, 2007). The present report gives an example of an antisense RNA that affects such traits through regulation of surface structures.

A biocomputational search algorithm suggested the *csgD* mRNA as a direct target of the two sequence-related sRNAs OmrA and OmrB. Overexpression of either of the two sRNAs resulted in white colony formation on Congo red plates, indicative of bacteria that fail to produce curli (Figure 1). On sRNA overexpression, chromosomally encoded *csgD* was downregulated at both the mRNA and protein level (Figure 2). Mutational analysis showed that, *in vivo* and *in vitro*, downregulation of *csgD* is direct and requires an antisense interaction between sRNA and mRNA (Figure 3), in line with *in silico* predictions (Supplementary Figure S1). Direct interaction was further substantiated by gel mobility shift assays (Supplementary Figure S3) and structure mapping of the interaction *in vitro* (Figure 6). Binding of the sRNA entails opening of a stem structure in the mRNA, rendering this segment single stranded.

A peculiarity of OmrA/B-*csgD* regulation is the unexpectedly long distance between the target sequence and the RBS of *csgD* (50 nt between target site and SD), which cannot be accounted for by sRNA-ribosome competition for an RBS. An initiating ribosome protects—through base-pairing to SD and AUG, or through backbone contacts— ≈ 50 –55 nt of mRNA (Hüttenhofer and Noller, 1994). Antisense-target RNA interactions within this region generally interfere with translation initiation; sRNAs often base-pair to SD and/or AUG sequences, but sRNAs that bind immediately upstream of an SD (Malmgren *et al*, 1996) or downstream, within the first five codons of the reading frame (Bouvier *et al*, 2008), also compete directly with ribosomes. As the OmrA/B-binding site is located too far upstream to plausibly explain direct interference with translation initiation, inhibition of *csgD* must involve an alternative mode of action. Several models that do not rely on a direct overlap between sRNA and the RBS can be excluded. Antisense RNA-driven transcriptional attenuation (as established in plasmids pT181 (Novick *et al*, 1989) and pIP501 (Brantl *et al*, 1993)) would entail lower levels of mRNA downstream of the termination site. Likewise,

sRNA-promoted enhanced mRNA decay, irrespective of the details of the mechanism (Masse *et al*, 2003; Morita *et al*, 2005; Pfeiffer *et al*, 2009), also causes decreased mRNA levels. We showed here that CsgD-GFP protein was strongly downregulated without corresponding effects on the mRNA level (Figure 5), that the sRNA decreased the synthesis rate of CsgD in an *in vitro* system in which neither transcription nor degradation occurs (Figure 3), and that it decreased the toeprinting signal at the *csgD* translation start site (Figure 4). Together, these results suggest that translational control can account for the observed regulation.

It should also be noted that sRNA induction decreased *csgD* mRNA from its chromosomal copy concomitantly with CsgD protein levels (Figure 2). As, by contrast, inhibition of the CsgD-GFP fusion protein is not accompanied by decreased mRNA levels (see above), translation control should be the primary effect, as observed earlier in a heterologous system (Morita *et al*, 2006). The sRNA-induced decrease in *csgD* mRNA from its chromosomal copy could be dominated by inhibition of OmpR, the activator required for *csgD* expression; inhibition of OmpR would cause lower transcription levels. Preliminary experiments using a chromosomal *csgD*-3xFLAG gene carrying the M4 mutation (Figure 3) indicates that most of the inhibition of CsgD synthesis is due to direct targeting of *csgD* rather than through OmpR; *csgD* mRNA levels were decreased to $\approx 50\%$ in the presence of OmrA-wt (which matches the *ompR* but not *csgD*-M4 target), but to $\approx 90\%$ by OmrA-M4 (data not shown). We infer that both direct inhibition of CsgD synthesis, and inhibition of *csgD* transcription through inhibition of *ompR*, contribute to the total regulation observed. In a few cases, translational control does involve far upstream targets. An sRNA can inhibit translation initiation either by sequestration of a ribosome standby site (Darfeuille *et al*, 2007), a translational enhancer element (Sharma *et al*, 2007), or by inhibition of leader ORF translation (Vecerek *et al*, 2007). OmrA/B inhibition of CsgD synthesis seems to be yet another case of 'upstream' regulation, although the available data do not appear to fit any of the above models. The upstream stem-loop structure, SL1, is not required for normal CsgD translation rates. Some truncations even increase the output of CsgD (e.g. pCsgD₋₁₉::GFP; Figure 7C), and thus SL1 does not contain a standby site or enhancer (Figure 7). The lack of expression of a putative leader peptide (pLP::GFP; Figure 7) suggests that indirect sRNA control through translational coupling is not involved either.

