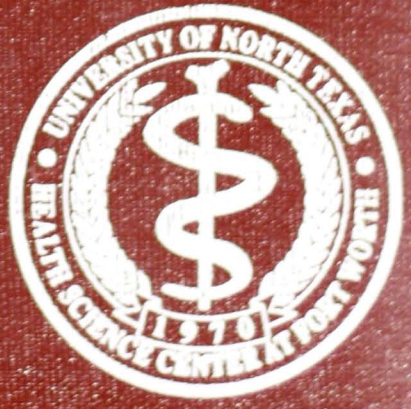
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Wei, Bangdong L., Positive Regulation of Acetate Metabolism and Motility by the RNA-binding Protein CsrA in Escherichia coli. Doctor of Philosophy (Biomedical Sciences), August, 2000, 118 pp., 5 tables, 19 illustrations, bibliography, 175 titles

The carbon storage regulatory (Csr) system consists of a small RNA-binding effector protein, CsrA, and non-coding RNA, CsrB. CsrA acts as a global regulator and modulates specific mRNA stability in *Escherichia coli*. It regulates central carbon metabolism, physiology, and cell surface properties on a broad scale. In this study, the regulatory roles of *csrA* in acetate metabolism and motility were examined. The *csrA* gene was demonstrated to positively regulate acetyl-CoA synthetase and isocitrate lyase, while it did not affect phosphotransacetylase, isocitrate dehydrogenase, or citrate synthase. As a result, growth of *csrA rpoS* mutant strains was very poor on acetate as a sole carbon source. Surprisingly, growth also was inhibited specifically by the addition of modest amounts of acetate to rich media. Cultures grown in the presence of ≥25 mM acetate consisted substantially of glycogen biosynthesis (*glg*) mutants, which were no longer inhibited by acetate. Several classes of *glg* mutations were mapped to known and novel loci. The TCA cycle intermediates or pyruvate, but not glucose, galactose or glycerol, restored growth and prevented the *glg* mutations in the presence of acetate. Furthermore, amino acid uptake was inhibited by acetate specifically in the *csrA rpoS* strain. Apparently, central carbon flux imbalance, inhibition of amino acid uptake, and a deficiency in acetate metabolism are combined to cause metabolic stress by depleting the TCA cycle.

The *csrA* gene was essential for motility and flagellum biosynthesis. Further studies elucidated the molecular mechanism by which CsrA positively regulates flagellum synthesis. Purified recombinant CsrA protein, which was isolated as a ribonucleoprotein

complex consisting of one single CsrB molecule and ~18 CsrA subunits, directly stimulated the coupled transcription-translation of *flhDC::lacZ* in S-30 extracts and bound specifically to the 5' non-coding segment of *flhDC* mRNA in mobility shift assay. The steady state level of *flhDC* mRNA was higher and its half-life was ~3-fold greater in a *csrA* wild type versus a *csrA::kanR* mutant strain, as shown by RT-PCR. Thus, CsrA is able to stimulate *flhDC* gene expression by a post-transcriptional mechanism that resembles its function in repression.

POSITIVE REGULATION OF ACETATE METABOLISM AND MOTILITY BY THE RNA-BINDING PROTEIN CsrA IN *Escherichia coli*

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DISSERTATION

Presented to the Graduate Council of the

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For the Degree of

DOCTOR OF PHILOSOPHY

By

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CHAPTER I

INTRODUCTION

Rationale and Specific Aims

Bacteria are frequently faced with changes in their environments to which they must respond in order to survive and reproduce. This adaptability is mediated primarily via the regulation of genetic expression. At the highest level of genetic control are the global regulatory systems, which simultaneously modulate numerous genes participating in various metabolic, physiological, genetic and morphological systems of the cell. A recently described gene, *csrA*, encodes a global regulator, CsrA, which is an RNA-binding protein and modulates specific mRNA stability. CsrA resides in a multisubunit ribonucleoprotein complex and interacts with a single RNA molecule, CsrB. Previous studies reveal that CsrA controls carbon metabolism, acting as an activator of glycolysis and a repressor of gluconeogenesis and glycogen metabolism, cell size and surface properties in *Escherichia coli*.

The key regulatory role of CsrA in central carbon metabolism prompted to examine the regulatory role of the *csrA* gene in acetate metabolism, including acetate activation, the glyoxylate shunt, and the tricarboxylic acid cycle (TCA cycle, or Krebs cycle). Cells of *E. coli* grown on various sugars or a mixture of amino acids excrete acetate during the exponential phase of growth and subsequently take up and metabolize this acetate. Growth on acetate is important for *E. coli* to survive in the mammalian intestine since a large amount of acetate is produced through fermentation of carbohydrate by enteric anaerobes. The glyoxylate shunt is essential for growth on carbon

sources such as acetate or fatty acids because it bypasses the two decarboxylation steps of the TCA cycle, allowing a net conversion of acetyl-CoA to metabolic intermediates.

The observation that the *csrA* gene homologs in several species are located within flagellar gene cluster led to hypothesize that the *csrA* gene may be involved in motility, which allows bacteria to move to favorable environments and is frequently required for bacterial pathogenesis. In fact, acetate metabolism and motility are connected through acetyl phosphate (AcP), which is an intermediate during the interconversion of acetyl-CoA to acetate. Phosphorylation of OmpR can be accomplished non-enzymatically by acetyl phosphate, allowing this protein to negatively regulate the flagellar master operon *flhDC* and thus repress motility.

The specific aims of this project are: 1) to further characterize the CsrA-CsrB complex, 2) to explore the regulatory role of CsrA in acetate metabolism, including the acetate activation pathways, the glyoxylate shunt, and the TCA cycle; 3) to examine the effects of CsrA on motility and flagellum biosynthesis, and 4) to determine the molecular mechanism by which CsrA regulates the expression of flagellum biosynthesis.

Background and Significance

Csr global regulatory system. In the environment, bacteria are believed to frequently exist in a physiological state which resembles the stationary phase of a batch culture in laboratory experiments. The transition from exponential phase into stationary phase represents a critical period for many species. During this time, cells become more stress-resistant, slowly-metabolizing and better able to survive starvation conditions (for review, see Huisman *et al.*, 1996; Romeo, 1998). Numerous changes in morphology, physiology, and genetic changes accompany the adaptation to stationary phase conditions (Huisman *et al.*, 1996). One of the metabolic pathways induced is the glycogen

biosynthesis pathway, depicted in Fig. 1. While the biological function of glycogen in bacteria is not rigorously established, it is believed that glycogen may provide carbon and energy to promote survival during starvation (Press and Romeo, 1994).

Biosynthetic reactions

1. ATP + Glucose-1-P ⟶ ADPglucose + PPi
2. ADPglucose + 1,4-Glucan ⟶ 1,4-Glucosyl-glucan + ADP
3. 1,4-Glucan ⟶ 1,6-Branched 1,4-Glucan (glycogen)

Enzymes	Structural genes
1. ADPglucose pyrophosphorylase	*glgC*
2. Glycogen synthase	*glgA*
3. Glycogen branching enzyme	*glgB*

Fig. 1. Reactions, enzymes and structural genes required for glycogen biosynthesis in *Escherichia coli.*

Glycogen synthesis is regulated via several global systems, including catabolite repression, stringent response (Romeo and Press, 1989; Romeo *et al.*, 1990), and RpoS (Hengge-Aronis and Fisher, 1992). Together with glycogen catabolism genes, *glgP(Y)* and *glgX*, the glycogen biosynthesis genes are organized in two operons, *glgCAP(Y)* and *glgBX*, respectively. The expression of *glgCAP(Y)*, but not *glgBX,* is positively regulated by cAMP-cAMP receptor protein (CRP) and ppGpp, which mediate the catabolite repression and stringent response global regulatory systems, respectively (Romeo and Preiss, 1989; Romeo *et al.*, 1990) Interestingly, the expression of a structural gene located outside of the *glg* gene cluster, *glgS*, is dependent on *rpoS*-encode alternative

sigma factor, σ^S, while the expression of the other *glg* genes is dependent on σ^{70} RNA polymerase (Hengge-Aronis and Fischer, 1992; Preiss and Romeo, 1994). Evidence that the *glg* genes may be negatively regulated led to the identification of a novel gene, *csrA* (carbon storage regulator A) in *E. coli* (Romeo *et al.*, 1993). The disruption of *csrA* caused ≥20 fold elevation of glycogen levels, overexpression of four *glg* biosynthetic genes (*glgC, glgA, glgB*, and *glgS*) located in three operons and overproduction of phosphoglucomutase, which generates the glycogen precursor glucose 1-phosphate (Romeo and Gong, 1993; Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Yang *et al.*, 1996).

The *csrA* gene encodes a 61-amino-acid RNA-binding protein, which is a global regulator and controls a variety of metabolic and physiological properties. First, CsrA is an activator of glycolysis and a repressor of gluconeogenesis (Sabnis *et al.*, 1995). Thus, a mutation in *csrA* exerts a dramatic effect on the flow of carbon into glycogen. Second, some gross phenotypic changes are observed in a *csrA* mutant of *E. coli*. The mutant cell is larger in size and adheres tightly to surfaces such as plastic or glass and forms a biofilm when it is grown in static culture (Romeo *et al.*, 1993). Third, by comparing genome sequences and DNA hybridization, it appears that *csrA* is widely distributed within the eubacterial domain (White *et al.*, 1996; Romeo, 1998). The plant pathogen *Erwinia carotovora*, a close relative of *E. coli*, uses the Csr (Rsm) system as a key regulator of factors which cause soft rot disease. In this species, the CsrA homolog represses the production of extracellular enzymes, tissue macerating enzymes, secondary metabolites, antibiotic activity, pigment production, and possibly the autoinducer, N-(3-oxohexanoyl)-L-homoserine lactone (Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Mukherjee *et al.*, 1996). Fourth, DNA gyrase activity is possibly regulated by CsrA (Murayama *et al.*, 1996).

Further investigation elucidates the second component of Csr system, CsrB, a non-coding RNA. This RNA consists of an approximately 360-nucletide transcript, which binds to affinity-purified CsrA in a complex consisting of a single CsrB RNA molecule and multiple CsrA protein subunits. CsrB is not required for CsrA to function, but in fact antagonizes CsrA *in vivo* (Liu *et al.*, 1997). The most striking feature of the *csrB* sequence is the presence of 18 imperfect repeats of the sequence 5'-CAGGA(U,C,A)G-3'. These repeats are found in loops of predicted hairpins distributed throughout the RNA molecule and in single-stranded segments between the hairpins. The hairpin loops are generally closed by the 5'-C:G-3' basepair of the consensus sequence (Fig. 2; Romeo, 1998). These repeated sequences are also observed in *csrB* homologs in *E. carotovora* and *Salmonella typhimurium* (Murata *et al.*, 1994; Altier *et al.*, 2000).

The mechanism by which CsrA negatively regulates glycogen biosynthesis has been studied in some detail. CsrA represses *glgC* gene expression by binding in the vicinity of the ribosome-binding site and facilitates the decay of *glg* mRNA. However, the positive regulatory mechanism of CsrA remains to be elucidated. A model for post-transcriptional regulation by CsrA and CsrB, depicted in Fig. 3 was proposed by Liu *et al.* (1997). The binding of CsrA to transcripts of negatively regulated genes within or close to the ribosome binding site may inhibit translation, resulting in ribosome clearance and mRNA decay, or involve direct endonucleolytic attack at the CsrA-mRNA complex, altered transcript conformation and secondary attack.

