The Csr system regulates genome-wide mRNA stability and transcription and thus gene expression in *Escherichia coli*

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Bacterial adaptation requires large-scale regulation of gene expression. We have performed a genome-wide analysis of the Csr system, which regulates many important cellular functions. The Csr system is involved in post-transcriptional regulation, but a role in transcriptional regulation has also been suggested. Two proteins, an RNA-binding protein CsrA and an atypical signaling protein CsrD, participate in the Csr system. Genome-wide transcript stabilities and levels were compared in wildtype *E. coli* (MG1655) and isogenic mutant strains deficient in CsrA or CsrD activity demonstrating for the first time that CsrA and CsrD are global negative and positive regulators of transcription, respectively. The role of CsrA in transcription regulation may be indirect due to the 4.6-fold increase in *csrD* mRNA concentration in the CsrA deficient strain. Transcriptional action of CsrA and CsrD on a few genes was validated by transcriptional fusions. In addition to an effect on transcription, CsrA stabilizes thousands of mRNAs. This is the first demonstration that CsrA is a global positive regulator of mRNA stability. For one hundred genes, we predict that direct control of mRNA stability by CsrA might contribute to metabolic adaptation by regulating expression of genes involved in carbon metabolism and transport independently of transcriptional regulation.

Organisms have developed multiple mechanisms of gene expression regulation to adapt their physiology and metabolism to changing environment in order to compete with other species. At any time in the adaptation process, the intracellular level of an mRNA is the direct result of gene expression regulation. For each gene, the balance of two independent cellular processes, synthesis and degradation of mRNA determines mRNA quantity. These two levels were previously demonstrated to contribute to adaptation processes in various organisms1–4.

At the genome-wide scale, global regulators allow coordinated expression control of a large set of genes after sensing environmental changes. Most of the known global regulators in Bacteria are transcriptional regulators (CRP, Fis, etc…) with well described mechanisms of action. They activate or repress initiation of mRNA transcription by the RNA polymerase, and control global metabolism such as carbon uptake and respiration5,6. Only a few global regulators act at the post-transcriptional level on mRNA stability. One example is the RNA binding protein Hfq, homolog of the Sm and Lsm proteins that form the core of splicing and mRNA degradation complexes in eukaryotes. Hfq facilitates base-pairing interactions of regulatory noncoding small RNAs (sRNAs) on multiple target mRNAs7 to destabilize mRNAs and regulate key bacterial metabolic pathways8,9.

The Csr/Rsm (carbon storage regulator/repressor of secondary metabolites) system is a multi-component global regulatory system, well conserved in Bacteria, that controls gene expression of many important cellular functions. The Csr system represses glycogen metabolism, gluconeogenesis, biofilm formation and quorum sensing while it activates glycolysis, cell motility, virulence and pathogenesis as demonstrated in γ-Proteobacteria such as *Escherichia, Salmonella, Erwinia, Pseudomonas* or *Vibrio*10. It is composed of two proteins, CsrA, an RNA

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binding protein, and CsrD, a putative signaling protein, and two regulatory sRNAs, CsrB and CsrC. The Csr system is mainly known for its post-transcriptional role in mRNA stability. In E. coli K-12 strains, mRNA binding of CsrA has been demonstrated to destabilize four transcripts (glgCAP, pgaABCD, ydcH and ycdT) and to stabilize one (flhDC). The CsrD protein stimulates the degradation of CsrB and CsrC. An originality of the Csr system is to combine multi-level regulation acting at the post-translational level but also at the transcriptional level. Besides its role in mRNA stability, the Csr system is indeed involved in post-translational regulation through the CsrA-dependent activation of enzyme activity. The CsrA activity is itself regulated via sequestration by the sRNA CsrB, CsrC and McaS. At the transcriptional level, CsrA interacts with the global stringent response and the BarA/UvrY two-component signal transduction system, two major actors in regulation of E. coli gene expression. The role of CsrD at the transcriptional level is not yet fully understood. Although CsrD contains GGDEF/EAL domains, CsrD is not directly involved in c-di-GMP metabolism. However, CsrD was demonstrated to regulate expression of a transcriptional regulator CsgD via mechanisms not yet fully elucidated.

How the Csr system combines transcriptional and post-transcriptional regulation at the genome-wide scale to control major physiological functions is not yet determined. There are a limited number of genomic studies of the Csr system, and they all focused on CsrA. Lists of CsrA targeted genes were provided by mRNA-protein interaction analysis, bioinformatics tools and with transcriptomic analysis. When changes in mRNA amounts were identified by transcriptomic analysis in response to CsrA overexpression, measurement of mRNA stability was not simultaneously performed impeding to discriminate between transcriptional and post-transcriptional regulations.

Here we have elucidated the regulation networks of CsrA and CsrD at both the transcriptional and post-transcriptional levels. We determined and compared genome-wide data of mRNA quantity and mRNA stability in a wild type MG1655 strain, in a strain containing a partial deletion of csrA, MG1655 ΔcsrA, and in a strain deleted of csrD, MG1655 ΔcsrD. Using an integrative approach, we show that in our experimental condition CsrA and CsrD are both involved in massive transcriptional regulation whereas genome-wide regulation of mRNA stability is only dependent on CsrA. We discuss these results in terms of direct and indirect effects, and show how the direct control of mRNA stability by CsrA contributes to metabolic adaptation.

