

Conditional Essentiality of the *csrA* Gene in *Escherichia coli*[∇]

Johan Timmermans and Laurence Van Melderen*

Laboratoire de Génétique et de Physiologie Bactérienne, Institut de Biologie et de Médecine Moléculaires, Faculté des Sciences, Université Libre de Bruxelles, 12 rue des Professeurs Jeener et Brachet, B:6041 Gosselies, Belgium

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CsrA is a global posttranscriptional regulator of numerous physiological processes, such as glycogenesis and glycolysis. Here, we show that the *csrA* gene of *Escherichia coli* is essential for growth on LB and on synthetic medium containing glycolytic carbon sources. However, *csrA* is not necessary for growth on synthetic medium containing pyruvate, showing that the Krebs cycle is functional in the *csrA::cat* deletion mutant. Deletion of the *glgCAP* operon in the *csrA::cat* mutant restored the ability to grow on LB and on synthetic medium containing glycolytic carbon sources, showing that growth inhibition is due to an excess of glycogen synthesis.

The *Escherichia coli* *csrA* (carbon storage regulator *A*) gene encodes a global regulator controlling a wide variety of biological processes, such as glycogen synthesis, glycolysis, and motility as well as cyclic-di-GMP synthesis and biofilm formation (2, 7, 14, 19, 21, 22, 26, 28). CsrA regulates gene expression both positively and negatively at the posttranscriptional level by modulating translation. It binds as a homodimer to a specific sequence (consensus sequence, ACANGGANGA) on both its mRNA targets and its small regulator RNAs (1, 2, 7, 18, 23, 26). Binding of CsrA to the leader sequence of the mRNA target blocks ribosome binding on the Shine-Dalgarno sequence (2, 7, 26) and most likely leads to its rapid degradation (18). As a positive regulator, CsrA enhances translation efficiency by stabilizing its mRNA targets (28).

Homologues of *csrA* have been identified, and their role in several bacterial species has been studied (for a review, see reference 15). These *csrA* homologues share regulatory networks with those identified in *E. coli*, such as motility (17) and biofilm formation (8). These homologues also appear to regulate other processes, such as virulence in *Salmonella enterica*, *Legionella pneumophila*, *Helicobacter pylori*, *Proteus mirabilis*, and *Yersinia pseudotuberculosis* (3, 9, 13, 17, 24); quorum sensing in *Erwinia carotovora* and *Vibrio cholerae* (5, 16); and oxidative stress resistance in *Campylobacter jejuni* (8).

In *E. coli*, the *csrA* gene was identified by transposon mutagenesis in a screen for mutants with altered glycogen accumulation. The kanamycin transposon insertion site is located at codon 51 in the *csrA* coding sequence (which encodes a protein of 61 amino acids). In addition to an increased glycogen accumulation, the *csrA::kan* mutant is affected for motility, biofilm formation, and glycolysis (2, 19, 21, 22, 28). In association with an *rpoS* defective mutation, the *csrA::kan* mutation led to an extended lag phase upon growth on acetate (27). Suppressor mutations restoring growth of the double mutant on acetate were mapped in the *glg* locus (27). This indicates that the

growth defect presented by the double *csrA::kan rpoS* mutant was associated with a high level of glycogen accumulation (27). Acetate sensitivity appears to require both the *csrA::kan* and the *rpoS* mutations since single mutants were not sensitive to acetate and did not evolve suppressor mutations (27).