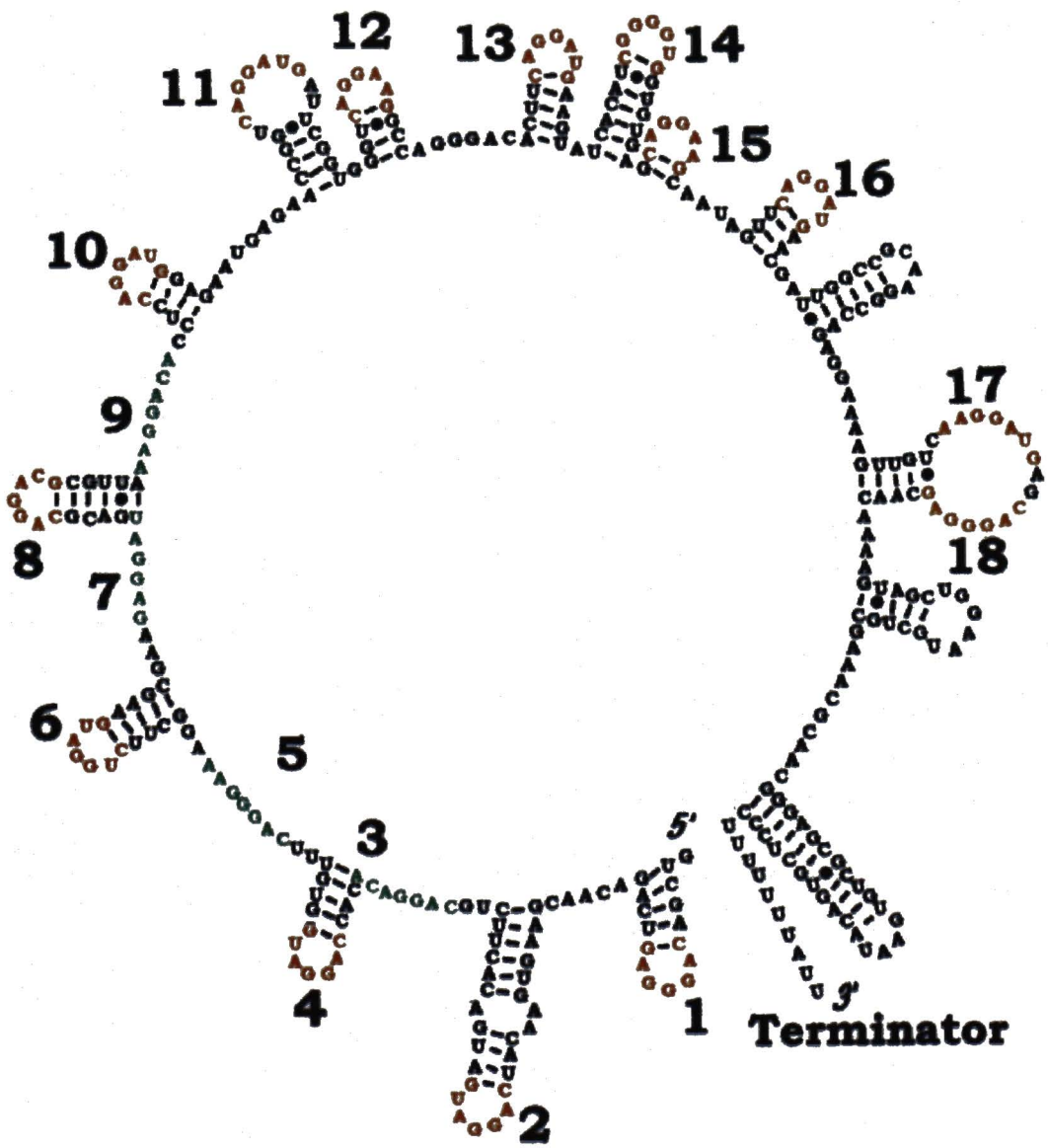

Fig. 2. Secondary structure of CsrB predicted using a stochastic folding algorithm (adapted from Romeo, 1998). The repeated elements, numbered from 1 through 18, are either located in loop (red) or in single-stranded (green) segments between the hairpins. The most prevalent sequences of the loop motif is 5'-CAGGAUG-3'.

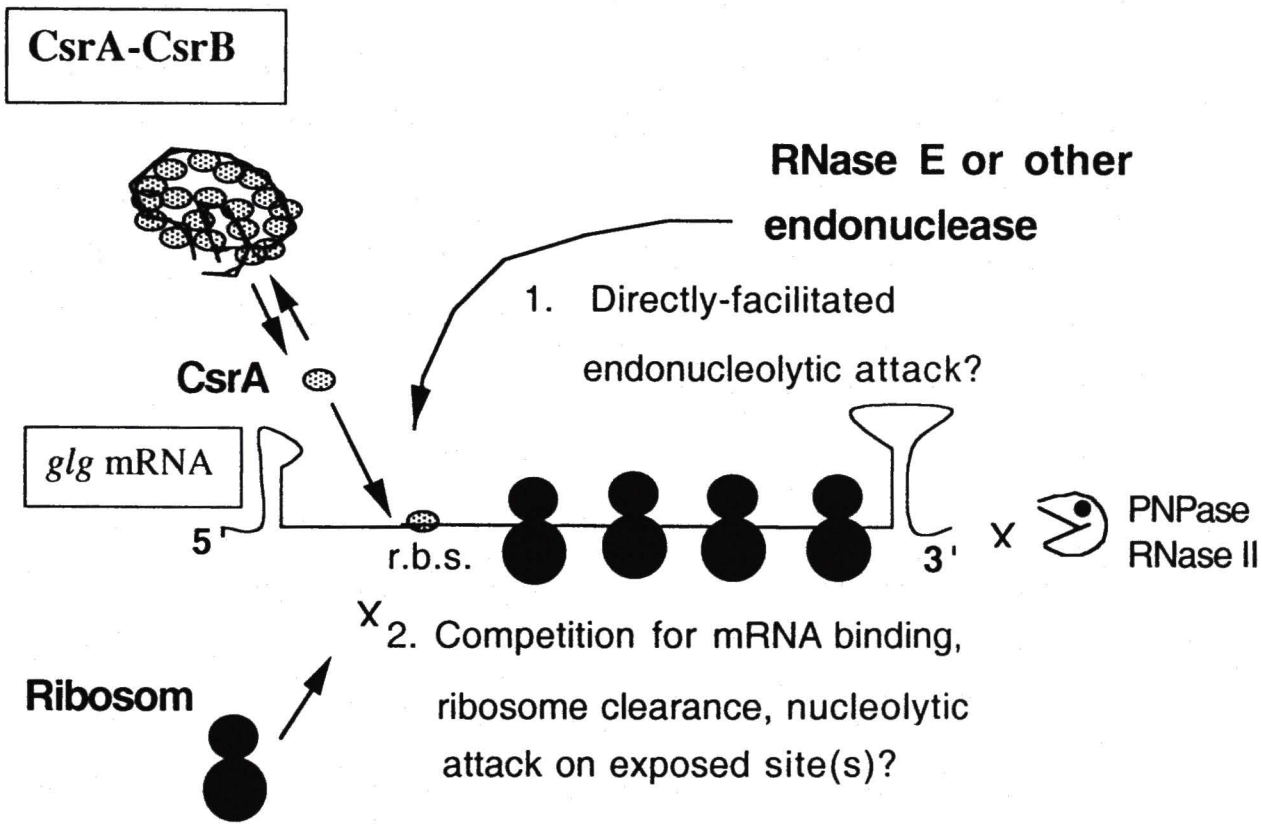

Fig. 3. A model for post-transcriptional regulation for gene expression by CsrA and CsrB (Adapted from Romeo, 1998). See text for discussion.

Acetate metabolism and the glyoxylate shunt. The ability to use acetate probably is important for survival of *Escherichia coli* in the mammalian intestine, since a large amount of acetate (up to 70 mM) is produced through fermentation of carbohydrate by enteric anaerobes (Cummings *et al.*, 1987). Growth of *E. coli* on glucose also produces large amount of acetate (up to 85 mM; Luli and Strohl, 1990) in the exponential phase. This waste of nutrients apparently is a result of an inefficient TCA cycle in which the α-ketoglutarate dehydrogenase complex limits the TCA cycle flux (Amarasingham and Davis, 1965). In contrast, growth in mixed amino acids (e.g., tryptone broth) leads to the secretion only a small amount of acetate (~ 1 to 2 mM) (Kumari, *et al.*, 1995; Shin *et al.*, 1997). This acetate is subsequently taken up and metabolized and the switch coincidence with the decrease of growth rate (Kumari *et al.*, 2000).

The uptake of acetate is presumed to occur by simple diffusion of the non-ionized form of the acid since no transporter for acetate has been identified (e.g., Salmond *et al.*, 1984). However, acetate uptake is saturable, suggesting that one may exist (Kay, 1972). Metabolism of acetate, whether for oxidation via the TCA cycle, or for replenishing intermediates of the TCA cycle via the glyoxylate shunt, requires its activation to acetyl-CoA. In *E. coli,* two pathways exist for the metabolic interconversion of acetate and acetyl-CoA. Acetyl-CoA synthetase (Acs pathway) produces acetyl-CoA directly from acetate, while acetate kinase and phosphotransacetylase (AckA-Pta pathway) produce acetyl phosphate as an intermediate (Fig. 4). The levels of acetate kinase and phosphotransacetylase in extracts of *E. coli* vary little with different carbon sources (Brown *et al.*, 1977; Kumari *et al.*, 1995), suggesting that the expression of the *ackA* and *pta* genes is neither induced by acetate nor catabolite repressed by glucose. In contrast, the Acs pathway is a catabolite-repressible, acetate-inducible, and high-affinity system, ideally suited for scavenging extracellular acetate present at physiological concentrations

(Brown *et al.*, 1977; Kumari *et al.*, 1995). The full expression of *acs* requires CRP and FNR (Shin *et al.*, 1997). Although *acs* transcription is dependent on σ^{70}, during the mid-exponential phase, the full expression of *acs::cat* and a small amount of Acs protein as determined by immunoblot analysis are dependent on σ^{S} (Shin et *al.*, 1997; Kumari *et al.*, 2000). This could be a result of some indirect mechanism of σ^{S}, perhaps by transcribing some accessory transcription factor that participated in the control of σ^{70}-dependent initiation of *acs* transcription (Kumari *et al.*, 2000).

The glyoxylate shunt, an anaplerotic bypass through the TCA cycle, is required in order for cells to grow on acetate, allowing net synthesis of biosynthetic precursors from acetate. As depicted in Fig. 4, in each turn of the TCA cycle, two carbons enter the cycle as acetyl-CoA and two are lost as CO_2. Thus, there is no means by the TCA cycle alone to replenish the dicarboxylic acids that are consumed in amino acid biosynthesis. The loss of carbons may be replenished by the glyoxylate shunt, which bypasses two of the decarboxylation steps of the TCA cycle. Consist with this observation, strains lacking this pathway fail to grow on acetate or fatty acids (Hughes *et al.*, 1988). Although the glyoxylate shunt prevents the loss of carbon through the TCA cycle, energy generation is much less efficient, with 4 or 12 ATP produced, respectively, from one molecule of acetyl-CoA. This requires that cells carefully regulate the relative flux through those pathways. Isocitrate is a critical substrate since it stands at the branch point between these two pathways. During growth on acetate, ~70% of isocitrate dehydrogenase (IDH) is maintained in the inactive phosphorylated form (LaPorte *et al.*, 1985). The phosphorylation and dephosphorylation of IDH are catalyzed by a single bifunctional enzyme, IDH kinase/phosphatase (LaPorte and Koshland, 1982).

The two enzymes of the glyoxylate shunt, isocitrate lyase and malate synthase A, encoded by *aceA* and *aceB*, respectively, are synthesized when *E. coli* is grown on

acetate. Isocitrate lyase catalyses the reaction from isocitrate to glyoxylate and succinate; and malate synthase A catalyses the reaction that condenses glyoxylate and acetyl–CoA to malate (Fig. 4). Malate synthase A should be distinguished from a second malate synthase, malate synthase G, encode by the *glcB* gene, which functions during growth on glycolate or glyoxylate as sole carbon sources (Molina *et al.*, 1994). The genes, *aceB*, *aceA*, and *aceK*, which encodes IDH kinase/phosphatase, form the *aceBAK* operon. Expression of *aceBAK* is affected by several regulatory factors, including IclR, FadR, IHF, and ArcAB (reviewed in Cronan and LaPorte, 1996). Transcriptional regulation is not the only mechanism by which cells modulate flux through the glyoxylate shunt. Post-translational modification of isocitrate dehydrogenase by the bifunctional enzyme IDH kinase/phosphatase is also important in allowing the glyoxylate shunt to compete effectively with the TCA cycle (Cronan and LaPorte, 1996).

Flagella and motility. Many bacteria are motile by means of flagellar propulsion. The benefits of motility include increased nutrient acquisition, avoidance of toxic substances, and the ability to access optimal colonization sites in host. Motility and flagella have been shown to play important roles at many points during the infectious cycles of a number of pathogenic bacterial species, such as *Vibrio Cholerae, Helicobacter pylori, Campylobacter jejuni*, *Pseudomonas aeruginosa*, and *Xenorhabdus nematophilus* (reviewed in Ottemann and Miller, 1997; Givaudan and Lanois, 2000). Motility is also required for biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa* under certain growth conditions (Pratt and Kolter, 1998; O'Toole and Kolter, 1998). Finally, it has been proposed that the flagellar pathway is a type III pathway and may be a general mechanism for transport of proteins involved in virulence (Macnab, 1999; Young *et al.*, 1999).