**Results**

**Construction and growth of the MG1655ΔcsrA** and **MG1655ΔcsrD** mutant strains. The csrA gene is essential for cell viability in E. coli K-12 strains grown on glycolytic sources. A hypomorphic mutant corresponding to a transposon insertion into the csrA gene is viable and has been used in numerous studies of the role of CsrA. CsrA is a dimeric 61 amino acid RNA binding protein. The transposon insertion results in the production of a 50 amino acid protein deleted of the C-terminal dimerization domain. In order to create isogenic strains in the MG1655 background, we engineered the MG1655ΔcsrA strain by inserting a stop codon at position 51. In this construct, the endogenous transcription termination signal downstream of the stop codon is conserved. As expected, the MG1655ΔcsrA strain produced much more glycogen than the MG1655 strain and this phenotype is abolished by complementation with an ectopic copy of the wild type csrA (Supplementary Fig. S1). The mutant strain also displayed significantly less motility and more biofilm formation (Supplementary Fig. S1) as expected. Measurement by Northern Blot of CsrB and CsrC levels in the MG1655ΔcsrA strain showed the absence of expression of the two sRNAs (Supplementary Fig. S1) confirming the role of CsrA in transcriptional activation of CsrB and CsrC. All these results confirmed that the activity of the C-terminal truncated variant of CsrA was drastically diminished in our MG1655ΔcsrA strain.

The MG1655ΔcsrD strain was constructed by total deletion of the csrD gene in E. coli MG1655. When cultured in batch in M9 minimal medium supplemented with glucose, MG1655ΔcsrA cells exhibited a two-fold reduced maximal growth rate (μmax = 0.31 ± 0.01 h⁻¹) compared to wild type (μmax = 0.63 ± 0.01 h⁻¹) (Fig. 1); normal growth was restored by complementation with an ectopic copy of the wild type csrA gene (Fig. 1). In contrast, the maximal growth rate was only slightly affected in MG1655ΔcsrD (0.52 ± 0.08 h⁻¹) compared to
involvement in c-di-GMP metabolism. All transcripts involved in glycogen synthesis were at least 3-fold up-regulated consistently with the high level of glycogen produced in MG1655ΔcsrA51 in our experimental condition (Table S2). In contrast to the massive destabilization of mRNA in the MG1655ΔcsrD strain compared to the MG1655 strain. We measured the glycogen content in the MG1655ΔcsrD strain and we did not find an increase of the glycogen content (Table 1). In our growth conditions, the presence/absence of CsrD did not affect mRNA stability neither via CsrD direct targeting nor via CsrA activity.

**Effect of the csrA51 and ΔcsrD mutations on mRNA levels.** To assess the transcriptional influence of CsrA and CsrD, we first measured the transcriptomic response in MG1655ΔcsrA51 and MG1655ΔcsrD mutant strains (Supplementary Table S2). In contrast to the massive destabilization of mRNA in the MG1655ΔcsrA51 strain, transcriptomic analyses showed a global up-regulation of mRNA amounts with 2195 mRNAs significantly higher compared to 249 mRNAs significantly lower (Fig. 4a). Up-regulated expressions were mainly related to biogenesis of cell wall and membrane and to signal transduction mechanisms while the down-regulated expressions were related to cell motility and carbohydrate transport and metabolism (Supplementary Table S3). These results are consistent with our phenotypic characterization and the literature (less motility, more biofilm formation).

| Strain        | Residual glucose (mM) | Produced acetate (mM) | $\vartheta_i$ (g glucose/g of dry cell weight h$^{-1}$) | $Y_{xy}$ (g of dry cell weight/g glucose) | Intracellular glycogen content (g glucose/g of dry cell weight) |
|---------------|-----------------------|-----------------------|------------------------------------------------------|----------------------------------------|---------------------------------------------------------------|
| MG1655        | ND                    | ND                    | 0.29 ± 0.03                                          | 0.36 ± 0.01                           | 0.034 ± 0.006                                                  |
| MG1655ΔcsrA51 | ND                    | ND                    | 0.26 ± 0.01                                          | 0.41 ± 0.02                           | 0.194 ± 0.006                                                   |
| MG1655ΔcsrD   | ND                    | ND                    | 0.29 ± 0.01                                          | 0.35 ± 0.01                           | 0.040 ± 0.007                                                   |

Table 1. Macro kinetics behavior and intracellular glycogen content of the strains MG1655, MG1655ΔcsrA51 and MG1655ΔcsrD in continuous cultures at 0.1 h$^{-1}$ in M9 minimal medium supplemented with 3 g/l glucose at 37°C. ND: not detected.

the MG1655 strain (Fig. 1). To eliminate the growth rate effects on mRNA half-lives and levels and to permit comparison between strains, the MG1655, MG1655ΔcsrA51 and MG1655ΔcsrD strains were cultivated in continuous culture at an equivalent growth rate of 0.10h$^{-1}$. The csrA51 or ΔcsrD mutations did not affect the macro kinetics behavior of the strains (Table 1). Glucose (3 g/l) was totally consumed and no acetate was produced. A slightly higher biomass yield was measured in the MG1655ΔcsrA51 strain. The csrA51 mutation was associated with a 5-fold higher intracellular glycogen content compared to the MG1655 strain (Table 1).