Thus, we tentatively conclude that sRNA binding to the upstream element affects the initiating ribosome's access to the translation start region of *csgD*. This does not seem to occur by a structural change induced downstream of SL1 (Figure 6). How binding of the sRNA to the upstream target site communicates with the activity state of the downstream element is so far elusive. However, it is apparent that the leader consists of two modules. SL1 confers regulation also to a heterologous, sequence- and structure-unrelated RBS (pCsgD-lucRBS::GFP; Figure 7), even though the fold regulation is less than for the wild-type fusion construct. The structure of the downstream SL2 affects translation efficiency. Its destabilization increases CsgD synthesis. Truncated *csgD* mRNAs whose 5'-ends are located downstream of SL1 are fully translation competent *in vivo* (Figure 7). The details of the inhibition mechanism are currently being investigated in detail.

As many sRNAs, and target mRNAs, *OmrA* and *OmrB* are avidly bound by Hfq (Wassarman *et al*, 2001; Supplementary Figure S3). The results in Figure 5 suggest that regulation of *csgD* is lost in the Δhfq strain. However, this experiment alone does not provide unequivocal support for an Hfq requirement for *OmrA/B*-dependent inhibition of *csgD*. In an *hfq* mutant strain, both *csgD::gfp* mRNA and *OmrA/B* were severely destabilized. This effect is post-transcriptional as expression of the *csgD-gfp* mRNA is driven by a constitutive $P_{\text{LtetO-1}}$ promoter, which is not affected by an *hfq* deletion (Urban and Vogel, 2007). Thus, loss of regulation in the Δhfq strain might reflect sRNA instability (Figure 5), as also observed by others (Guillier and Gottesman, 2008). The higher *csgD-gfp* mRNA levels in the *hfq*-proficient strain could either be indicative of a protection from endonucleolytic cleavages, an Hfq-dependent stimulation of translation, which as a consequence would stabilize the mRNA, or the effect of an (unknown) sRNA that promotes stabilization of *csgD* mRNA. However, sRNA-dependent inhibition of *csgD* mRNA translation *in vitro* is also aided by Hfq (Figure 5C), which suggests that the effect of this protein occurs both on the level of RNA–RNA association and on RNA stabilization.

What biological significance can be attributed to *OmrA/B* regulation of *csgD*? Under sRNA overexpression conditions, *csgD* is severely repressed. When the *csgD* gene is in its normal chromosomal context, this repression (Figure 3) must reflect the combination of at least two effects: one is the direct targeting of the *csgD* mRNA, the other inhibited synthesis of *OmpR* (see above). When $\Delta omrA/B$ and wild-type strains lacking overexpression plasmids are compared, steady-state levels of CsgD are only about two-fold higher (Supplementary Figure S5). Thus, sRNAs from single gene copies have fairly modest effects in bacteria grown at 28°C. This raises the important question when and under which conditions sRNA regulation of CsgD synthesis occurs. In LB medium at 37°C, *omrA* expression is highest in stationary phase, and *omrB* peaks slightly earlier (Argaman *et al*, 2001; Wassarman *et al*, 2001; Zhang *et al*, 2003). In *E. coli* K-12 and its derivatives, curli are only produced at temperatures below 30°C, and the highest expression levels are observed in low-salt medium. However, other *E. coli* strains express *csgD* and produce curli also at higher temperature. To understand the physiological conditions and the time window in which the sRNAs exert their effects on *csgD* and ultimately curli formation, extensive characterization of sRNA expression is needed. The complex transcriptional regulation of the *csgDEFG* operon will also have to be considered, which occurs in stationary phase and involves several global regulators such as RpoS, HNS, IHF and *OmpR* (Barnhart and Chapman, 2006).