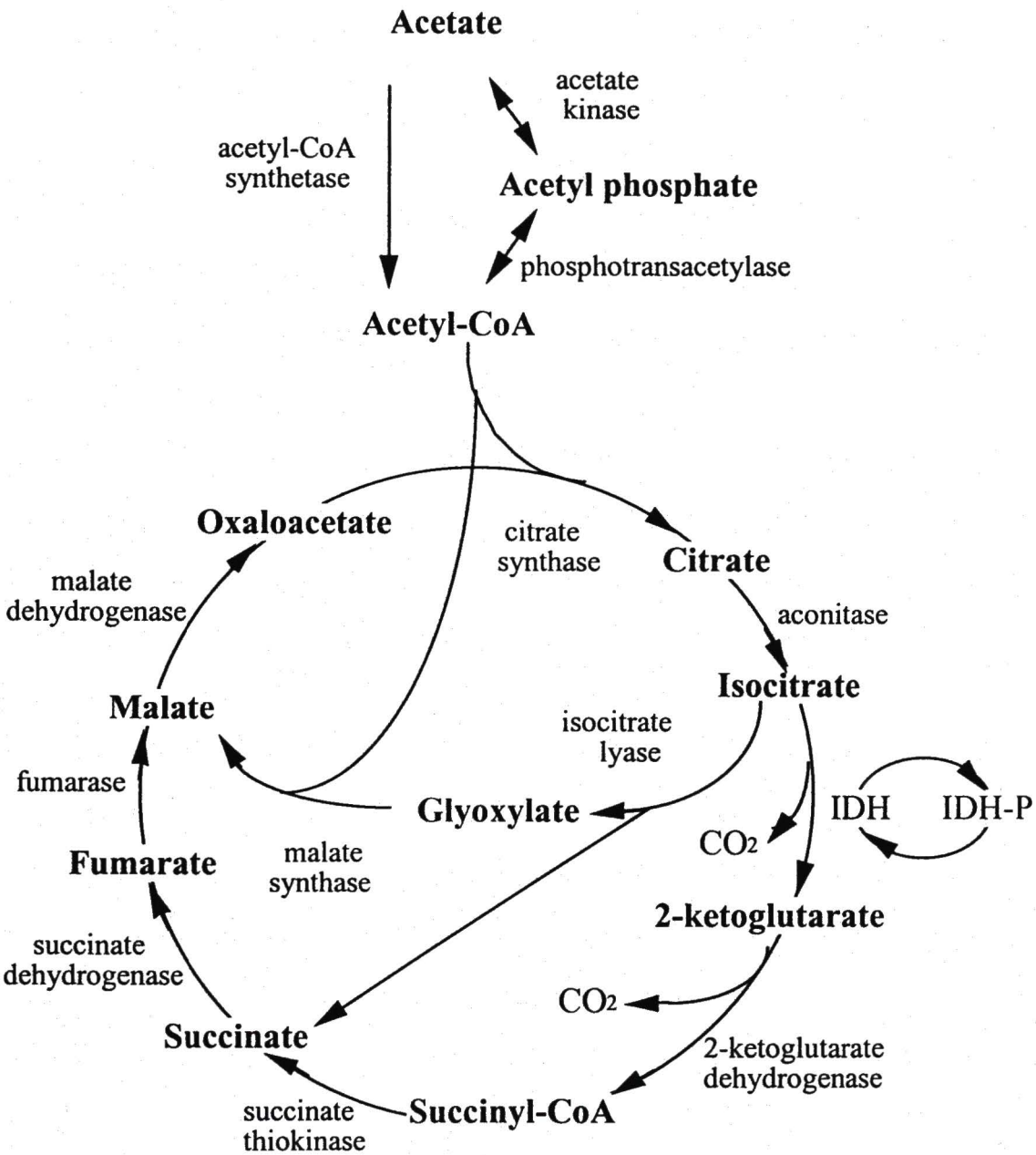

Fig. 4. Acetate metabolic pathways, including acetate activation, the glyoxylate shunt, and the TCA cycle. IHD and IDH-P represent the dephosphorylated and phosphorylated forms of isocitrate dehydrogenase, respectively.

A bacterial flagellum is composed of three parts: the helical filament, the hook and the basal body. The basal body is embedded in the cell membrane while the hook and filament extend into extracellular space. The helical filament is a propeller; the hook is a universal joint; and the basal body servers as a motor using proton motive force as an energy source. The synthesis of flagella in *E. coli* requires at least 14 operons encoding more than 50 genes. These genes are organized in a 3-tiered hierarchy, in which the expression of genes in lower tier is dependent upon the expression of higher tier genes (reviewed in Macnab, 1996). The top of this hierarchy is the master operon, *flhDC*, which directly or indirectly controls the expression of all of the remaining genes in the regulon. The second level of the hierarchy encodes proteins for the basal body and the hook. It also encodes the flagellar sigma factor, FliA, which is required for the expression of level 3 genes. These genes are required for the assembly of flagellar filaments, motor activity, chemotaxis, and an anti-sigma factor, FlgM, which binds to FliA and modulates its activity (Hughes *et al.*, 1993; Kutsukake and Iino, 1994).

Flagellum biosynthesis requires a large amount of energy. It is estimated that ~2% of total cellular energy for its synthesis and rotation (Macnab, 1996). The flagellin alone comprises about 8% of the total cell protein when the flagellar operons are expressed optimally (Li *et al.*, 1993). Thus, cells of bacteria must strictly control the expression of flagellar genes in response to environmental challenges. A number of environmental conditions affect flagellum biosynthesis in *E. coli*. Adverse conditions that cause the loss of motility include the presence of high concentration of inorganic salts, high concentrations of carbohydrates, high concentrations of alcohols, high temperature, or the presence of gyrase inhibitors (Adler and Templeton, 1967; Li *et al.*, 1993). The ability to shut down this costly process under adverse conditions may help bacteria to survive (Li *et al.*, 1993). Thorough investigation indicates that the regulation

of motility primarily takes place at the level of *flhDC* transcription (reviewed in Pruβ, 2000). The expressions of *flhDC* in *E. coli* and *Salmonella typhimurium* are dependent on numerous regulators, including cyclic AMP receptor protein (Kutshukake, 1997; Soutourina *et al.*, 1999), histone-like nucleoid-structuring (H-NS) protein (Bertin *et al.*, 1994; Soutourina *et al.*, 1999), the heat shock response network proteins DnaK, DnaJ, and GrpE (Shi *et al.*, 1992), Pss and Psd which are involved in the synthesis of phosphatidylethanolamine in cell membranes (Shi *et al.*, 1993), and inorganic polyphosphate (Rashid *et al.*, 2000). In addition, phosphorylated OmpR negatively regulates the expression of *flhDC* (Shin and Park, 1995). The phosphorylation of OmpR is mediated either by acetyl phosphate or by the osmoregulation sensor protein EnvZ, which, together with OmpR, forms a two-component regulatory system (Forst *et al.*, 1989).

Motility and flagellum biosynthesis are growth phase dependent, increasing as cells progress through the exponential phase, reaching a maximum during post-exponential phase (Amsler *et al.*, 1993). This regulation is achieved at least partially by phosphorylation of OmpR via AcP (Pruβ, 2000). Synthesis of AcP from acetyl-CoA and Pi is catalyzed by phosphotransacetylase; from ATP and acetate is catalyzed by acetate kinase. Since both reactions are readily reversible, the main determinant of intracellular AcP is the balance between intracellular acetyl-CoA and acetate. When either acetyl-CoA or acetate accumulates the level of AcP increases. During early exponential phase, cells possess a large amount of AcP as the result of synthesis of acetate via Pta-AckA pathway (Pruβ and Wolfe, 1994). After the primary carbon sources are depleted, cells switch to the consumption of TCA cycle degradable amino acids and the level of AcP decreases due to the uptake of acetate from the medium and its conversion back to acetyl-CoA by Acs (Pruβ and Wolfe, 1994; Kumari, *et al.*, 1995). AcP acts as a phosphodonor

in the autophosphorylation of OmpR and phosphorylated OmpR binds to and inhibits the transcription from the *flhDC* promoter (Pruβ and Wolfe, 1994; Shin and Park, 1995; Pruβ, 2000).

Post-transcriptional regulation. Gene expression in bacteria is controlled at many levels, including transcription initiation, mRNA stability, and the efficiency of translation (reviewed in Grunberg-Mangago, 1999). However, most of studies on bacterial gene regulation focus on the level of transcription initiation. Regulation of gene expression at the level of mRNA stability and translation were once considered to be restricted to a limited number of genes. However, recent studies have shown that several global regulators act at the post-transcriptional level, including Host Factor 1 (HF-1), CsrA, cold-shock proteins (reviewed in Nogueira and Springer, 2000), indicating that post-transcriptional regulation is far more common than once believed (e.g., Liu *et al.*, 1995; Tang and Guest, 1999). Although procaryotic mRNAs are generally unstable relative to those of eucaryotes, their decay rates can differ considerably within a single cell. The chemical half-life of mRNA in *E. coli* can range from a fraction of a minute to half an hour (for review see Belasco, 1993; Kushner, 1996).

The decay of many mRNAs is initiated by a primary endonucleolytic cleavage, often by RNase E or by RNase III. This cleavage is followed by exonucleolytic degradation at the new 3' end. Two enzymes, polynucleotide phosphorylase (PNPase) and RNase II, are involved in this process (Grunberg-Mangago, 1999). A multienzyme complex called the degradosome contains RNase E, PNPase, an ATP-dependent helicase, RhlB, which is a member of a DEAD box family, and enolase, a glycolytic enzyme (Py *et al.*, 1994). If the degradosome, whose existence has only been biochemically examined,

is involved in mRNA degradation *in vivo*, mRNA decay may be linked to intermediary metabolism (Grunberg-Manago, 1999).

While no bacterial nuclease is known to sequentially remove nucleotides from the 5' end of bacterial RNAs, net degradation often occurs with a 5' to 3' polarity. This paradox may be explained by sequential RNase E attack, which apparently requires a free 5' end to perform the first downstream endonucleolytic cleavage. This liberates an upstream fragment that is exonucleolytically degraded and a downstream fragment with a new 5' end that is recognized by RNase E and further cleaved (Grunberg-Manago, 1999). This processive endonucleolytic cleavage followed by a 3' to 5' exonucleolytic degradation process may explain the net 5' to 3' polarity of decay.

The regulation of mRNA decay is likely to involve a variety of molecular mechanisms, including both *cis*- and *trans*-acting factors. In addition, translation status of a message also affects its half-life. RNA secondary structural features present in the 5' untranslated region (5'-UTR) dramatically influence mRNA stability and translation initiation. Furthermore, the rate-limiting step in mRNA decay is usually an initial endonucleolytic cleavage that often occurs at the 5' extremity (Kushner, 1996; Jackson and Wickens, 1997; Yamanaka *et al.*, 1999). The longevity of *papA* mRNA is determined primarily by its 5'-UTR. A stem-loop near the 5' end of this UTR helps to protect the message from degradation (Bricker and Belasco, 1999). In the case of *ompA* mRNA, it appears that stabilization is most likely caused by a highly ordered structure consisting of three stem-loops that prevent access to the nuclease normally initiating decay at the 5' terminus (Emory, *et al.*, 1992; Kushner, 1996). The growth rate-dependent manner of *ompA* mRNA degradation is likely related to the regulatory role of host factor 1 (Hfq; Vytvytska *et al.*, 1998). In contrast to the situation observed in many eukaryotic systems, ribosomes are actively involved in mRNA stabilization in

prokaryotic cells. The presence of ribosomes at or near the ribosome-binding site is often particularly important for mRNA stability, probably protecting the 5' terminal extremity from initiation of degradation.

Significance. Our findings will help to elucidate a novel regulation that allows bacteria to adapt and survive under various environmental and nutritional conditions. Since *csrA* homologues are present in diverse species and play a central regulatory role in directing carbon flux and other physiological functions in *E. coli* and related species, our investigation is expected to have a very broad impact on both fundamental and applied microbial research. Bacterial motility is regarded as a virulence factor. In addition, the Csr system controls virulence genes of both plant and mammalian pathogens, and regulates stationary phase processes. Therefore, an understanding of the mechanism by which CsrA regulates flagellum biosynthesis could lead to the development of therapies for bacterial diseases, which decrease virulence and/or stationary phase-associated resistance properties of pathogens.

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CHAPTER II

Part of the original results presented in this chapter were published in the *Journal of Biological Chemistry*, 1997, **272**:17502-17510.

ESTIMATION OF THE NATIVE MOLECULAR MASS OF CsrA-CsrB COMPLEX

Introduction

In order to facilitate further studies on the mechanism of CsrA, a biologically active recombinant CsrA protein containing a metal-binding affinity tag (6 carboxy- terminal histidine residues) was over-expressed and purified using Ni-nitrilotriacetic acid (NTA) affinity chromatography. The affinity-purified product consisted of a single polypeptide with a molecular mass of 7.6 kDa and was authenticated by SDS- polyacrylamide gel electrophoresis (PAGE), immunoblotting, N-terminal sequencing, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

Although affinity-purified CsrA-H6 was free of other proteins, preliminary characterization indicated that it was contaminated with nucleic acids. Analysis of the CsrA-H6 preparation by 7.5% native PAGE revealed a single major product that stained with Coomassie Blue, Western blot analysis, and acridine orange, indicative of a discrete ribonucleoprotein complex. The complex yielded red fluorescence with acridine orange treatment, a characteristic of single-stranded RNA, and in contrast to DNA, which fluorescens green (McMaster and Carmichael, 1977). The RNA and protein components of the complex were constantly found to be tightly bound under native conditions. Phenol-purified RNA from the complex consisted of a major band of ~360 nucleotides. The reactivity of the complex with orcinol versus bicinchoninic acid (BCA) suggested a mass ratio of 0.8 RNA:protein, or ~ 20 CsrA subunits per RNA molecule. More sensitive approach is needed to precisely determine the native mass of this complex.

Nondenaturing PAGE of multimeric proteins on a set of gels of various polyacrylamide concentration is a sensitive and reliable determination of protein molecular weights, irrespective of charge (Ferguson, 1964; Hedrick and Smith, 1968; Bryan, 1977). Since the CsrA-CsrB appears to be a spherical complex as determined by

negative staining electron microscopy, its molecular mass should be accurately estimated by this approach.