The *E. coli* *csrA* gene is essential under certain growth conditions. In an attempt to construct a *csrA* deletion mutant of *E. coli*, large series of homologous recombination experiments were carried out with the DJ624 strain (MG1655 *lacX74 malP::lacI^q*) (12) to replace the *csrA* open reading frame (ORF) with a chloramphenicol resistance cassette (6). Despite many attempts under various conditions, no deletion mutants were obtained, suggesting that *csrA* is essential for growth. To test this hypothesis, various rescue plasmids were constructed. The *csrA* gene was cloned in the pBAD33 (11) and pKK223-3 (4) expression vectors. The expression of the rescue copy of *csrA* was induced in LB medium by the addition of arabinose (0.4%) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM). These constructs were checked by sequencing, and their ability to complement a *csrA::kan* mutation was tested. Using these plasmids as *csrA* rescue copy, attempts to delete *csrA* in DJ624 were not successful, indicating that high expression levels of CsrA might be deleterious under these conditions. To bypass this problem, the *csrA* gene was cloned in the low-copy-number pWSK129 vector under the control of the *lac* promoter (25). DJ624 was transformed by this construct, and *csrA* expression was induced by the addition of 0.1 mM IPTG in the culture medium. Under these conditions, *csrA* deletion mutants were obtained on LB plates containing chloramphenicol (20 μg/ml) and kanamycin (50 μg/ml) at 37°C ($\Delta csrA::cat$). The replacement of the *csrA* ORF with the chloramphenicol resistance cassette was checked by PCR using primers flanking the *csrA* ORF. The $\Delta csrA::cat$ mutant strain containing the pWSK-*csrA* plasmid was able to grow in the presence of 0.1 mM IPTG. No growth was observed on LB plates without IPTG, showing that the *csrA* gene is essential for growth in LB medium (Fig. 1, upper panels).

The CsrA₁₋₅₀ peptide is partially active. The *kan* transposon is inserted at codon 51 in the *csrA* gene (21). Since this insertion mutant is viable on LB medium, it leaves the possibility that the 50 amino-terminal amino acids might retain some partial activity as proposed by Romeo and coworkers (21). To

* Corresponding author. Mailing address: Laboratoire de Génétique et de Physiologie Bactérienne, Institut de Biologie et de Médecine Moléculaires, Faculté des Sciences, Université Libre de Bruxelles, 12 rue des Professeurs Jeener et Brachet, B:6041 Gosselies, Belgium. Phone: 32 2 650 97 78. Fax: 32 2 650 97 70. E-mail: lvmelderen@ulb.ac.be.

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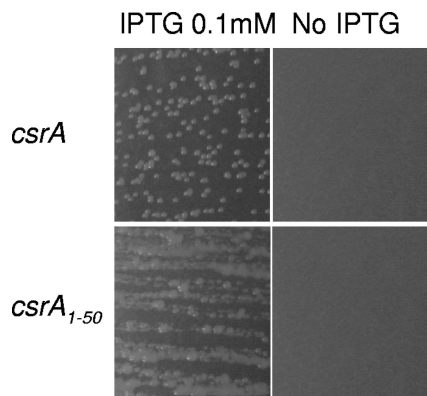


FIG. 1. The *csrA* gene is essential in *E. coli*. The $\Delta csrA::cat$ mutant containing either the pWSK-*csrA* (upper panels) or the pKK-*csrA*₁₋₅₀ (lower panels) plasmids were grown on LB plates containing the appropriate antibiotics with 0.1 mM IPTG (left panels) and without IPTG (right panels).

test this hypothesis, the first 150 5' nucleotides of the *csrA* coding sequence, followed by a stop codon, were cloned in pKK223-3. The *csrA* deletion mutant containing the pWSK-*csrA* plasmid was transformed with the pKK-*csrA*₁₋₅₀ plasmid. To displace the pWSK-*csrA* plasmid, we used the incompatibility properties of pWSK129 and pMLO59 (both plasmids have a pSC101 replication origin but different antibiotic resistance genes; pWSK129 confers resistance to kanamycin and pMLO59 to spectinomycin). The $\Delta csrA::cat$ pWSK-*csrA* pKK-*csrA*₁₋₅₀ strain was transformed by the pMLO59 vector, and the resulting transformants were plated on LB plates containing spectinomycin (100 μ g/ml), chloramphenicol (20 μ g/ml), and 0.1 mM IPTG to induce the expression of the *csrA*₁₋₅₀ polypeptide. Transformants were checked for kanamycin sensitivity (loss of the pWSK-*csrA* plasmid). The $\Delta csrA::cat$ mutant containing the pKK-*csrA*₁₋₅₀ plasmid was able to form colonies on LB plates containing 0.1 mM IPTG but not on plates without IPTG (Fig. 1, lower panels), showing that the CsrA₁₋₅₀ peptide is functional. On IPTG plates, the colonies had an abnormal phenotype (forming heterogeneous and "sticky" colonies). The molecular mechanisms underlying this phenotype are unknown. This phenotype does not appear to be dependent on the CsrA₁₋₅₀ amount, since it has also been observed at higher IPTG concentrations (data not shown). As the carboxy-terminal part of CsrA (amino acids 51 to 54) is involved in RNA binding (20), the truncated form of CsrA might have a reduced affinity for RNA (another RNA binding site has been mapped in the amino-terminal region of CsrA). This might provide an explanation for the phenotype of the *csrA::kan* mutant.