The targeting of TF mRNAs by sRNAs, exemplified here by *csgD* mRNA and *OmrA/B*, leads to a so far unexplored possibility. *OmrA/B* from single copy genes give relatively small effects on CsgD levels (Supplementary Figure S5), but the question of possible stochastic effects has not yet been addressed. Through regulatory crosstalk, genes involved in flagella synthesis and motility are inversely regulated with *csgD* expression, which leads to curli production and a predominantly sessile lifestyle in biofilms (Pesavento *et al*, 2008). However, stochastic fluctuations of gene expression are known to occur in individual cells. As several TFs converge on *csgD* expression, bacteria would benefit from

inhibiting inappropriate synthesis of CsgD when transcription is inadvertently ON under physiological conditions that call for motility. In addition, even under biofilm conditions when *csgD* expression is considered to be ON, the population consists of cells with *csgD* expression ON or OFF (Grantcharova *et al*, 2009). Under such circumstances, an inhibiting function could be provided by *OmrA/B*. A role for sRNAs as a scavenger of miscontrolled TF mRNAs might also be applicable to other sRNA/TF mRNA systems. At this point, such a model has not yet been tested. We have initiated experiments that involve single cell monitoring of GFP-tagged CsgD in populations, in dependence of *omrA/B*.

Several important questions remain. What is the relationship between the effects of *OmrA* and *OmrB* on its different targets? For example, does transcriptional upregulation of OMP target mRNAs outtitrate the sRNAs so that other targets (like *csgD*) become less inhibited? A second issue concerns *OmrA/B* redundancy. Whether the two homologues are functionally distinguishable under some conditions remains to be elucidated. Finally, the major challenges lie in the overall physiological significance of *OmrA/B* in the regulation of curli and its associated traits, and in the details of the mechanism of inhibition.

Materials and methods

Chemicals, reagents and oligodeoxyribonucleotides

Chemicals and reagents were purchased from Sigma-Aldrich and Fermentas unless otherwise specified. All oligodeoxyribonucleotides (see Supplementary Table S1) were purchased from Sigma-Genosys.

Bacterial strains, media and growth conditions

All strains used in this study are listed in Supplementary Table S2. Cells were grown aerobically at 28°C or 37°C in L broth, and growth was monitored by measuring optical density at OD₆₀₀. When required, antibiotics were added at 50 µg/ml (ampicillin or kanamycin), 30 µg/ml (chloramphenicol) or 17 µg/ml (tetracycline). Curli expression was monitored on Congo red plates (LA plates without NaCl, 20 µg/ml Congo red, 10 µg/ml Coomassie brilliant blue G). Cellulose expression was monitored on LA plates without NaCl supplemented with 50 µg/ml calcofluor. Congo red and calcofluor plates were incubated at 28°C for 48 h.