Methods

Affinity–purified CsrA-CsrB (4 μg of protein) and standard proteins, α-lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45.0 kDa), bovine serum albumin (monomer 66 kDa, dimer 132 kDa), and urease (trimer 272 kDa, hexamer 545 kDa), were analyzed by PAGE on a series of native gels containing 5%, 5.5%, 6%, 7%, 8%, and 9% acrylamide. Proteins were detected after electrophoresis by staining with Coomassie brilliant blue as describe by Chrambach *et al.* (1967). Their relative mobility (R_f) values were calculated as the ratio of migration distance relative to the dye bromophenol blue. The values of 100 [Log (R_f x 100)] for each of the proteins were plotted versus gel concentrations as percent, and the logarithm of negative slope of each curve (retardation coefficient, K_R) was then plotted against the logarithm of known molecular mass of each protein to generate a curve from which the molecular mass of CsrA-CsrB was estimated.

Results and Discussion

Fig. 1 presents a typical electrophoretic banding patterns of standard proteins and the CsrA-CsrB complex at 7% gel concentration. Relative mobility (R_f) of each protein at different gel concentrations was generated from this experiment. The molecular mass of the CsrA-CsrB complex was estimated to be 256 kDa the standard curve (Fig. 2). Subtraction of the RNA mass (~120 kDa) from this value indicated that there are approximately 18 CsrA subunits per complex, in close agreement with the rough estimation of 12~20 CsrA subunits per RNA determined by the mass ratio of

protein:RNA. This size also corresponds well to the large structures observed by electron microscope.

The *csrB* gene encoding CsrA RNA was later identified and sequenced. Located within the CsrB sequences are 18 imperfect repeats of the consensus sequences 5'-CAGGA(U, C, A)G-3'. When the RNA secondary structure was predicted, these repeats were found in loops of predicted hairpins distributed throughout the RNA molecule and in single-stranded segments between the hairpins. The data from this experiment that ~18 CsrA proteins interact with a single CsrB molecule in the CsrA-CsrB complex strongly suggest that the repeated sequences constitutes the CsrA binding element.

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Fig. 1. Electrophoretic analysis of CsrA-CsrB and standard proteins on a 7% native gel. Lanes 1 through 6 depict 7.5 μg of α-lactalbumin (14.2 kDa), 10 μg of carbonic anhydrase (29 kDa), 10 μg of chicken egg albumin (45.0 kDa), 7.5 μg of bovine serum albumin (monomer 66 kDa, dimer 132 kDa), 4 μg of affinity-purified CsrA- CsrB protein, and 10 μg of urease (trimer 272 kDa, hexamer 545 kDa), respectively.

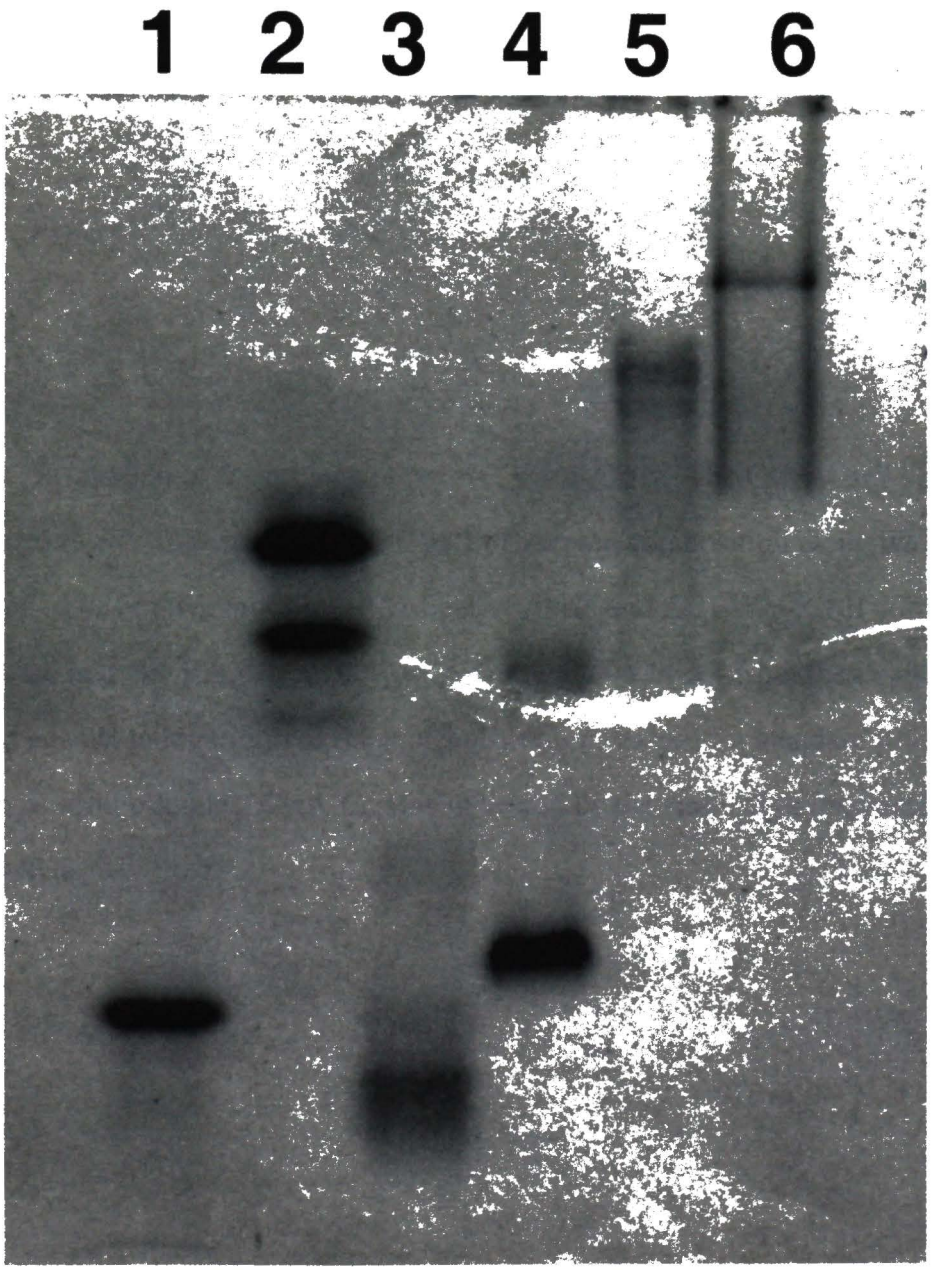
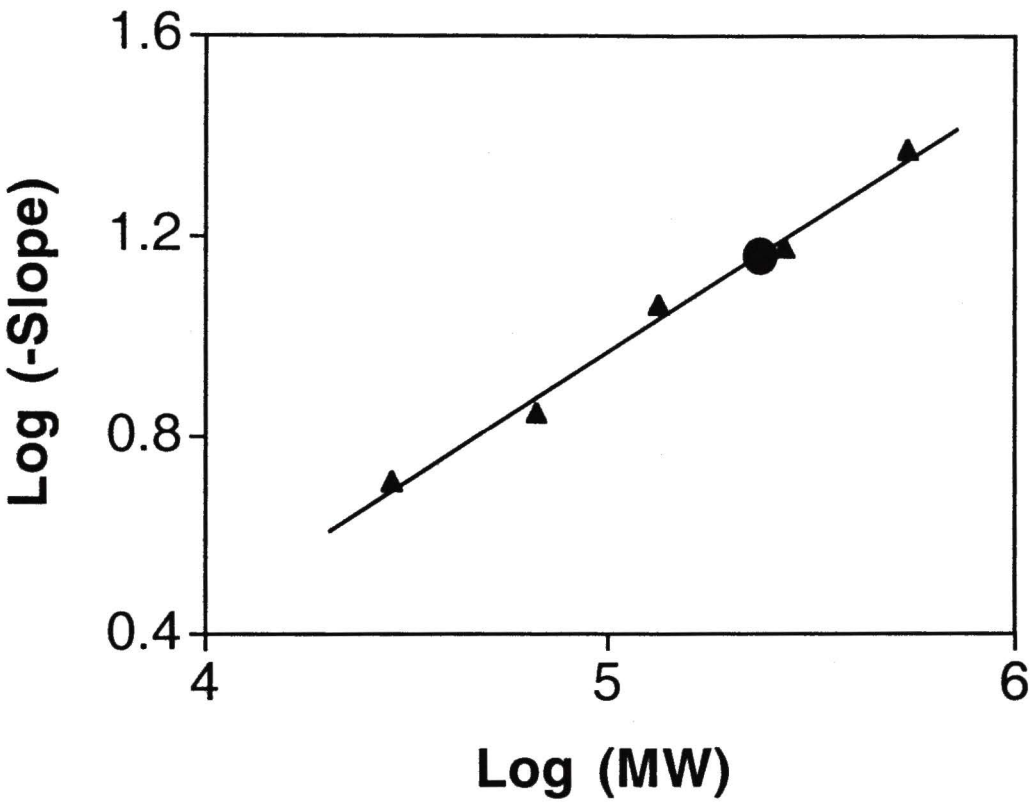

1 2 3 4 5 6

Fig. 2. Estimation of the molecular mass of CsrA-CsrB. The CsrA-CsrB complex was subjected to electrophoresis in native gels of varying composition along with the standard proteins. The relative mobility (R_f) of each protein and the CsrA-Csr B complex was calculated and 100 [Log(R_f x 100)] values of negative slopes from theses graphs were plotted against to logarithm of the molecular mass. Values for standard proteins are shown as solid triangles; CsrA-CsrB is indicated by the circle at the representative mass of 256 kDa.


1.6
1.2
0.8
0.4
Log (-Slope)
4
5
6
Log (MW)

CHAPTER III

The following manuscript was published in the *Journal of Bacteriology*, 2000, **182**:1632-1640.

GLOBAL REGULATORY MUTATIONS IN *csrA* AND *rpoS* CAUSE SEVERE CENTRAL CARBON STRESS IN *Escherichia coli* IN THE PRESENCE OF ACETATE

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Running title: *csrA* and acetate-induced metabolic stress

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ABSTRACT

The *csrA* gene encodes a small RNA-binding protein, which acts as a global regulator in *Escherichia coli* and other bacteria (Romeo, Mol. Microbiol. 29:1321-1330, 1998). Its key regulatory role in central carbon metabolism, both as an activator of glycolysis and as a potent repressor of glycogen biosynthesis and gluconeogenesis, prompted us to examine the involvement of *csrA* in acetate metabolism and the TCA cycle. We found that growth of *csrA rpoS* mutant strains was very poor on acetate as a sole carbon source. Surprisingly, growth also was inhibited specifically by the addition of modest amounts of acetate to rich media (e.g. tryptone broth). Cultures grown in the presence of $\geq$ 25 mM acetate consisted substantially of glycogen biosynthesis (*glg*) mutants, which were no longer inhibited by acetate. Several classes of *glg* mutations were mapped to known and novel loci. Several hypotheses were examined to provide further insight into these effects of acetate on growth and metabolism in these strains. We determined that *csrA* positively regulates *acs* (acetyl-CoA synthetase, Acs) expression and isocitrate lyase activity without affecting key TCA cycle enzymes or phosphotransacetylase. TCA cycle intermediates or pyruvate, but not glucose, galactose or glycerol, restored growth and prevented the *glg* mutations in the presence of acetate. Furthermore, amino acid uptake was inhibited by acetate specifically in the *csrA rpoS* strain. We conclude that central carbon flux imbalance, inhibition of amino acid uptake, and a deficiency in acetate metabolism apparently are combined to cause metabolic stress by depleting the TCA cycle.

INTRODUCTION

Acetate metabolism probably is important for survival of *Escherichia coli* in the mammalian intestine, since a large amount of acetate (up to 70 mM) is produced through fermentation of carbohydrate by enteric anaerobes (5). In the laboratory, growth in liquid media, such as tryptone broth, leads to the secretion of ~ 1 to 2 mM acetate in the late exponential phase. This acetate is subsequently taken up and metabolized (14; 36). No transporter for acetate has been identified (34; 3), although acetate uptake is saturable, suggesting that one may exist (11). Metabolism of acetate requires its activation to acetyl-CoA. In *E. coli* , two pathways exist for the metabolic interconversion of acetate and acetyl-CoA. Acetyl-CoA synthetase [EC 6.2.1.1] (Acs pathway) produces acetyl-CoA directly from acetate, while acetate kinase [EC 2.7.2.1] and phosphotransacetylase [EC 2.3.1.8] (AckA-Pta pathway) produce acetyl phosphate as an intermediate. The Acs pathway is a catabolite-repressible, acetate-inducible, and high-affinity system, ideally suited for scavenging extracellular acetate present at physiological concentrations. On the other hand, the reversible AckA-Pta pathway functions primarily in generating acetate (2). The AckA-Pta pathway is considered to be constitutive (2; 14), while *acs* requires σ^S, CRP, and FNR for full expression (36; unpublished observations).

Growth on acetate also requires the glyoxylate shunt, which bypasses the decarboxylation steps of the TCA cycle, allowing net synthesis of biosynthetic precursors from acetate. The two enzymes of the glyoxylate shunt, isocitrate lyase [EC 4.1.3.1] and malate synthase [EC 4.1.3.2], are synthesized when *E. coli* is grown on acetate. These two proteins are encoded by *aceA* and *aceB*, respectively. These genes, together with *aceK*, which encodes isocitrate dehydrogenase (IDH) kinase/phosphatase, form the *aceBAK* operon. Expression of *aceBAK* is affected by several regulatory factors, including IclR, FadR, IHF, and ArcAB (reviewed in 4). Transcriptional regulation is not

the only mechanism by which cells modulate flux through the glyoxylate shunt. Post-translational modification of isocitrate dehydrogenase [EC 1.1.1.42] by the bifunctional enzyme IDH kinase/phosphatase is also important in allowing the glyoxylate shunt to compete effectively with the TCA cycle (4).