**Effect of the csrA51 and ΔcsrD mutations on mRNA stability.** CsrA has been demonstrated to regulate the stability of a few mRNAs. However, many more putative targets are suggested by interaction study and bioinformatics analysis. CsrD is involved in the turn-over of the two sRNA CsrB and CsrC but its involvement in mRNA stability regulation has not yet been investigated. To explore CsrA and CsrD involvement in mRNA stability regulation, we compared genome-wide measurements of mRNA stability in the MG1655, MG1655ΔcsrA51 and MG1655ΔcsrD strains grown in continuous culture at 0.1 h$^{-1}$ (Supplementary Table S1). Transcripts were globally less stable in MG1655ΔcsrA51 than in the MG1655 strain, the median half-life decreasing from 4.5 min in the MG1655 to 2.9 min in the mutant strain (Fig. 2a). More precisely, a statistical test (P-value < 0.1) identified 1672 messengers with differential half-life between the MG1655ΔcsrA51 and MG1655 strains: 1618 were significantly destabilized in the mutant strain whereas 54 were significantly stabilized (Fig. 2b). Interestingly, these results show a genome-wide involvement of CsrA in mRNA stability regulation and more particularly a massive destabilization of mRNA in the MG1655ΔcsrA51 mutant strain.

The median mRNA half-life in MG1655ΔcsrD was only slightly higher than in the MG1655 strain (Fig. 2a). However, applying the same statistical test used for the MG1655ΔcsrA51 strain (P-value < 0.1), no mRNA with a significant variation of stability was found between the MG1655ΔcsrA51 and MG1655ΔcsrD strains (Fig. 2c) suggesting a small systematic increase in global mRNA stability. This observation is quite surprising since according to the established regulatory network of the Csr system, CsrD is believed to positively regulate CsrA activity via CsrB and CsrC destabilization. Consequently, a destabilization as already observed for MG1655ΔcsrA51 (Supplementary Fig. S2) but their levels were not modified accordingly, suggesting a transcriptional retro-control: indeed the CsrB level was only increased around two fold in our experimental condition whereas the CsrC level was even decreased around two fold (Fig. 3). Since CsrD regulation of CsrB and CsrC turnover depends on the physiological status of the cells, the CsrB and CsrC sRNA stabilization shows that CsrD was active in our growth condition. The opposite regulation of CsrB and CsrC levels is expected to only slightly modify the amount of CsrA sequestrated by the two sRNAs in the MG1655ΔcsrD strain compared to the MG1655 strain. Therefore, CsrA should exhibit a rather similar activity in the two strains. This conclusion was supported at the phenotypic level by the absence of a CsrA-related glycogen response in the MG1655ΔcsrD strain compared to the MG1655 strain. We measured the glycogen content in the MG1655ΔcsrD strain and we did not find an increase of the glycogen content (Table 1). In our growth conditions, the presence/absence of CsrD did not affect mRNA stability neither via CsrD direct targeting nor via CsrA activity.
A strong transcriptomic response was observed in the MG1655(ΔcsrD) strain compared to the MG1655 strain (Fig. 4b). A large majority of the genes were down-regulated (3345/4254) and only 61 were significantly up-regulated. In the MG1655(ΔcsrA51) strain, a connection between CsrA and CsrD expression is expected due to the negative regulation of csrD gene expression by CsrA. Our results show that csrD mRNA quantity was strongly enhanced in the MG1655(ΔcsrA51) strain (4.6-fold increase). Considering this result, we have compared the transcriptomic responses in the MG1655(ΔcsrA51) and MG1655 strains (n = 3028 mRNAs) and between the MG1655(ΔcsrD) and MG1655 strains (n = 3333 mRNAs). A P-value ≤ 0.1 was required for fold change significance (above the horizontal dashed line). The significantly stabilized mRNAs in the mutant strain compared to the MG1655 strain are colored in red whereas the significantly destabilized mRNAs are colored in green.

Figure 2. Effect of the csrA51 and ΔcsrD mutations on mRNA half-life. (a) Box plots of transcript half-life for the MG1655, MG1655(csrA51) and MG1655(ΔcsrD) strains (n = 3351 mRNAs). Values are separated into four quartiles by horizontal bars. The central bar (in the middle of the rectangle) represents the median value, which is given above the bar. VolcanoPlot of the log, fold change (Log2 FC) of mRNA half-lives (b) between the MG1655(ΔcsrA51) and MG1655 strains (n = 3028 mRNAs) and (c) between the MG1655(ΔcsrD) and MG1655 strains (n = 3333 mRNAs). A P-value ≤ 0.1 was required for fold change significance (above the horizontal dashed line). The significantly stabilized mRNAs in the mutant strain compared to the MG1655 strain are colored in red whereas the significantly destabilized mRNAs are colored in green.

CsrA and CsrD are involved in massive transcriptional regulation. To decipher the global roles of CsrA and CsrD at the transcriptional level, we integrated datasets of mRNA stability and mRNA quantity measured in the MG1655, MG1655(ΔcsrA51) and MG1655(ΔcsrD) strains. A difference in mRNA concentration...
between each mutant and the wild type strain can be the consequence of change in mRNA synthesis (transcription), degradation and/or mRNA dilution as cells divide not necessarily at the same rate. In our experiments, all the strains were cultivated at the same growth rate, so the effect of mRNA dilution on gene expression can be eliminated. Therefore, all changes in mRNA level in our study can only be explained by transcriptional and/or mRNA stability regulation.

In the MG1655(ΔcsrD) strain, no individual transcript stability was significantly modified whereas a negative transcriptomic response was observed for 3345 genes. Therefore, these changes in mRNA levels result mainly from down regulation of transcription. Similarly, in MG1655(csrA), 542 mRNAs were regulated at the mRNA level (positively for 82%), but not in stability. This demonstrates that mutating csrA provokes a transcriptional response, which is generally positive. These results show that CsrD and CsrA regulate a large number of genes by acting only at the transcriptional level. Molecular validation of widespread transcriptional action of CsrA and CsrD was provided by transcriptional fusions using a lacZ reporter gene. We confirmed higher lacZ expression under the control of the glgB and ydeH promoters in the MG1655(csrA) strain compared to the MG1655 strain, and the lower lacZ expression under the control of the frdA and csrC promoters in the MG1655(ΔcsrD) compared to the MG1655 strain (Supplementary Table S4).