The *omrAB* locus was deleted by λ red-mediated homologous recombination (Yu *et al*, 2000) in strain NC397, using a DNA fragment with an FRT-bordered Tc^R cassette PCR-amplified from plasmid pCP16 with primers EHO-245 and EHO-246 (all primers are listed in Supplementary Table S3). The $\Delta omrAB$ locus was moved into strain MC4100 *relA*⁺ by P1-mediated transduction selecting for Tc^R. Removal of the Tc^R cassette was achieved with the helper plasmid pCP20 (temperature-sensitive replication, thermal induction of FLP synthesis; Datsenko and Wanner, 2000). Deletion of the *hfq* gene was carried out similarly, but with the following modifications. Strain E352 (MC4100 *relA*⁺ $\Delta omrAB$) was transformed with plasmid pSIM5, which carry the λ red genes (Datta *et al*, 2006) and electroporated with a PCR fragment containing a Tc^R cassette amplified from pCP16; the PCR primers EHO-317/EHO-318 were used and contained appropriate flanking sequences. Chromosomal C-terminal 3xFLAG-tagging of *csgD* and *ompA* was done using a PCR product (primers EHO-302/EHO-303 and EHO-208/EHO-209, respectively) amplified from pSUB11 according to Uzzau *et al* (2001). The *csgD*-3xFLAG strain is phenotypically identical to the parental strain with respect to plating on Congo red plates. All deletions or insertions were verified by DNA sequencing and Northern blots.

Plasmids

pOmrA and pOmrB were constructed using PCR amplified and *Xba*I-digested pZE12-luc as described (Urban and Vogel, 2007). Inserts were generated by PCR using primers EHO-226 and EHO-227

(*omrA*) or EHO-224 and EHO-225 (*omrB*) and digested with *Xba*I. pOmrA-M4 and pOmrB-M4 were constructed by site-specific mutagenesis (Stratagene, #200518) with primers EHO-365/EHO-366 and EHO-369/EHO-370, respectively.

To construct pCsgD::GFP, a fragment of the *csgD* gene containing positions -148 to +45 (relative to the ATG start codon) was PCR amplified with primers EHO-355/EHO-356. The resulting fragment was digested by *Nsi*I and *Nhe*I and inserted into plasmid pXG-10 as described (Urban and Vogel, 2007). pCsgD-M4::GFP was constructed by site-specific mutagenesis (Stratagene, #200518) with primers EHO-373/EHO-374.

To truncate the 5'-UTR of *csgD* on plasmid pCsgD::GFP, the entire plasmid was linearized by PCR and religated. The forward primer was in all cases EHO-428. Reverse primers were EHO-426 (pCsgD_{-83::GFP}), EHO-481 (pCsgD_{-60::GFP}), EHO-475 (pCsgD_{-41::GFP}), EHO-476 (pCsgD_{-29::GFP}) and EHO-477 (pCsgD_{-20::GFP}). pLP::GFP, pSLM1 and pCsgD-lucRBS::GFP were constructed similarly using primers EHO-440/EHO-482, EHO-423/EHO-425 and EHO-443/EHO-445, respectively.

Northern blot and quantitative RT-PCR

Bacterial cultures were grown to the desired cell density and mixed with 0.2 volumes of RNA stop solution (5% phenol, 95% ethanol) and frozen in liquid nitrogen to stop growth. After thawing on ice, cells were pelleted by 5 min centrifugation at 6000 r.p.m. at 4°C. Total RNA was prepared by the hot acid-phenol method essentially as described (Blomberg *et al*, 1990). The Turbo DNA-free kit (Ambion, #AM1907) was used to remove contaminating DNA. For Northern blotting, 1 µg total RNA was mixed with 2 × RNA loading buffer (95% [v/v] formamide, 0.025% [w/v] bromophenol blue, 0.025% [w/v] xylene cyanol, 5 mM EDTA at pH 8.0, 0.025% [w/v] SDS) and heated 4 min at 95°C. After electrophoresis on 8% polyacrylamide gels with 7 M urea, the gels were electroblotted (Bio-Rad Trans-Blot cell) onto Nylon N+ membranes (GE Healthcare). Prehybridization and hybridization was performed in modified Church and Gilbert hybridization buffer (Church and Gilbert, 1984). Probing with 5'-end-labelled oligodeoxyribonucleotides was carried out at 42°C. For detection of *OmrA*, *OmrB* and 5S rRNA, probes EHO-406, EHO-407 and EC-5S-RNA-GW were used, respectively. Hybridized radioactive probes were visualized with a PhosphorImager model 400S (Molecular Dynamics). Signal intensities were quantified using the ImageQuant software, version 4.2a (Molecular Dynamics).