Previously, we elucidated a novel bacterial global regulator, a small RNA-binding protein called CsrA (carbon storage regulator A) (17-19; reviewed in 27). In *E. coli,* CsrA represses a number of stationary phase functions and activates certain exponential phase functions. CsrA represses gluconeogenesis, glycogen biosynthesis and glycogen catabolism; it activates glycolysis (30; 44; 33). Thus, a mutation in *csrA* exerts a dramatic effect on the flow of carbon into glycogen, causing mutant cells to accumulate ≥ 20-fold higher levels of glycogen than the wild type cells. Glycogen can constitute greater than 50% of the dry weight of a *csrA* mutant harvested in the early stationary phase of growth (44). Glycogen synthesis in *E. coli* requires three essential enzymes, ADP-glucose pyrophosphorylase [EC 2.7.7.27], glycogen synthase [EC 2.4.1.21], and glycogen branching enzyme [EC 2.4.1.18], encoded by *glgC*, *glgA*, and *glgB*, respectively. These genes are clustered in two tandem operons, *glgBX* and *glgCAP* , which also include genes encoding the catabolic enzymes glycogen phosphorylase [EC 2.4.1.1] (*glgP*) and glycogen debranching enzyme [EC 3.2.1.-] (*glgX*) (reviewed in 24; 44). CsrA negatively regulates these three *glg* biosynthetic genes, *glgP*, and the monocistronic gene *glgS*, which stimulates glycogen synthesis by an undefined mechanism (30; 44). A mutation in *csrA* results in decreased adenylate energy charge and altered levels of the central carbon metabolites fructose-1, 6-bisphosphate and phosphoenolpyruvate (PEP) (33). Nevertheless, in a variety of media, the growth rate of a *csrA* mutant is indistinguishable from its isogenic parent.

In this study, we examined the regulatory role of the *csrA* gene in acetate metabolism, including its effects on the acetate activation pathways and the glyoxylate shunt. We demonstrated that the *csrA* gene positively regulates Acs and the glyoxylate shunt enzyme isocitrate lyase, but does not affect Pta or certain TCA cycle enzymes. Interestingly, modest levels of acetate cause a dramatic growth defect in *csrA rpoS* mutant strains. Suppressor mutations that restored growth in the presence of acetate were isolated in abundance, and generally were found to decrease glycogen biosynthesis. Insight into the nature of this surprising stress caused by acetate was sought by examining genetic factors and metabolites that either favor or suppress the acetate-induced growth defect and the appearance of glycogen mutations. Our results indicate that the central problem is insufficient TCA cycle flux. This is apparently caused by greatly enhanced carbon flux away from the TCA cycle and towards glycogen biosynthesis in conjunction with decreased uptake of amino acids.

(Experiments described herein were conducted in partial fulfillment of the Ph.D. degree by B. Wei at the University of North Texas Health Science Center at Fort Worth.)

MATERIALS AND METHODS

Chemicals and reagents. Isopropyl-β-D-thiogalactopyranoside (IPTG), *o*-nitrophenol-β-D-galactopyranoside (ONPG), L-amino acids, coenzyme A, acetyl coenzyme A, acetyl phosphate, propionic acid, benzoic acid, 2,4-dinitrophenol, malate dehydrogenase [EC 1.1.1.37], β-nicotinamide adenine dinucleotide (β-NAD), and β-nicotinamide adenine dinucleotide phosphate (β-NADP), and palmitic acid were purchased from Sigma Chemical Co. (St. Louis, Mo.). The palmitic acid was suspended in 10% Brij 58, saponified with KOH, and filter sterilized before use (38). Citrate synthase [EC 4.1.3.7] was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). The

compound 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) was from U.S. Biochemical Corp. (Cleveland, Ohio). [^{14}C]-Radiolabeled L(U)-amino acids (54.2mCi/mmol) were purchased from NEN Life Science Products, Inc. (Boston, MA). All other biochemical reagents were purchased from commercial sources and were of the highest quality available.

Bacterial strains and plasmids. Table 1 lists the strains, plasmids, and phages that were used in this study, their sources, and relevant genotypes.

Growth conditions. LB medium [1% tryptone, 1% NaCl, 0.5% yeast extract, 0.2% glucose, pH 7.4 (22)], was used for routine laboratory cultures. Kornberg medium [1.1% K_2HPO_4, 0.85% KH_2PO_4, 0.6% yeast extract, pH 6.8, and 0.5% glucose for liquid, or 1% glucose for solid media] was used for evaluating the capacity of colonies to synthesize glycogen after staining over iodine vapor (e.g. 8; 18). Tryptone broth contained 1% tryptone and 0.5% NaCl, pH 7.4. Potassium morpholinopropane sulfate (MOPS) medium (23) supplemented with L-amino acids (2 mg/l), nitrogenous bases (0.2 mM), and vitamins (0.01 mM) was used in studies on the glyoxylate shunt enzymes and gene expression experiments. All organic acids were added as sodium salts. Media were supplemented with following compounds as required: kanamycin, 100 μg/ml; tetracycline, 10 μg/ml; ampicillin, 100 μg/ml; X-gal, 40 μg/ml. Sodium acetate was added to media at a final concentration of 50 mM, unless otherwise indicated. Cultures were inoculated with 1 volume of overnight culture per 500 volumes of freshly prepared medium, and were grown at 37 °C on a gyratory shaker at 250 rpm.

Preparation of cell extracts. Cell-free extracts for assays of isocitrate lyase, isocitrate dehydrogenase, and citrate synthase were prepared from the mid-exponential phase cultures according to Maloy et al. (20). Extracts for assays of acetate kinase and phosphotransacetylase were prepared from late-exponential phase cultures according to

Brown et al. (2), except that a French pressure cell was used to disrupt cells, instead of sonication.

Enzyme assays. Acs and Pta were assayed according to Brown et al. (2). The reaction of acetyl-CoA with oxaloacetate to form citrate was coupled to the oxidation of malate, with the concomitant production of NADH, which was monitored spectrophotometrically. Acs activity was determined in an *ackA pta* genetic background to avoid interference by AckA and Pta. These reaction mixtures contained 100 mM Tris/HCl at pH 8.0, 0.5 mM $MgCl_2$, 0.5 mM β-NAD, 0.5 mM coenzyme A, 50 mM L-malate, 12.5 μg crystalline malate dehydrogenase (5,300 units/mg protein), 25 μg crystalline citrate synthase (110 units/mg protein), cell free extract, 10 mM acetate and 10 mM ATP for the Acs assay, or 10 mM lithium acetyl phosphate instead of acetate and ATP, for the Pta assay.

Isocitrate lyase was assayed at 25 °C by the method of Maloy et al. (20). The reactions contained 100 mM potassium phosphate buffer, pH 7.0, 6 mM $MgCl_2$, 4 mM phenyl hydrazine HCl, 12 mM cysteine HCl, 8 mM trisodium DL-isocitrate, and cell-free extract. Isocitrate dehydrogenase was assayed at 25 °C according to LaPorte et al. (15). The reaction mixtures contained 25 mM MOPS at pH 7.5, 250 μM β-NADP, 500 μM DL-isocitrate, 5 mM $MgCl_2$, and cell-free extract.

Citrate synthase [EC 4.1.3.7] was assayed by the method of Stitt (41). The reactions contained 80.6 mM triethanolamine, 3 mM L-malate, 0.22 mM acetylpyridine-adenine dinucleotide (APAD), 12.9 kU/l malate dehydrogenase, 0.18 mM acetyl-CoA, and cell-free extract.

Values for enzyme activities were determined within the linear range with respect to the amount of cell extract added, which was experimentally determined for each enzyme. One unit of activity (U) in each case is defined as 1 μmole of product generated

per min under the given reaction conditions. Each activity was determined in at least two independent experiments to assure reproducibility.

Uptake of a mixture of amino acids. Cells were grown in tryptone broth to exponential phase (approximately 0.3 OD_{600}) and 0.95 ml aliquots were removed and added to sterile tubes containing 0.05 ml of 1 M sodium acetate or water. After a 5-min incubation (37°C, 250 rpm), 5 μCi of the labeled amino acid mixture (54.2 mCi/mmol) was added to each tube. At 0, 1, 2, and 4 min thereafter, 0.2 ml of culture was transferred to centrifuge tubes containing 1 ml tryptone broth and 200-fold excess unlabeled amino acids. Cells were immediately washed twice in tryptone broth, and the cell pellet resuspended in 10 μl SET buffer (20% sucrose; 50 mM EDTA, pH 8.0; 50 mM Tris-HCl, pH 8.0) and lysed with 50 μl lysis solution (0.2 M NaOH, 1% SDS). Radioactivity was determined by liquid scintillation counting, and values were corrected for cell mass at the times of harvest (adjusted to 0.3 OD_{600}) and for nonspecific binding (using "0" time of incubation). Each experiment was conducted at least twice to assure reproducibility.

Protein and β-galactosidase assays. Total cell protein was measured by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard (40). β-galactosidase specific activity was assayed and calculated as described previously (28).

Genetic and molecular biology techniques. P1*vir* transduction mapping of *glg* genotypes, and standard molecular biology approaches such as plasmid isolation and transformation were conducted as described previously (29; 31).

Construction of gene fusions. Single copy chromosomal *'lacZ* transcriptional fusions were constructed for *ackA-pta, pta,* and *acs* in strain W3110 using pRS415 and bacteriophage λRS45 (37). Clone 405 of the Kohara library (12) was the source of a 2079 bp *Pvu*II-*Pvu*II fragment containing the upstream region of the *ackA-pta* operon and 84 codons of *ackA* and of a 1776 bp *Sca*I-*Hpa*I fragment containing the putative *pta*

promoter and 171 codons of *pta* (10). A 1397 bp Klenow-filled *Xho*I-*Cla*I fragment from pSK122, which contained the *acs* promoter region, was used to construct the *acs::lacZ* fusion (36). Dideoxy nucleotide sequencing with M13/pUC forward primer, CCCAGTCACGACGTTGTAAAACG, was used to confirm the '*lacZ* junctions present in the plasmid clones. The single copy gene fusions present in the λ lysogens were verified by PCR amplification. The above primer was used along with the primer ATCCGGCGATCATCTTCCACC, TATCCAGTTGTTTGAAGGCGCG, or TTTACCAATGGCTTCCATCGCG to amplify the *pta, ackA or acs* fusion, respectively.

RESULTS

Growth of *csrA rpoS* strains is inhibited by acetate. During initial studies to examine the possible regulatory role of *csrA* in acetate metabolism, we observed that *csrA* mutants grew poorly in liquid media containing acetate as a sole carbon source, and exhibited an extended (up to several hours) and quite variable lag phase (data not shown). Further studies revealed that acetate was not only a poor carbon source for the *csrA* mutant, but also selectively inhibited the growth of *csrA* mutants when added to rich media. Whereas the parent strain BW3414, which we now know carries an *rpoS*(Am) mutation, and the isogenic *csrA::kanR* mutant TR1-5BW3414 grew equally well in tryptone broth, 50 mM acetate specifically increased the doubling time of the mutant approximately 2-fold. In contrast, it had no effect on the growth of the wild type parent. Although other poor sole carbon sources such as pyruvate and palmitate also supported slower growth of the *csrA* mutant strain relative to its parent, they did not inhibit growth on rich media (data not shown). Clearly, the effects of acetate in rich medium could not be explained by a simple

inability to metabolize acetate, since sufficient carbon and energy for growth were already available in the rich medium.

In order examine the role of *csrA* in acetate metabolism more fully, we decided to further investigate the stress that is caused by acetate on the *csrA* mutant. After 24 hours in the presence of acetate, each strain was streaked onto Kornberg agar and the resulting colonies stained with iodine vapor to detect endogenous glycogen. When cultured in tryptone broth, *csrA* mutants (TR1-5BW3414) yielded uniform dark-brown staining colonies (17). However, in tryptone plus acetate, they yielded primarily glycogen mutants of a variety of striking phenotypes. These included colonies that stained medium brown, yellow, or blue with iodine vapor (e.g. Fig. 2A). These phenotypes indicate moderate synthesis of glycogen, little or no synthesis of glycogen, or synthesis of unbranched glycogen due to the loss of glycogen branching enzyme (6), respectively. The resulting phenotypes were all stable upon repeated subculture (e.g. Fig. 2B). In numerous repetitions of this experiment, glycogen mutations always evolved from the *csrA* mutant grown in the presence of ≥25 mM acetate, but never from either the parent strain treated with acetate or the *csrA* mutant grown in tryptone broth without added acetate. Similar results were observed using medium prepared with 1% casamino acids in place of 1% typtone (data not shown).

By plating cells exposed to acetate and harvested at various time points along the growth curve, we observed that glycogen mutants accumulated during the exponential phase of growth (data not shown). Furthermore, the addition of acetate to mid-exponential phase cultures (~0.3 OD_{600}) caused an immediate decrease in the growth rate (data not shown). These experiments demonstrated that acetate inhibits cell growth, and not simply the exit from stationary phase. Incubation of strains for 2 hours in the

presence of 1mM acetate, which itself did not result in the appearance of glycogen mutants, did not permit adaptation to 50 mM acetate stress (data not shown).