To go deeper in the transcriptional regulation, we searched for changes in mRNA levels of genes related to the functional category “Transcription, DNA-dependent” in both MG1655(ΔcsrD) and MG1655(csrA). As expected, transcriptional regulators that are supposed to be under transcriptional control were down-regulated in the MG1655(ΔcsrD) strain but up-regulated in MG1655(csrA) (Table 2). These genes correspond to RNA polymerase (β and β’ subunits, rpoB and rpoC), sigma factors (rpoD, rpoE, rpoH, rpoN, rpoS and fecI), factors involved in the stringent response (relA, spoT and dksA) and global transcriptional regulators. More precisely, the transcriptional regulators were crr, cyaA and fruR/cra for regulating the carbon flow, fur and arca in involved in regulation of the aerobic/anaerobic metabolisms and ihfA and ihfB encoding the IHF factor involved in chromosomal structure. Taken together with the up-regulated expression of csrD mRNA in the MG1655(csrA) strain, these results support the conclusion that CsrD-mediated transcriptional regulation contributes to the transcriptional response observed in MG1655(csrA).

**CsrA is a global positive regulator of mRNA stability.** To decipher the precise role of CsrA in large scale mRNA stability regulation, we considered variations in both mRNA stability and concentration between MG1655(csrA) and MG1655. Stability and level variations in MG1655(csrA) compared to MG1655 were schematically classified in three groups (Fig. 3a and Supplementary Table S5). Group I corresponded to a variation in mRNA amount not associated with a variation in stability. This group contained the above mentioned 542 mRNAs whose levels were under transcriptional control. In the two other groups, both the stability and quantity differed in MG1655(csrA) compared to MG1655. In group II (n = 1217), stability and quantity varied in opposite direction (lower stability associated with a higher amount in MG1655(csrA) compared to MG1655 or vice versa) whereas group III (n = 455) included mRNAs exhibiting stabilization with similar or a higher level and destabilization with a similar or lower level.

In a previous study, we have demonstrated in E. coli at the genome-wide scale that the quantity of an mRNA is the major determinant of its stability41. Plotting mRNA half-life as a function of its amount in MG1655(csrA) and MG1655 showed a strong negative correlation (Fig. 5b). This indicates that for a given mRNA, a change in stability between MG1655(csrA) and MG1655 can potentially be linked to a change in concentration. In other words, mRNA stabilization in MG1655(csrA) may result from a lower mRNA amount whereas a destabilization may be due to a higher amount. This relationship has been validated at the molecular level (Nouaille et al.,
manuscript in preparation). Therefore, for the 1217 transcripts of group II with antagonistic regulation of stability and quantity in MG1655(csrA51), the regulation of mRNA stability could correspond to a direct effect of CsrA on mRNA stability or an indirect effect due to change in mRNA level depending on transcription regulation. The regulation of stability of these mRNAs by CsrA is thus complex due to overlapping direct and indirect effects. Most of the mRNAs (1215/1217) were destabilized with up-regulated level and functional classes such as "DNA replication" and "signal transduction" were enriched in this group (Supplementary Table S5). The presence of a consensus CsrA binding site RUACARGGAUGU27 in the −100/+100 nt region (+1 corresponding to the start codon) of the 1217 transcripts. 635 displayed at least one putative CsrA binding site.

For the 455 mRNAs of group III with stability and quantity varying in the same directions, variation in half-life cannot be explained by variation in amount according to the correlation shown in Fig. 5b. Consequently, it is likely that their stability is directly controlled by CsrA; these mRNAs were therefore predicted as putative direct post-transcriptional targets of CsrA. Many were novel CsrA targets, since cross-comparisons of our list with previous studies showed that only 10 and 93 were already in silico predicted28 or shown to co-purify with CsrA23, respectively (Supplementary Table S6). As previously, the presence of a consensus CsrA binding site was investigated in the 455 transcripts. We found that 180 mRNAs displayed unique putative CsrA binding site while 78 had at least 2 sites with hot spot localization in the 5′ UTR region (Supplementary Table S6). Taken together, more than 65% (297/455) were predicted to experimentally bind CsrA23 and/or to have a putative CsrA binding site. These results suggest that most of the identified putative CsrA direct post-transcriptional targets are likely to interact physically with the CsrA protein although false positive experimental binding23 and degenerated

Figure 4. Effect of the csrA51 and ΔcsrD mutations on mRNA levels. VolcanoPlot of the log, fold change (Log, FC) of mRNA amounts (n = 4254 mRNAs) (a) between the MG1655(csrA51) and MG1655 strains and (b) between the MG1655(ΔcsrD) and MG1655 strains. A P-value ≤ 0.01 (above the horizontal dashed line) and a log2 FC higher than 0.5 or lower than −0.5 (outside the vertical dashed lines) were required for fold change significance. Between the mutant strain and the MG1655 strain, the significantly up-regulated amounts were colored in red whereas the significantly down-regulated levels were colored in green.
Table 2. Effect of the ΔcsrD and csrA51 mutations on mRNA level of genes involved in the biological process “Transcription, DNA-dependent”. GeneId corresponds to the gene Blattner number55, GeneSym to the gene name, FC to the fold change of mRNA amounts between the mutant strain and the MG1655 strain and P-value is the associated adjusted P-value.