cDNA was produced using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) followed by quantitative real-time PCR using SYBR[®] Green PCR Master Mix (Applied Biosystems, #4309155) on a 7300 Real-Time PCR System (Applied Biosystems). Relative mRNA levels were determined using the Standard Curve Method (Livak and Schmittgen, 2001) using tmRNA levels for normalization. Primers used were EHO-257/EHO-258 (*csgD*), EHO-253/EHO-254 (*csgA*), EHO-403/EHO-404 (*gfp*) and EHO-269/EHO-270 (tmRNA). All primers were designed using the PRIMER EXPRESS software (Applied Biosystems).

Western blot

Overnight cultures were grown in LB medium with appropriate antibiotics. At the desired optical density, 1 ml of culture was withdrawn and centrifuged for 2 min at 13 000 r.p.m. at 4°C. Pelleted cells were resuspended in sample loading buffer (Fermantas, #R0891) and heated at 95°C for 4 min. Aliquots corresponding to 0.05 OD were separated on 10% SDS-PA gels and transferred to Immobilon-P PVDF membranes (Millipore, #IPVH00010) at 35 mA o/n at 4°C. Membranes were blocked in 10% dry milk in PBST₂₀ (PBS with 0.1% Tween20). Incubation with primary antibodies (monoclonal anti-GFP [Roche, #11814460001], anti-GFP-HRP [Miltenyi Biotec # 130-091-833], anti-FLAG [Sigma #F1804] or HRP-linked anti-GroEL [Sigma #A8705]) was performed at 1:2000 (anti-GFP and anti-FLAG), 1:10 000 (anti-GFP-HRP) or 1:20 000 (anti-GroEL) dilutions in PBST₂₀ with 3% BSA for 1 h at room temperature. Blots with anti-GFP or anti-FLAG antibodies were washed 2 × for 15 min in PBST₂₀ and incubated for 1 h with HRP-conjugated anti-mouse IgG (Dako A/S, #P0161) diluted 1:20 000 in PBST₂₀ with 3% BSA. Membranes were washed 8 × 6 min in PBST₂₀. Blots were developed for 1 min in ECL Western Blotting Detection Reagent (GE Healthcare, #RPN2109) and exposed X-ray films were developed on an OPTIMAX 1170-1-0000 X-ray film processor (Protec GmbH).

RNA in vitro transcription, purification and labelling

To generate *csgD* mRNAs for structure probing or translation, DNA templates containing a T7 promoter sequence were generated by PCR using primers EHO-244/EHO-345 or EHO-244/EHO-346 followed by *in vitro* transcription as described in Hjalt and Wagner (1995). A *csgD*-M4-3xFLAG DNA template was generated by a two-step PCR protocol. First, the *csgD*-3xFLAG DNA fragment served as template in PCR reactions with primers EHO-244/EHO-374 or EHO-373/EHO-346, respectively. The obtained PCR products then were purified from a gel, pooled and served as template in a second PCR reaction using primers EHO-244/EHO-346. An *ompA*-3xFLAG DNA template with a T7 promoter sequence was generated by PCR using primers T7-ompA/EHO-346 and DNA from strain E388 as template. To generate *omrA* and *omrB* DNA templates, oligonucleotides EHO-348/EHO-349 (*omrA*) and EHO-350/EHO-351 (*omrB*) were used. Full DNA duplexes were obtained by annealing the partially complementary oligodeoxyribonucleotides followed by a fill-in reaction using the Klenow fragment (Fermantas, #EP0421). The generated DNA fragments contain a T7 RNA polymerase promoter sequence and a sequence derived from the hammerhead ribozyme followed by the *omrA* or *omrB* sequences. *In vitro* transcription and ribozyme self-cleavage was done as described in Fechter *et al* (1998). The RNA of correct length was electrophoresed, excised from the gel, and purified by phenol-chloroform extraction. Purified RNAs were dephosphorylated and 5'-end-labelled with γ -³²P-ATP according to the manufacturer's protocol (GE Healthcare or Perkin Elmer).