Growth of the mutants that failed to synthesize glycogen or synthesized unbranched glycogen was no longer inhibited by acetate (e.g. Fig. 3A); and mutants that accumulated intermediate levels of glycogen exhibited intermediate growth rates (data not shown). P1*vir* transduction of a transposon mutation (from strain EG3-153), which disrupts glycogen biosynthesis, into the *csrA* mutant also generated a strain that was insensitive to acetate inhibition (Fig. 3B). Strain EG3-153 was isolated as a glycogen-deficient transposon mutant of BW3414 using previously described methodology (30). The transposition mutation was localized by P1*vir* transduction and Southern blot analysis to a 3.4 Kb *EcoR*I fragment containing '*glgBXCA*' (see reference 32 for the genomic restriction map). Together, these experiments clearly demonstrated that the growth defect in the presence of acetate occurred, at least in part, because of excessive glycogen synthesis by the *csrA* mutant.

In an attempt to extend these experiments to other *E. coli* strains, a *csrA* mutant of the prototrophic strain MG1655 was observed to be genetically stable in the presence of acetate. Because the *csrA* mutant TR1-5BW3414 is now known to also contain an *rpoS*(Am) mutation, we constructed single and double *csrA::kanR* and *rpoS::*Tn*10* derivatives of MG1655. The resultant double mutant grew poorly in the presence of acetate and evolved numerous glycogen mutants. In contrast, the single *csrA* or single *rpoS* mutants did not (data not shown). These experiments revealed that sensitivity to acetate requires defects in both *csrA* and *rpoS*.

Effects of other compounds. A variety of other compounds were tested for the ability to inhibit growth of the *csrA* mutant strain TR1-5BW3414 in tryptone broth and to generate glycogen mutations. Pyruvate, α-ketoglutarate, succinate, fumarate, malate,

palmitate, ribose or glycerol did not yield glycogen mutations at 50 mM concentrations. In contrast, propionate was inhibitory and also caused glycogen mutants to accrue, although not as effectively as acetate. Whereas 100 mM propionate was required to give rise to a significant proportion (more that 70%) of apparent glycogen mutants, 50 mM yielded a significantly smaller proportion of glycogen mutants ≤10%), and 25 mM propionate yielded no mutants (data not shown). Benzoate inhibited the growth of *csrA::kanR* mutant; at concentrations greater than 25 mM, no growth occurred. However, benzoate failed to yield glycogen mutations at any concentration. Likewise, the uncoupling agent 2,4-dinitrophenol inhibited growth, but did not yield glycogen mutants at any concentration tested. These experiments provided evidence that the observed acetate stress does not result from decreased intracellular pH nor from depletion of ATP pools, respectively.

Mapping of glycogen mutations to three different loci. P1*vir* transduction mapping was used to localize several of the glycogen mutations. All 16 independently isolated yellow-staining, glycogen-deficient mutations and two blue-staining, apparent branching enzyme mutations mapped to ~0.4 min clockwise from the *tetR* marker of strain CAG18450 (39), which is located at 76.5 min on the most recent *E. coli* genomic map (32). This result provides evidence that all 18 mutations reside within the *glgBX-glgCAP* gene cluster. P1 transduction of the 77 min region of the chromosome from CAG18450 restored to the glycogen-deficient mutants both the parental glycogen phenotype and the sensitivity to inhibition by acetate (data not shown).

Since *glgC* and *glgA* are both essential for glycogen biosynthesis, mutations completely lacking glycogen could be defective in either *glgC*, *glgA*, or both. A complementation experiment was conducted by introducing plasmids carrying wild type alleles of either *glgC* or *glgA* into the mutant strains and testing for restoration of

glycogen synthesis. Surprisingly, all 16 of the yellow-staining mutations were complemented by either *glgC* or *glgA*. This demonstrated that the underlying mutations did not fully inactive either of these two genes, but might have decreased the expression of both genes (e.g. as would be observed for a cis-acting mutation upstream from the *glgCAP* operon). Furthermore, none of the 16 glycogen-deficient mutations affected the expression of the *glgCAP* operon *in trans* (data not shown), as determined using a *glgC::lacZ* translational fusion (28).

P1*vir* transduction mapping with a collection of Tn*10*-marked donor strains (39) also determined the genomic locations of 13 independently-isolated, medium brown-staining mutations. Six of these mutations mapped to 77 min (the region of the *glg* gene cluster), five mutations mapped to ~42.7 min, and two remaining mutations map to ~54.0 min. The latter two regions of the chromosome do not contain any genes previously known to affect glycogen synthesis.

Effects of *csrA* on acetate activation enzymes. Because strain BW3414 grew well with acetate as the sole carbon source, while the *csrA* mutant did not, it was conceivable that *csrA* may affect either the acetate activation pathway or the glyoxylate shunt. When grown in tryptone broth, wild type cells (MG1655) exhibited 2 to 3-fold higher Acs specific activity than did an isogenic *csrA* mutant (Table 2). This difference was not observed in the BW3414 background, in which Acs was extremely low in both the *csrA*$^+$ and *csrA* mutant strains, likely because *acs* expression also depends upon *rpoS* (36). In contrast, the *csrA* mutation exerted little or no effect on the specific activity of Pta. Thus, a *csrA* mutant was defective in the primary pathway needed for the conversion of acetate to acetyl-CoA. To determine whether this effect occurs at the level of transcription, we measured β-galactosidase activity expressed from transcriptional fusions *acs::lacZ*, *pta::lacZ*, and *ackA-pta::lacZ*. Induction of *acs* occurred in the mid-

exponential phase and increased to maximal levels during the transition to stationary phase. The expression of the *acs::lacZ* fusion was higher throughout the growth phase in *csrA* $^{+}$ strain relative to the *csrA* mutant, and the difference during the transition to stationary phase was 2- to 3-fold (Fig. 4). In contrast, *csrA* did not affect expression of *pta::lacZ* or *ackA::lacZ* fusions (data not shown).

Effects of *csrA* on enzymes of the glyoxylate shunt and Krebs cycle. Studies to assess the role of *csrA* in the regulation of the glyoxylate shunt were originally conducted in MOPS supplemented medium containing 50 mM acetate. In this medium, the generation time of the *csrA* parent strain BW3414 and its isogenic *csrA* mutant strain was ~4 h. Unlike the parent strain, the *csrA* mutant exhibited an extended lag phase of variable duration, and when stationary phase cultures were plated onto Kornberg agar, they were found to contain numerous glycogen mutants (data not shown). In mid-exponential phase, isocitrate lyase activities in BW3414 and the isogenic *csrA* mutant were 0.22 and 0.13 U/mg protein, respectively. The addition of acetate (25 mM) and succinate (25 mM) to MOPS supplemented medium improved the growth properties of the *csrA* mutant and prevented the appearance of glycogen mutations. Although the wild type levels of isocitrate lyase were lower than in 50 mM acetate medium, the relative levels of this enzyme were still ~2-fold higher in the parent strain than in the *csrA* mutant (Table 3). Isocitrate lyase activity was extremely low in media containing glucose (Table 3). The expression of β-galactosidase activities from *aceB::lacZ* and *iclR::lacZ* transcriptional fusions in cells growing in MOPS medium supplemented with acetate and succinate (25 mM each) exhibited little or no effects of *csrA* (data not shown), suggesting that *csrA* may affect isocitrate lyase activity post-transcriptionally. Finally, the specific activities of two key Krebs cycle enzymes, citrate synthase and isocitrate dehydrogenase, were found to be unaffected by the *csrA* mutation (Table 3).

Disruption of the glyoxylate shunt, Acs pathway or Pta/Ack pathway in a *csrA rpoS* strain. Significantly fewer ATPs are synthesized when carbon is metabolized through the glyoxylate shunt instead of the Krebs cycle (one acetyl-CoA molecule yields 4 versus 12 ATPs, respectively). If this lower capacity for ATP synthesis were to be involved in acetate stress in *csrA rpoS* strains, then disruption of the glyoxylate shunt should prevent the appearance of glycogen mutations. However, a *csrA rpoS* mutant also defective in the glyoxylate shunt remained sensitive to acetate-dependent inhibition of growth and still gave rise to glycogen mutants when grown in the presence of acetate (data not shown). Clearly, diversion of carbon through the glyoxylate shunt was not responsible for acetate stress. Similarly, knocking out the Acs or Pta-AckA pathways in a *csrA rpoS* mutant did not prevent the appearance of glycogen mutants (data not shown). These experiments, and the observation that palmitic acid, which is metabolized to acetyl-CoA, does not mimic acetate stress, indicated that metabolism of acetate to acetyl-CoA is not required for it to cause metabolic stress. These studies also showed that the effects of acetate are not mediated through its conversion to the intracellular signal molecule acetyl-phosphate, which cannot be synthesized by strains deficient in the Ack-Pta pathway.

Metabolic suppression of acetate-derived glycogen mutations. We hypothesized that increased gluconeogenesis and glycogen synthesis and decreased glycolytic flux in the *csrA* mutant may predispose the strain to depletion of the TCA cycle in the presence of acetate. This was further suggested by the finding that succinate plus acetate no longer gave rise to glycogen mutants in MOPS medium. To test this hypothesis more directly, the *csrA* mutant was cultured in the presence of 50 mM acetate plus 50 mM Krebs cycle intermediates or pyruvate. Each of the following compounds, α-ketoglutarate, succinate, fumarate, malate, or pyruvate suppressed the appearance of

glycogen mutants, while glucose, galactose or glycerol failed to do so. This provided strong evidence that acetate was depleting the TCA cycle in the *csrA rpoS* strains.

Uptake of amino acids from the growth medium. The major carbon and energy source of cells growing on tryptone broth is amino acids (25), which are metabolized via the TCA cycle (21). Thus, we hypothesized that acetate may affect amino acid uptake in the *csrA rpoS* strain. Fig. 5 shows that the parent strain MG1655 and *csrA* and *rpoS* single mutants exhibited slight inhibition of amino acid uptake, ranging from no inhibition up to ~20%. However, in four separate experiments, we consistently observed that uptake by the *csrA rpoS* strain was more sensitive to acetate, exhibiting 35 to 45% inhibition when preincubated for 5 min with 50 mM sodium acetate. Essentially the same results were obtained when pyruvate was added to these strains rather than acetate (data not shown).

DISCUSSION

Genetic adaptation of bacteria to their external environment can manifest in striking ways and reveal new insights into the physiological complexity of these organisms. The results of the present study show that endogenous stress, resulting from defective regulation of central carbon metabolism, can provide very strong selective pressure for adaptation.

E. coli K-12 strains defective in the *csrA* and *rpoS* genes were inhibited by the addition of acetate to the growth medium, which resulted in the rapid appearance of suppressor mutations that disrupt glycogen biosynthesis. Several of the mutations isolated in this study identify two novel genes affecting glycogen synthesis, which we are currently characterizing.

Because central carbon pathways are interconnected, excesses or deficiencies in one pathway should impact upon others (Fig. 6). Considerable evidence indicates that

the major metabolic problem caused by adding acetate to *csrA rpoS* strains is the depletion of the TCA cycle. TCA cycle intermediates or pyruvate, which is a direct precursor for the TCA cycle, restored the growth rate and prevented the appearance of glycogen mutants in the presence of acetate. Furthermore, the contribution of *csrA* to the underlying stress appears to be readily explained. The *csrA* gene encodes an RNA-binding protein that is a potent repressor of glycogen synthesis and gluconeogenesis, and is an activator of glycolysis (reviewed in 27). In a *csrA* mutant, central carbon metabolism is shifted to favor carbon flow away from the TCA cycle and toward the synthesis of glycogen, which act as a metabolic sink for carbon and energy (30; 44). The effect of *csrA* on glycogen synthesis was shown to be a necessary component of acetate-induced stress. Normal growth in the presence of acetate was restored by mutations that decrease glycogen synthesis, and restoration of glycogen synthesis made the latter strains again sensitive to acetate inhibition. Importantly, the *csrA* mutation did not alter levels of key TCA cycle enzymes.

Unlike TCA cycle intermediates, compounds that enter the glycolysis as glucose-6-phosphate (glucose or galactose) or as dihydroxyacetone phosphate (glycerol) (16), could not suppress the effects of acetate. These observations also are consistent with the regulatory effects of a *csrA* mutation on carbon flux. A *csrA* mutant is deficient in triose phosphate isomerase, pyruvate kinase F, and other glycolytic enzymes, while it greatly overproduces phosphoglucomutase, glycogen biosynthetic enzymes and gluconeogenic enzymes (33). Thus, metabolism of glucose-6-phosphate or dihydroxyacetone phosphate is diverted away from the TCA cycle and towards glycogen biosynthesis in a *csrA* mutant.

Acetate contributes to depletion of the TCA cycle specifically in the *csrA rpoS* strain by decreasing the rate of uptake of amino acids, which are metabolized primarily

via the TCA cycle (21). Paradoxically, pyruvate also inhibited amino acid uptake in this strain, and both pyruvate and acetate enter the TCA cycle by direct conversion to acetyl-CoA. However, pyruvate also served as a sole carbon source in the *csrA rpoS* strain without causing the appearance of glycogen mutants, and therefore must be able to replenish the TCA cycle. Acetate itself does not serve as a sole carbon source, unless suppressor mutations occur, perhaps because the *csrA rpoS* double mutant is extremely deficient in acetyl-CoA synthetase activity. In addition, the *csrA* mutant is deficient in isocitrate lyase of the glyoxylate shunt, which is needed for growth on acetate but not pyruvate. However, it is not clear that the latter more modest effect of *csrA* is enough to inhibit growth.