An excess of glycogen accumulation impairs the growth of the *csrA::cat* mutant. CsrA controls the intracellular carbon flux by positively regulating the glycolysis pathway and negatively regulating the gluconeogenesis pathway and glycogen synthesis (Fig. 2) (22). In the absence of CsrA, glycogen synthesis is strongly favored. Indeed, the *csrA::kan* insertion mutation resulted in 20-fold-higher levels of glycogen (21). The *csrA::cat* mutant might thus be unable to direct the carbon flux to the Krebs cycle, because of a reduced glycolysis and an enhanced glycogenesis. To test this hypothesis, the $\Delta csrA::cat$ mutant was plated on Ceria synthetic medium (10) containing

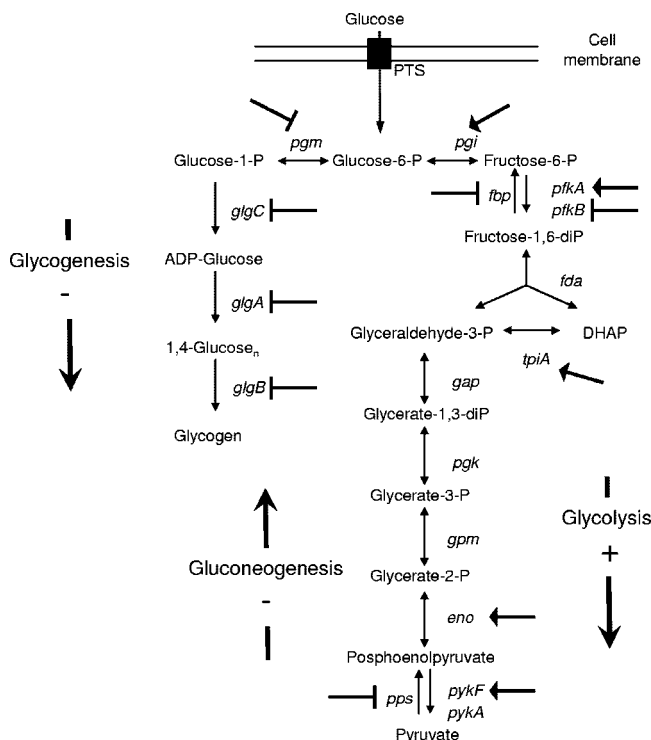


FIG. 2. CsrA-mediated regulation of the central carbon flux. CsrA positively regulates glycolysis and negatively regulates gluconeogenesis and glycogen synthesis. Negative and positive regulations of target genes are indicated by blocked arrows (⊥) and arrows, respectively. Adapted from reference 22.

amino acids and glycolytic carbon sources. The $\Delta csrA::cat$ mutant was unable to grow on glucose and on fructose-6-phosphate as sole carbon sources (Fig. 3B and C). Interestingly, it was able to grow on pyruvate, which is the carbon source at the junction between glycolysis and the Krebs cycle (Fig. 3D). On fructose-6-phosphate plates, very small colonies appeared after 16 h at 37°C (too small to be seen in Fig. 3C). These data indicate that the early steps of glycolysis are not fully functional in the $\Delta csrA::cat$ mutant. With glucose and fructose-6-phosphate as sole carbon sources, the carbon flux might be essentially directed to glycogen synthesis, thereby impairing glycolysis and the Krebs cycle. We tested whether the deletion of the *glgCAP* operon, which is involved in the first two steps of glycogen synthesis, would restore the ability to grow on glycolytic carbon sources. The *glgCAP* operon was deleted in the DJ624 strain by using the method described in reference 6. The *csrA::cat* deletion was then transduced by P1 in the $\Delta glgCAP$ strain containing the pWSK-*csrA* plasmid in the presence of 0.1 mM IPTG. Transductants were obtained and checked by PCR with primers flanking the *csrA* ORF. Interestingly, the double $\Delta glgCAP \Delta csrA::cat$ pWSK-*csrA* mutant was able to form colonies on LB plates without IPTG (Fig. 3E) as well as on glucose and fructose-6-phosphate plates (Fig. 3F and G), unlike the $\Delta csrA::cat$ pWSK-*csrA* mutant (Fig. 3A, B, and C). Both strains were able to grow in the presence of pyruvate as the sole carbon source (Fig. 3D and H). Our data show that in the *csrA::cat* mutant, glycogen synthesis is favored and that glycogen accumulation impairs viability. In the absence of the gly-