| GeneId | GeneSym | MG1655(ΔcsrD) FC | P-value | MG1655(csrA51) FC | P-value |
|--------|---------|------------------|---------|------------------|---------|
| b0080  | fruR/cra | 0.50 2.4E−07     | 1.40    | 1.6E−07          |         |
| b0145  | dksA    | 0.53 7.4E−06     | 1.67    | 3.3E−05          |         |
| b0912  | ilvB    | 0.60 8.2E−06     | 1.43    | 9.9E−05          |         |
| b1334  | fnr     | 0.55 3.0E−05     | 1.31    | 4.1E−03          |         |
| b1712  | ilvA    | 0.58 3.0E−05     | 1.30    | 2.8E−05          |         |
| b2573  | rpoE    | 0.51 3.7E−05     | 1.36    | 4.3E−03          |         |
| b2741  | rpoS    | 0.49 4.0E−05     | 1.28    | 1.9E−02          |         |
| b2784  | relA    | 0.53 1.6E−05     | 2.05    | 1.0E−05          |         |
| b3067  | rpoD    | 0.55 1.8E−07     | 1.26    | 3.5E−05          |         |
| b3202  | rpoN    | 0.54 2.5E−07     | 2.06    | 1.3E−07          |         |
| b3357  | crp     | 0.48 4.0E−07     | 1.39    | 4.1E−05          |         |
| b3461  | rpoH    | 0.51 3.0E−05     | 2.28    | 1.2E−03          |         |
| b3650  | spoT    | 0.58 1.2E−06     | 2.31    | 1.2E−05          |         |
| b3806  | cyaA    | 0.53 2.2E−07     | 1.73    | 4.9E−07          |         |
| b3987  | rpoB    | 0.69 5.8E−07     | 1.13    | 5.5E−04          |         |
| b3988  | rpoC    | 0.53 3.7E−06     | 1.14    | 2.9E−02          |         |
| b4293  | fecI    | 0.54 6.6E−07     | 2.13    | 2.3E−05          |         |
| b4401  | arcA    | 0.50 3.3E−06     | 1.16    | 2.7E−02          |         |

Figure 5. (a) Variations in mRNA stability and quantity between the strains MG1655(csrA51) and MG1655. mRNAs with variation in level but not in stability in MG1655(csrA51) compared to MG1655 are in group I, those with variations in stability and quantity in opposite directions are in group II, and those with variations in stability and quantity not in opposite directions are in group III. (b) Plots of transcript half-life (in min) as a function of transcript quantity (in arbitrary units per mg of dry cell weight) in the MG1655 strain (n = 3074 mRNAs) and MG1655(csrA51) (n = 4098 mRNAs). All the values were log-transformed and centered. The Pearson correlation coefficient is r = 0.81 with a P-value < 2.2E−16.
CsrA binding motif cannot be totally excluded. We found that more than 88% (403/455) of the CsrA direct post-transcriptional targets were destabilized in the MG1655(csrA51) strain (Supplementary Table S6). Together with the results shown in Fig. 2b, our work suggests that CsrA has an important role in the stabilization of a large number of messengers.

**Metabolic impact of the direct regulation of mRNA stability by CsrA.** The predicted direct regulation by CsrA of the stability of 455 mRNAs is not expected to contribute in the same way to the control of metabolic adaptation. For 356 mRNAs, a change in stability between the two strains was not associated with a significant change in mRNA level, which is generally required to provoke protein concentration variations. For these mRNAs, stability regulation was counteracted by transcription regulation. These mRNAs were mainly related to translation and carbohydrate transport and metabolism (Supplementary Table S5). On the contrary, for 99 transcripts (Supplementary Table S7), changes in stability were associated with changes in mRNA amounts and might therefore contribute to metabolic differences between the two strains.

More precisely, 64 mRNAs were destabilized with down-regulated level whereas 35 were stabilized with up-regulated level between the MG1655(csrA51) and MG1655 strains (Supplementary Table S7). Among the destabilized and down-regulated mRNAs in the MG1655(csrA51) strain, we found tpiA in agreement with a previous result showing that the triose-phosphate isomerase activity was positively regulated by CsrA activity42. We also identified the zwf transcript, previously reported to co-purify with CsrA23, but its stability and amount have never previously been shown to be regulated by the Csr system. Our results clearly showed down-regulated mRNA stability and quantity of genes encoding carbohydrate transport (araF, malE, alsB, idhT, melB, glpF, srhAEB and agaVW) and certain metabolic pathways, e.g. idonate/gluconate metabolic process (idhDOT) and galacturonate catabolic process (uxaCA) in MG1655(csrA51) (Supplementary Table S5). mRNA stability and level of a large number of metabolic enzymes (aldA, ldhA, udp, yfaU, hdhA, glpK, damA, glob, aed, fabF, speB, tpx, thiH, yddB, ilvC, pta, elvG and friB) were found to be also negatively regulated in MG1655(csrA51).

In the stabilized and up-regulated mRNAs in the MG1655(csrA51) strain, we identified the transcript, previously reported to co-purify with CsrA 23, but its stability and amount were not regulated by the Csr system (Supplementary Table S7). Among the 75 mRNAs whose level and stability were regulated in the MG1655(csrA51) strain, we found the stability and amount related to translation and carbohydrate transport and metabolism (Supplementary Table S5). The expression and/or activity of some of these genes/proteins were known to be negatively controlled by CsrA36,42. Our results provide a better understanding of the CsrA effect on these targets, the regulator acting at the level of both mRNA stability and quantity. Expression (stability and level) of several genes of the Krebs cycle and the amino acid metabolism were found to be upregulated in the MG1655(csrA51) strain: acnB, fumA, sdhC, leuB, leuC, gltA and asd. We observed that the messengers of the mannose PTS permease (manXYZ), of the pyridine nucleotide transhydrogenase (pntAB) and of the fatty acid synthesis (fabD and fabF) were stabilized and exhibited higher level in the MG1655(csrA51) strain. The csrA51 mutation also stabilizes and increases the amount of mRNAs related to transcription (rpoD encoding the vegetative sigma factor σ70 of RNA polymerase) and to translation (ribosomal rplP, rplN and rpsS transcripts). This and the accumulation of glycogen (Table 1) could partially explain the slightly higher biomass yield in the MG1655(csrA51) strain compared to the MG1655 (Table 1).