We also have provided evidence to exclude several potential explanations for the observed acetate stress. It is not caused by bypassing energy generating steps of the TCA cycle via induction of the glyoxylate shunt, since glyoxylate shunt mutants are still sensitive to acetate stress and generate glycogen mutants. It does not involve conversion of acetate to the intracellular signaling molecule acetyl-phosphate (43), since *ack-pta* mutants still generated glycogen mutants. Since neither benzoate nor 2,4-dinitrophenol treatment resulted in the appearance of glycogen mutants, acidification of the cytoplasm (as discussed in 35) or decreasing the cellular capacity for ATP synthesis do not explain the effects of acetate, respectively. Conversion of acetate to acetyl-CoA is apparently not required, because *acs* and *ack-pta* mutants still gave rise to glycogen mutations, and palmitic acid and pyruvate, which also are metabolized to acetyl-CoA, neither inhibited growth nor yielded glycogen mutants.

The specific requirement for the *rpoS* defect in the observed acetate stress is unclear, but in part involves sensitization of amino acid uptake to acetate inhibition in the *csrA* mutant. Interestingly, a variety of connections among *rpoS* and acetate metabolism

have been previously established. Accumulation of acetate in the growth medium has been reported as a signal for increasing *rpoS* transcription (35). In contrast, acetyl-phosphate appears to be a signal for proteolysis of RpoS, via the direct covalent modification of the protease RssB (1). Therefore, σ^S levels appear to respond to acetate metabolism in a complex and dynamic fashion. In addition, *rpoS* directly or indirectly induces *acs* expression, and therefore promotes acetate metabolism (36).

Glycogen biosynthesis is also stimulated by *rpoS* via effects on the transcription of *glgS*, while the *glgCAP* operon is not regulated via *rpoS* (9; 24). In this respect, *rpoS* promotes glycogen synthesis, and acts oppositely to *csrA*. Thus, it cannot be argued that the *rpoS* mutation is required for acetate stress by enhancing glycogen biosynthesis. Furthermore, the effects of *rpoS* on glycogen synthesis are modest in a *csrA* mutant, and the *csrA rpoS* double mutant TR1-5BW3414 accumulates very high levels of glycogen (44). It is intriguing to consider that *rpoS* may have effects on central carbon metabolism that contribute to its involvement in acetate stress, but such a role for *rpoS* has not been examined.

Finally, it seems unlikely that *rpoS* specifically affects the mutagenic process, as opposed to the selective process, although this has not been tested. In fact, *rpoS* has been implicated in the formation of certain types of adaptive mutations that occur in stationary phase (7). However, the acetate-induced mutations in the present study occurred during exponential growth, and of course, a functional *rpoS* gene actually prevented the appearance of these mutants.

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TABLE 1. Bacterial strains, plasmids and phages used in this study

Strain, plasmid, Phage	Description	Source or reference
E. coli strains		
BW3414	*ΔlacU169 rpoS*(Am)	Barry Wanner
EG3-153	BW3414 *glg::kanR*, a polar insertion in a 3.4 Kb *Eco*RI fragment, *'glgBXC"*	This study
MG1655	Prototrophic	Michael Cashel
CAG strains	Strain collection used for transduction mapping	(39)
CAG18450	MG1655 (Tn*10* at 76.5 min, near *glg*CAP)	(39)
CAG18500	MG1655 (Tn*10* at 92 min, near *aceBAK*)	(39)
RH106	*rpoS::*Tn*10*	(9)
MG1655*rpoS*	MG1655 *rpoS*::Tn*10* (from RH106)	This study
W3110	F^- prototrophic	Richard Wolf
SS364	W3110 *Δ(lac)58*	This study
SS412	SS364 *pta::lac*	This study
SS413	SS364 *ackA-pta::lac*	This study
SS414	SS364 *acs::lac*	This study
BW*acs*	BW3414 *acs::lac*	This study
CP875	*ΔlacX74 thi-1 thr-1(Am) leuB6 metF159(Am) rpsL136 λlacY*	(26)
AJW803	CP875 ϕ(*Δacs*::Km-1)	(14)
AJW-BW	BW3414 ϕ(*Δacs*::Km-1)	This study
CP911	CP875 *Δ (ackA pta hisJ hisP dhu)*	(14)

PLWT	*aceB::lacZ* (at *lac*) *zah-281*::Tn*10*	David LaPorte
BW3414*ace*	BW3414 *aceB::lacZ zah-281*::Tn*10*	This study
K8-5m	*aceA3 iclR13*	(42)
TR1-5 [A]	*csrA::kanR*	(30)
Mutants isolated from TR1-5BW or TR1-5BW*ace* after acetate treatment:		
B1-TR	TR1-5BW3414 *glgB*	
L9-1	TR1-5BW3414 No glycogen	
MD-1	TR1-5BW*ace* Medium glycogen	
BL-1	TR1-5BW*ace glgB*	
LT-1	TR1-5BW*ace* No glycogen	
Plasmids		
pUC19	Cloning vector; Amp^R	(45)
pPOP245	*glgA* in pBR322, Tet^R	(13)
pPRC1	*glgC* in pUC19, Amp^R	This study
pCZ3-3	*φglgC::lacZ* in pMLB1034, Amp^R	(28)
pRS415	For construction of *'lacZ* operon fusions, Amp^R	(37)
Bacteriophages		
P1*vir*	Strictly lytic P1; forms clear plaques	Carol Gross
λRS45	For transferring *'lac* fusions to single copy	(37)
λSS118	λRS45 carrying *pta::lacZ* fusion	This study
λSS120	λRS45 carrying *ackA:lacZ* fusion	This study
λSS121	λRS45 carrying *acs::lacZ* fusion	This study

[A] Throughout the manuscript, strain designations that contain the prefix TR1-5 indicate that the wild-type *csrA* allele has been replaced by the TR1-5 mutant allele (*csrA::kanR*) by P1*vir* transduction.

TABLE 2. Specific activities of acetyl-CoA synthetase and phosphotransacetylase from *csrA*$^+$ and *csrA::KanR* strains [A]

Strains	Genotype	Specific activity (U/mg protein ± SD, n≥3)	
		Acs	Pta
BW3414	*rpoS csrA*$^+$	0.008 ± 0.002	1.09 ± 0.33
TR1-5BW3414	*rpoS csrA::kanR*	0.005 ± 0.002	0.88 ± 0.05
MG1655	*csrA*$^+$	0.037 ± 0.004	0.97 ± 0.25
TR1-5MG1655	*csrA::kanR*	0.011 ± 0.001	1.31 ± 0.31

[A]Cultures were grown aerobically in 1% of tryptone broth to the transition to stationary phase. Enzyme assays were conducted as described in Materials and Methods.

TABLE 3. Specific activities of isocitrate lyase, isocitrate dehydrogenase, and citrate synthase from *csrA*$^+$ and *csrA::kanR* strains grown under different conditions [A]

Medium	Specific activity (U/mg protein ± SD, n≥3)					
	Isocitrate lyase		Isocitrate dehydrogenase		Citrate synthase	
	csrA$^+$	*csrA:kanR*	*csrA*$^+$	*csrA:kanR*	*csrA*$^+$	*csrA:kanR*
MOPS						
Ace.&Suc.	0.083 ± 0.006	0.041 ± 0.006	1.23 ± 0.22	1.27 ± 0.05	0.61 ± 0.05	0.51 ± 0.05
Glucose	0.008 ± 0.000	0.010 ± 0.000	1.27 ± 0.12	1.24 ± 0.08	0.20 ± 0.01	0.16 ± 0.00
Kornberg						
Glucose	0.003 ± 0.000	0.003 ± 0.000	0.47 ± 0.03	0.49 ± 0.04	0.03 ± 0.00	0.03 ±0.00

[A] Cultures were grown in MOPS supplemented media containing the indicated carbon sources or Kornberg medium to mid-exponential phase. Enzyme assays were conducted as described in Materials and Methods.

FIG. 1. Growth of *csrA*$^+$ and *csrA:: kanR* strains in 1% tryptone broth or tryptone broth plus acetate (50 mM). Open circles and squares represent BW3414 (*csrA*$^+$) grown in tryptone broth with or without acetate, respectively. Solid circles and squares represent TR1-5BW3414 (*csrA::kanR*) grown in tryptone broth with or without acetate, respectively.

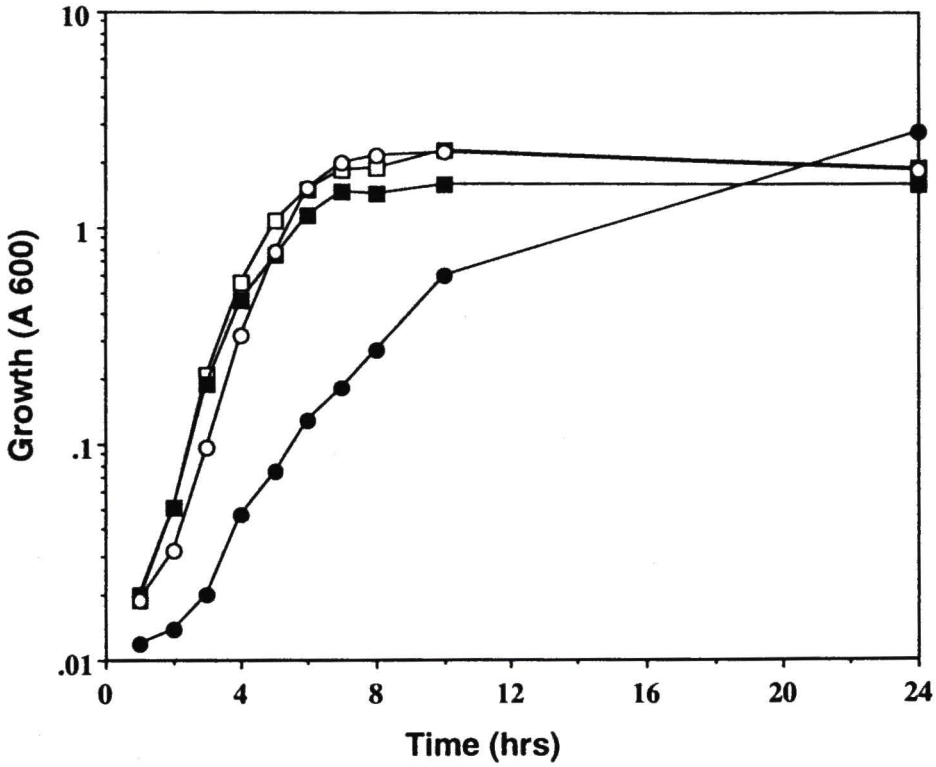

10
1
.1
.01
Growth (A 600)
0
4
8
12
16
20
24
Time (hrs)

FIG. 2. Genetic instability of a *csrA rpoS* strain grown in the presence of acetate. Panel A. A 24 hour culture of TR1-5BW*acs* was streaked directly from tryptone broth plus 50 mM sodium acetate onto Kornberg agar. This plate was incubated overnight at 37°C and intracellular glycogen was stained using iodine vapor. Panel B. Stable glycogen mutants isolated from the plate shown in panel A were streaked onto Kornberg medium and stained with iodine vapor. Strain identities are as follows: 1, TR1-5BW*acs*; 2, MD-1; 3, BL-1; 4, LT-1.