Enzymatic and chemical probing of RNAs

5'-labelled *csgD* leader RNA was incubated in 1xTMN buffer at 37°C for 30 min in the presence of 1 µg yeast tRNA in 10 µl total volume. Reactions for RNA-RNA complex detection also contained 1 µM unlabelled *OmrB* RNA. The same conditions were used in the reverse experiment where 5'-labelled *OmrB* was studied except that unlabelled *csgD* leader was added at concentrations of 0.125, 0.250, 0.500 and 1.00 µM. Enzymatic digestions were done by adding 2 µl (0.02 units) of RNase T1 followed by 5 min continued incubation. Chemical probing was done by adding 2 µl of a 25 mM lead(II) acetate solution and continue incubation for 1 min. Reactions were stopped by adding 5 µl 0.1 M EDTA and EtOH precipitation. The RNA samples were dissolved in loading buffer and separated on 8% polyacrylamide sequencing gels.

In vitro translation reactions

The *csgD*-3xFLAG mRNAs were denatured for 3 min at 95°C, chilled 2 min on ice, and diluted in TMN buffer to a volume of 5 µl. The sRNAs were treated the same and diluted to 2.5 µl. Incubation was for 5 min at 37°C. After mixing sRNAs and mRNAs for 10 min at 37°C, 17.5 µl PureSystem classic II (Cosmo Bio Co.) was added and incubated for 30 min. Final RNA concentrations were 50 nM (mRNA) and 1 µM (sRNA). Translation of CsgD-3xFLAG protein was analysed by western blot using anti-FLAG antibodies. For the experiments in Figure 5C, *csgD*-3xFLAG and *ompA*-3xFLAG mRNAs in TMN buffer were denatured for 3 min at 95°C and chilled 2 min on ice. Purified Hfq and renatured sRNA was mixed and incubated for 5 min at 37°C. The mRNAs were added to the sRNA/Hfq mix and incubated for 5 min at 37°C, followed by addition of PureSystem classic II as above. Final concentrations were 80 nM mRNAs each, 50 nM/100 nM/500 nM of sRNA and 20 nM Hfq (calculated as hexamer).

Toeprinting assay

Toeprinting assays were performed essentially as described (Udekku *et al*, 2005). Annealing mixes contained 0.5 pmol *csgD*-3xflag mRNA and 0.5 pmol of 5'-end labelled primer EHO-524. After denaturation, followed by renaturation at 37°C, annealing mixes were incubated 30 min ± sRNAs. When applicable, incubation with 30S was for 1 min, then tRNA-fMet was added for 2 min. Reverse transcription was started by addition of SuperScript II (Invitrogen) and dNTPs for 5 min and stopped by EDTA. We noted that *OmrA/B*-dependent inhibition of toeprinting was more pronounced at short incubation times with 30S ribosomes, reflecting reversibility in *OmrA/B* binding on *csgD* mRNA (data not shown). Final amounts/reaction: 0.04 pmol *csgD*-3xflag mRNA, 5 pmol sRNA, 15 pmol 30S ribosomes, 8 pmol tRNA-fMet. The cDNAs and sequencing reactions were run on polyacrylamide gels, and signals detected using a PhosphorImager.

Supplementary data

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

Acknowledgements

We are grateful to A Fender for purification of Hfq, and C Mandava for kindly providing 30S ribosomal subunits. This work was supported by The Swedish Science Research Council (VR) and the

European Commission (EU-STREP BacRNAs). EH planned and performed most of the experiments and co-wrote the paper, JR carried out all bioinformatics, MS conducted *in vitro* translation assays, NG did plate assays, and UR and EGHW supervised the project and co-wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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