For the 99 mRNAs whose level and stability were regulated in the MG1655(csrA51) strain, we examined the contribution of stability regulation to the control of the mRNA amount. Regulatory coefficients were calculated between MG1655(csrA51) and the MG1655 strain. This quantifies the respective effects of transcription regulation and stability regulation on mRNA amount1. For a large majority of these mRNAs (85/99), the regulation of stability predominantly controlled mRNA level. This indicates that it was a change in stability that provoked differential mRNA amount in the MG1655(csrA51) strain and this is true even if transcription regulation is present; we called this type of regulation degradational control of the mRNA quantity (Supplementary Table S7). Altogether these results provide the first demonstration of how direct regulation (positive/negative) of mRNA stability by CsrA regulates a hundred mRNA amounts independently of transcription regulation and therefore contributes to control the global metabolism.

**Discussion**

In this study, we applied for the first time an integrative biology approach to decipher the transcriptional and post-transcriptional roles of CsrA and CsrD. Although the Csr system is generally believed to be involved in a post-transcriptional response, the protein CsrD was demonstrated here to be involved in a massive positive transcriptional regulation. In contrast, mutating the csrA gene induces both transcriptional and post-transcriptional responses. Transcription of more than 500 genes and stability of more than 1600 mRNAs differ between the MG1655(csrA51) and MG1655 strains. Opposite transcriptional responses were obtained in the MG1655(csrA51) and MG1655(ΔcsrD) strains. Since we confirmed the strong up-regulation of csrD expression in MG1655(csrA51), it is likely that the transcriptional response observed in MG1655(csrA51) was at least partially mediated by CsrD. The importance of the negative feedback created by CsrA regulation of CsrD expression was previously highlighted in the dynamic response of the Csr system41. The time of turning on and off expression of CsrA target proteins was dependent on the CsrD expression level. Our study shows that the regulatory control of the Csr system involves genome-wide transcriptional and post-transcriptional regulation of gene expression connected by CsrA and CsrD (Fig. 6).

Several mechanisms of CsrD action at the transcriptional level can be proposed. This may occur indirectly via transcriptional factors whose expressions are CsrD-dependent. Many transcriptional regulators are down-regulated in the absence of CsrD. The actions of CsrD on transcriptional regulators could involve regulation of enzymes of the c-di-GMP signaling pathway, as shown for CsgD26. Another possibility is that CsrD regulates the turnover of sRNAs other than CsrB and CsrC17, which in turn control transcriptional regulator expression at the post-transcriptional level. Finally, we cannot exclude a direct transcriptional effect of CsrD on targeted genes through a still unknown mechanism.
A strong negative correlation between mRNA half-life and its amount has been observed here for the whole genome, indicating that as a general trend mRNA stabilization can be due to a decrease in mRNA amount and vice versa. The regulation of mRNA stability by CsrA was complex for the 1217 transcripts with opposite variations of stability and quantity in MG1655(csrA51). For these genes, direct effect of CsrA on stability and the potential indirect effect of CsrA on stability due to change in mRNA level cannot be discriminated (Fig. 6). Nevertheless for more than 400 mRNAs with stability varying in the same direction as mRNA quantity or not associated with mRNA quantity variation, we predicted that CsrA regulates directly their stability. For the majority of these mRNAs, control may be attributed to a physical interaction between CsrA and the mRNA molecule as indicated by the prediction of a putative binding site and/or high throughput binding analysis. For one third of these mRNAs, there was no experimental proof of CsrA binding and we did not identify any consensus CsrA binding site. Either the sequences of the CsrA binding site were highly degenerated compared to the consensus in these mRNAs or CsrA may act on their stability through other post-transcriptional regulators (such as the RNA-binding protein Hfq) whose expression is CsrA-dependent. About 88% of the half-lives directly regulated by CsrA were shorter in the MG1655(csrA51) strain than in the MG1655 strain. This result is surprising because, to date, most of the known targets of CsrA are repressed and/or destabilized, and at the molecular level, examples of repression are more numerous than positive regulation. Thus, contrary to earlier studies on selected targets, our genome-wide analysis shows that CsrA has a significant role as a positive regulator of mRNA stability. The protective effect of CsrA binding on mRNA by sequestering RNase E cleavage sites, only described until now in the case of flhDC, might be common.

In terms of gene expression regulation, for one hundred mRNAs, the direct regulation of mRNA stability by CsrA led to a significant variation in mRNA concentration. We confirmed previously identified metabolic targets of CsrA such as glgB, pgm, tpiA and pck mRNAs. In addition, many new direct mRNA targets of CsrA were discovered with a potential metabolic impact via mRNA level changes. Functional analysis of these transcripts underlined that the Csr system is strongly involved in the control of E. coli metabolism by regulating expression of enzymes involved in the central carbon metabolism and carbohydrate transport but also in other specific metabolic pathways (syntheses of amino acids and fatty acids). In the MG1655(csrA51) strain, mRNA stability regulation favors gluconeogenesis especially anaerobic reactions (e.g. malic enzyme, PEP carboxykinase), whereas the transport of glycolytic substrates (such as sorbitol, melibiose, gluconate, allose, maltose and arabinose) is down-regulated. Therefore, compared to MG1655, the MG1655(csrA51) strain is more likely to efficiently metabolize gluconeogenic substrates. Consequently, our results provide the first evidence at the genome-wide scale of the role of CsrA-dependent regulation of transcript stability in metabolic adaptation.