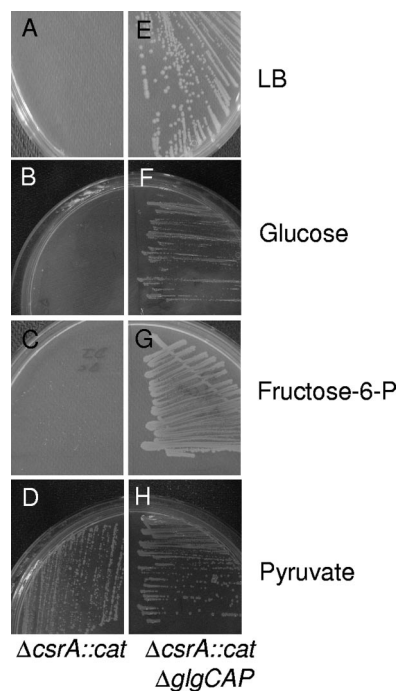


FIG. 3. Excessive glycogen accumulation in the $\Delta csrA::cat$ mutant impairs viability on glycolytic carbon sources. The $\Delta csrA::cat$ (left panels) and the $\Delta csrA::cat \Delta glgCAP$ (right panels) mutants containing the pWSK-*csrA* plasmid were streaked without IPTG on LB medium (A and E) or on minimal medium containing amino acids, with either glucose (B and F), fructose-6-phosphate (C and G), or pyruvate (D and H) as the sole carbon source.

cogen synthesis pathway, the carbon flux is most likely redirected to glycolysis and/or pentose phosphate pathways, thereby enabling the Krebs cycle to be functional.

Conclusion. We have shown that the *csrA* gene is essential in *E. coli* under growth conditions that lead to an excess of glycogen accumulation. Deletion of the glycogen synthesis pathway restored the growth of the $\Delta csrA::cat$ mutant, most likely by forcing the carbon flux to enter glycolysis and the Krebs cycle.