Our study provides new insights into the Csr regulatory network. We determined the extent, the nature and the metabolic effect of the CsrD and CsrA regulations. Our results highlight the superimposition at the genome-wide scale of the transcriptional and post-transcriptional controls that likely contribute to the robustness and dynamics of gene expression reprogramming during bacterial adaptation.
Methods

Bacterial strains and plasmids. E. coli K-12 MG1655 strain (λ− F− rph−) was the genetic background for mutation of csrA and csrD genes. Mutagenesis was carried out using λ red recombination27 and primer pair OKT-24/OKT-25. As the csrA gene is essential29,30, the ORF was mutated using the csrA mutant obtained by Romeo and coworkers as a model38. The MG1655Δ csrA strain expresses a truncated variant of CsrA in which 11 amino acids at the C-terminal end of the protein have been deleted. The MG1655Δ csrD strain has a total deletion of the csrD gene, which was obtained from the KEIO collection29. The mutations were transferred into E. coli K-12 MG1655 wild type background by λ P1 phage-mediated transduction before removing the antibiotic-resistance cassette by FLP recombination. MG1655Δ csrA strain was complemented by a low copy number plasmid containing the wild type csrA gene. Briefly, csrA was amplified using primer pair OCT-29/OCT-30 and cloned in pSAB1148 at EcoRI site. To perform gene expression assays, csrC-lacZ, glgB-lacZ, frdA-lacZ and ydeH-lacZ transcriptional fusions were constructed. The transcriptional fusions were cloned in pYB79 (Cmr), a plasmid that was constructed by inserting the omegon kan from pHP45-kan into pACYC184 using the restriction site BamHI and subsequently by cloning at PstI site a PCR amplified fragment containing plac-lacZ from E. coli. 5′-UTR sequence containing a ribosome binding site and a start codon (GAATTCGCGGGGATCCTAAGTAAGTAAGGAG AAAAAAATT GCTGATCCC) was fused to lacZ 10th codon. Then, the vector was amplified by PCR with primers MBO-200/–199 and assembled with the promoter regions using In-Fusion HD cloning kit (Clontech). The promoters upstream of the transcription starts of csrC (249 nt), glgB (200 nt), frdA (205 nt) and ydeH (421 nt) were cloned on MG1655 genomic DNA with oligonucleotide pairs MBO-137/138, MBO-203/–204, MBO-207/–208 and MBO-197/–198, respectively. For glgB, frdA and ydeH, the promoter regions that were chosen using information available on the EcoCyc E. coli Database listing previously characterized transcriptional regulators. For csrC, the promoter region is as used in Suzuki et al.17. The strains and plasmids were validated by sequencing. The oligonucleotides are listed in Table S8.

Culture conditions. For RNA extraction and microarray analysis, cells were grown in continuous culture at 37 °C in M9 minimal medium supplemented with glucose as described3. The MG1655, MG1655Δ csrA and MG1655Δ csrD strains were cultured at the same growth rate, μ = 0.10 h−1, which corresponds to doubling time of 6.9 h. Each culture was repeated three times to provide independent biological replicates. Biomass was estimated from absorbance at 600 nm (Libra S4, Biochrom): 1 unit of absorbance corresponding to 0.42 g of dry cell weight 1−1 for the MG1655 strain, 0.27 g of dry cell weight 1−1 for the MG1655Δ csrA strain and 0.44 g of dry cell weight 1−1 for the MG1655Δ csrD strain. To determine the maximal growth rate of each strain, the cells were grown in batch culture (identical medium, oxygen concentration, pH and temperature) and the rates were determined in exponential growth phase.

Sampling and RNA extraction. Sampling and RNA extraction were conducted as described3 with additional cell samplings after rifampicin addition at 9, 15, 20 and 30 min.

Northern Blot. For each sample, 10 μg of total RNA was denatured for 5 min at 95 °C in RNA loading buffer (95% [v/v] formamide, 0.1% [w/v] xylene cyanole, 0.1% [w/v] bromphenol blue, 10 mM EDTA), separated on a 7 M urea/6% polyacrylamide gel at 250 V and transferred to Hybond-XL membranes (GE Healthcare) by electroblotting (1 h, 50V, 4 °C) in 1X TBE. After UV crosslinking, the membranes were hybridized overnight in Roti®.Hybri-Quick (Roht) at 65 °C with [32P] body-labeled riboprobes specific of CsrB (primers OCT-50/OCT-51) or CsrC (primers OCT-52/OCT-53) or at 42 °C with a 55 specific [32P] end-labeled oligonucleotide (MBO-59). Hybridization with CsrB or CsrC riboprobe is followed by 15 min washes in 2X, 1X and 0.1X SSC/0.1% SDS solutions at 65 °C. Hybridization with 5S oligonucleotide is followed by 15 min washes in 5X, 1X and 0.1X/0.1% SDS solutions at 42 °C. Hybridization signals were quantified on a PhosphorImager (Typhoon Trio – GE Healthcare) with MultiGauge software (Fujifilm).

Microarray procedures. A double-stranded cDNA synthesis kit (Invitrogen) was used to produce cDNA from 2μg of total RNA. cDNA (1μg) was labeled using the one color DNA labeling kit (Nimblegen – Roche) and labeled cDNA (2μg) was hybridized onto E. coli K-12 gene expression arrays (Nimblegen – Roche) for 17 h at 42 °C according to the manufacturers’ instructions. Arrays were scanned with a MS200 Microarray Scanner (Nimblegen – Roche). The images were analyzed with DEVA 1.2.1 software. Only raw data were used for further analyses. All array procedures were performed by the GeT-Biopuces platform (http://get.genotoul.fr).

mRNA level and half-life determination. mRNA quantity determination by transcriptomic analysis was conducted as described3. Intensity values were multiplied by the total RNA extraction yield (1μg total RNA per μg of dry cell weight) to provide mRNA amount value in arbitrary units per μg of dry cell weight. RNA extraction yields were 13.1 ± 2.2, 8.7 ± 0.9 and 8.9 ± 1.5μg RNA per μg of dry cell weight for the MG1655, MG1655Δ csrA and MG1655Δ csrD strains, respectively. Differences in mRNA level were evaluated as described in3. The P-values were adjusted for multiple testing by the ‘Benjamini and Hochberg’ (BH) false discovery rate method49. Differences in mRNA quantity were considered as significant for adjusted P-values lower than 1% and log2 fold change (Log2 FC) higher than 0.5 or lower than –0.5. mRNA half-life determination was performed as previously described3. The statistical significance of differences in half-life was evaluated using the probability value of interaction between time and the type of strain (MG1655, MG1655Δ csrA) or MG1655ΔcsrD in a global model of linear regression. A statistical threshold of 10% was used for adjusted P-values by the “BH” false discovery rate method49.
Determination of mRNA level regulatory coefficients. For the selected mRNAs, the coefficient $p_D$ corresponding to the relative contribution of the mRNA stability regulation in the control of mRNA level was calculated as previously described using data of mRNA stability and quantity obtained in the MG1655(csrA51) and MG1655 strains. Regulatory classes were defined according to the $p_D$ value: 0 $< p_D < 0.6$, mRNA quantity mainly controlled by transcription or controlled by both transcription and mRNA stability; $0.6 < p_D$, mRNA quantity mainly controlled by mRNA stability.

Enrichment methods. R free statistical software (www.r-project.org) was used for enrichment methods. Functional categories enriched in transcript subgroups were determined by a hypergeometric test on data using the Biological Process of Gene Ontology annotation database (GO project; http://www.geneontology.org/). Only GO terms with associated P-value $< 0.05$ were considered as significant.

In silico research of CsrA binding sites. To determine if an mRNA contained a potential CsrA binding site, the software PatScan was used. The SELEX-derived consensus RUACARGGAUGU was searched in a $-100/+100$ nt window (+1 corresponding to the start codon) allowing a maximum of 5 mismatches. Subsequently, only sequences that included a conserved GGA motif were considered as a potential CsrA binding site.

Analytical methods. Glucose and acetate concentrations were measured by HPLC coupled to a refractometer and with UV detection. The device was equipped with a Bio-Rad HPX87H column maintained at a temperature of 48 °C and 5 mM H2SO4 was used as the eluent, at a flow rate of 0.5 ml min$^{-1}$. Glycogen staining by iodine vapor, biofilm formation by crystal violet staining and motility assays on 0.3% agar plate were carried out as previously described. The intracellular glycogen quantification was done as previously described. Briefly, the cells were lysed to extract the glycogen which was then hydrolyzed by amyloglucosidase to glucose subunits. The glucose subunits were then quantified using glucose oxidase coupled to the colorimetric reagent o-dianisidine dihydrochloride. Three independent experiments were performed for each assay.

The csrC-lacz, gglb-lacZ, frdA-lacZ and ydeH-lacZ gene expression assays were performed using cells grown in batch culture at 37 °C in M9 minimal medium supplemented with fructose (2.7 g L$^{-1}$), asasmino acids (0.2%) and chloramphenicol (25 μg ml$^{-1}$). Under these conditions, the MG1655, MG1655(csrA51) and MG1655(ΔcsrD) strains have similar growth rates (0.39 h$^{-1}$). In exponential growth, the cells were centrifuged, suspended in Tris (6 mM)/Tricarballylate (1.8 mM) buffer pH 7.2 and lysed mechanically with glass beads. β-galactosidase activity was assayed using 2.5 mM orthonitrophenyl-β-D-galactoside. For the selected mRNAs, the coefficient $\rho_D$ corresponding to the relative contribution of the mRNA stability regulation in the control of mRNA level was calculated as previously described using data of mRNA stability and quantity obtained in the MG1655(csrA51) and MG1655(ΔcsrD) strains have similar growth rates (0.39 h$^{-1}$, 0.39 h$^{-1}$ and 0.31 h$^{-1}$ respectively). These conditions aimed at mimicking growth in continuous cultures. In exponential growth, the cells were centrifuged, suspended in Tris (6 mM)/Tricarballylate (1.8 mM) buffer pH 7.2 and lysed mechanically with glass beads. β-galactosidase activity was assayed using 2.5 mM orthonitrophenyl-β-D-galactoside. Total protein was measured by the Bradford method with bovine serum albumin as the protein standard. Three independent experiments were performed for each assay.

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Author Contributions

T.E., C.T. and M.B. experimental work and data acquisition; T.E., M.B., A.J.C., L.G. and M.C.B. designed the research, analyzed the data and wrote the paper.
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