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REFERENCES

- Babitzke, P., and T. Romeo. 2007. CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr. Opin. Microbiol.* **10**:156–163.
- Baker, C. S., I. Morozov, K. Suzuki, T. Romeo, and P. Babitzke. 2002. CsrA regulates glycogen biosynthesis by preventing translation of glgC in *Escherichia coli*. *Mol. Microbiol.* **44**:1599–1610.
- Barnard, F. M., M. F. Loughlin, H. P. Fainberg, M. P. Messenger, D. W. Ussery, P. Williams, and P. J. Jenks. 2004. Global regulation of virulence and the stress response by CsrA in the highly adapted human gastric pathogen *Helicobacter pylori*. *Mol. Microbiol.* **51**:15–32.
- Brosius, J., and A. Holy. 1984. Regulation of ribosomal RNA promoters with a synthetic lac operator. *Proc. Natl. Acad. Sci. USA* **81**:6929–6933.
- Cui, Y., A. Chatterjee, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee. 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. *J. Bacteriol.* **177**:5108–5115.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Dubey, A. K., C. S. Baker, K. Suzuki, A. D. Jones, P. Pandit, T. Romeo, and P. Babitzke. 2003. CsrA regulates translation of the *Escherichia coli* carbon starvation gene, *cstA*, by blocking ribosome access to the *cstA* transcript. *J. Bacteriol.* **185**:4450–4460.
- Fields, J. A., and S. A. Thompson. 2008. *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. *J. Bacteriol.* **190**:3411–3416.
- Forsbach-Birk, V., T. McNealy, C. Shi, D. Lynch, and R. Marre. 2004. Reduced expression of the global regulator protein CsrA in *Legionella pneumophila* affects virulence-associated regulators and growth in *Acanthamoeba castellanii*. *Int. J. Med. Microbiol.* **294**:15–25.
- Glandsdorff, N. 1965. Topography of cotransducible arginine mutations in *Escherichia coli* K-12. *Genetics* **51**:167–179.
- Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**:4121–4130.
- Hansen, A. M., H. Lehnher, X. Wang, V. Mobley, and D. J. Jin. 2003. *Escherichia coli* SspA is a transcription activator for bacteriophage P1 late genes. *Mol. Microbiol.* **48**:1621–1631.
- Heroven, A. K., K. Bohme, M. Rohde, and P. Dersch. 2008. A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of *Yersinia pseudotuberculosis* through RovM. *Mol. Microbiol.* **68**:1179–1195.
- Jackson, D. W., K. Suzuki, L. Oakford, J. W. Simecka, M. E. Hart, and T. Romeo. 2002. Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J. Bacteriol.* **184**:290–301.
- Lapouge, K., M. Schubert, F. H. Allain, and D. Haas. 2008. Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol. Microbiol.* **67**:241–253.
- Lenz, D. H., M. B. Miller, J. Zhu, R. V. Kulkarni, and B. L. Bassler. 2005. CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol. Microbiol.* **58**:1186–1202.
- Liaw, S. J., H. C. Lai, S. W. Ho, K. T. Luh, and W. B. Wang. 2003. Role of RsmA in the regulation of swarming motility and virulence factor expression in *Proteus mirabilis*. *J. Med. Microbiol.* **52**:19–28.
- Liu, M. Y., and T. Romeo. 1997. The global regulator CsrA of *Escherichia coli* is a specific mRNA-binding protein. *J. Bacteriol.* **179**:4639–4642.
- Liu, M. Y., H. Yang, and T. Romeo. 1995. The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. *J. Bacteriol.* **177**:2663–2672.
- Mercante, J., K. Suzuki, X. Cheng, P. Babitzke, and T. Romeo. 2006. Comprehensive alanine-scanning mutagenesis of *Escherichia coli* CsrA defines two subdomains of critical functional importance. *J. Biol. Chem.* **281**:31832–31842.
- Romeo, T., M. Gong, M. Y. Liu, and A. M. Brun-Zinkernagel. 1993. Identification and molecular characterization of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J. Bacteriol.* **175**:4744–4755.
- Sabnis, N. A., H. Yang, and T. Romeo. 1995. Pleiotropic regulation of central carbohydrate metabolism in *Escherichia coli* via the gene *csrA*. *J. Biol. Chem.* **270**:29096–29104.
- Schubert, M., K. Lapouge, O. Duss, F. C. Oberstrass, I. Jelezarov, D. Haas, and F. H. Allain. 2007. Molecular basis of messenger RNA recognition by the specific bacterial repressing clamp RsmA/CsrA. *Nat. Struct. Mol. Biol.* **14**:807–813.
- Teplitski, M., R. I. Goodier, and B. M. Ahmer. 2003. Pathways leading from BarA/SirA to motility and virulence gene expression in *Salmonella*. *J. Bacteriol.* **185**:7257–7265.
- Wang, R. F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
- Wang, X., A. K. Dubey, K. Suzuki, C. S. Baker, P. Babitzke, and T. Romeo. 2005. CsrA post-transcriptionally represses *pgaABCD*, responsible for synthesis of a biofilm polysaccharide adhesin of *Escherichia coli*. *Mol. Microbiol.* **56**:1648–1663.
- Wei, B., S. Shin, D. LaPorte, A. J. Wolfe, and T. Romeo. 2000. Global regulatory mutations in *csrA* and *rpoS* cause severe central carbon stress in *Escherichia coli* in the presence of acetate. *J. Bacteriol.* **182**:1632–1640.
- Wei, B. L., A. M. Brun-Zinkernagel, J. W. Simecka, B. M. Pruss, P. Babitzke, and T. Romeo. 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol. Microbiol.* **40**:245–256.