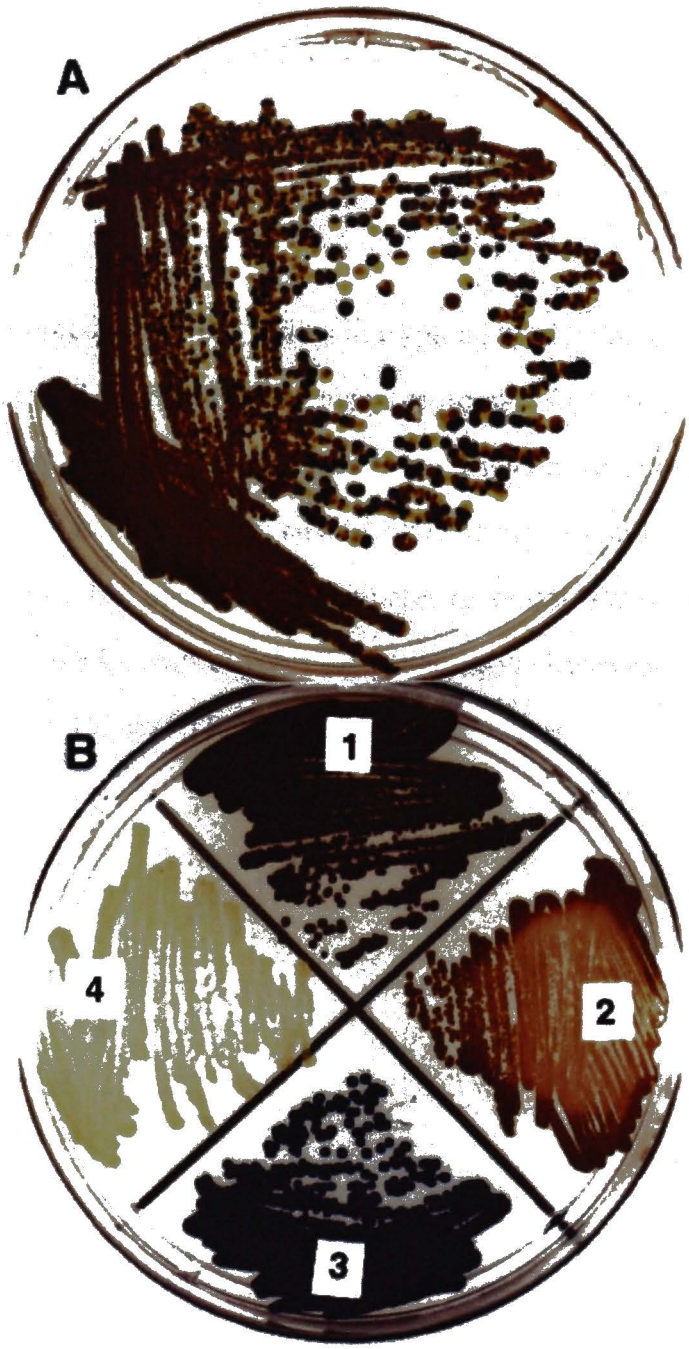

A
B
1
2
3
4

FIG. 3. Growth of glycogen-deficient strains in tryptone broth supplemented with acetate (50 mM). Panel A, open and solid squares represent BW3414 (*csrA*$^{+}$) and TR1-5BW3414 (*csrA:: kanR)*, respectively. Open and solid triangles represent two glycogen-deficient mutants, B1-TR and L9-1, respectively, isolated after exposure of TR1-5BW3414 to acetate. Panel B, open and solid squares represent BW3414 (*csrA*$^{+}$) and TR1-5BW3414 (*csrA:: kanR)*, respectively. Open and solid circles represent the *glg* transposon mutants EG3-153 and TR1-5EG3-153, respectively.

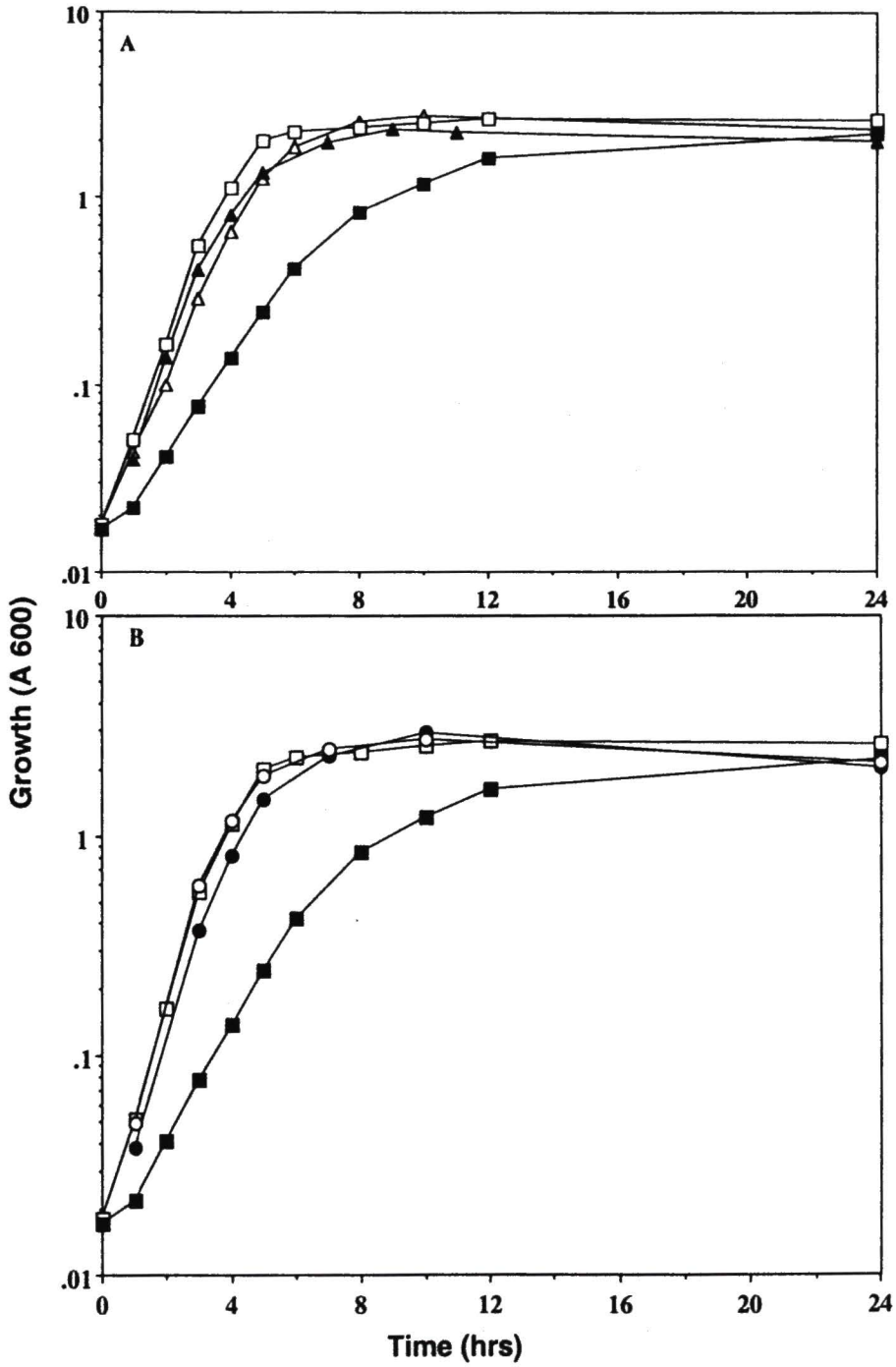

A
B
10
1
.1
.01
0
4
8
12
16
20
24
Growth (A 600)
Time (hrs)

FIG. 4. Effects of *csrA* on the expression of a *acs::lacZ* transcriptional fusion throughout the growth curve. Cultures were grown in 1% tryptone broth. Turbidity readings of cultures of strain SS414 and its isogenic *csrA::kanR* mutant are indicated by open and solid circles, respectively. β-galactosidase activities of these two strains are shown as open and solid squares, respectively. Essentially identical results were observed for this experiment using the BW3414 strain background.

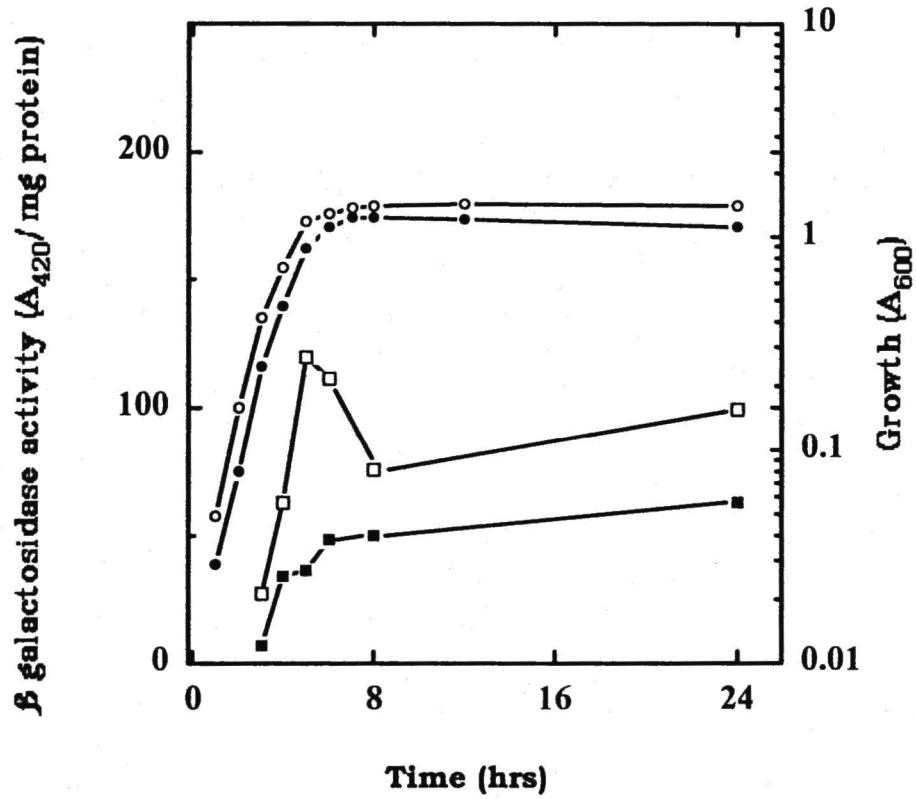

ß galactosidase activity (A_{420}/ mg protein)
200
100
0
0
8
16
24
Time (hrs)
10
1
0.1
0.01
Growth (A_{600})

FIG. 5. Uptake of amino acids by mid-exponential phase cultures in the presence or absence of acetate. Panels A-D depict [^{14}C] amino acid uptake experiments using strains MG1655, TR1-5MG1655 (*csrA::kanR*), RHMG1655 (*rpoS::*Tn*10*), and RHTR1-5MG1655 (*csrA::kanR*, *rpoS::*Tn*10*), respectively, as described in the Materials and Methods. Open and solid triangles represent control and acetate treatments, respectively.

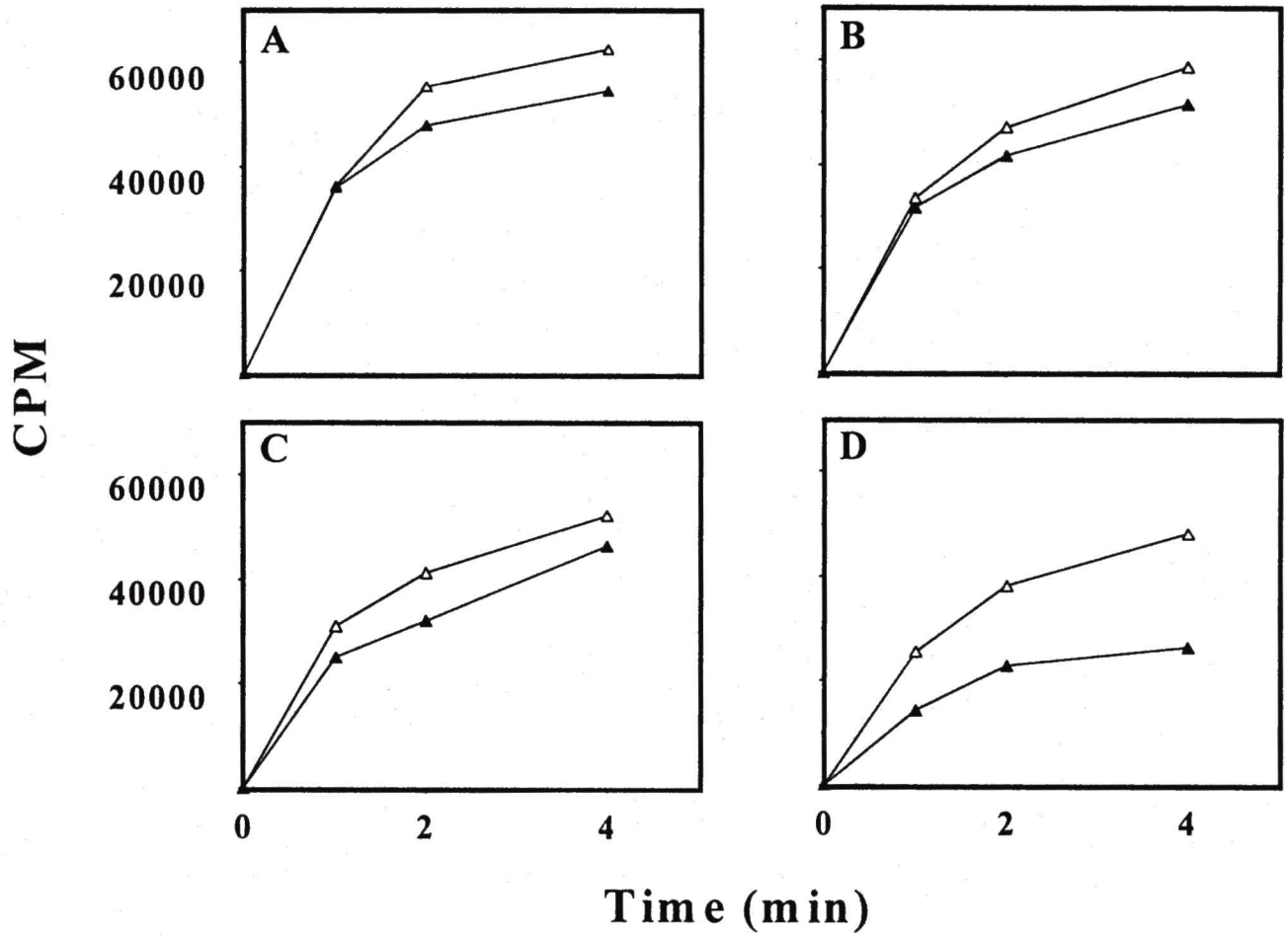
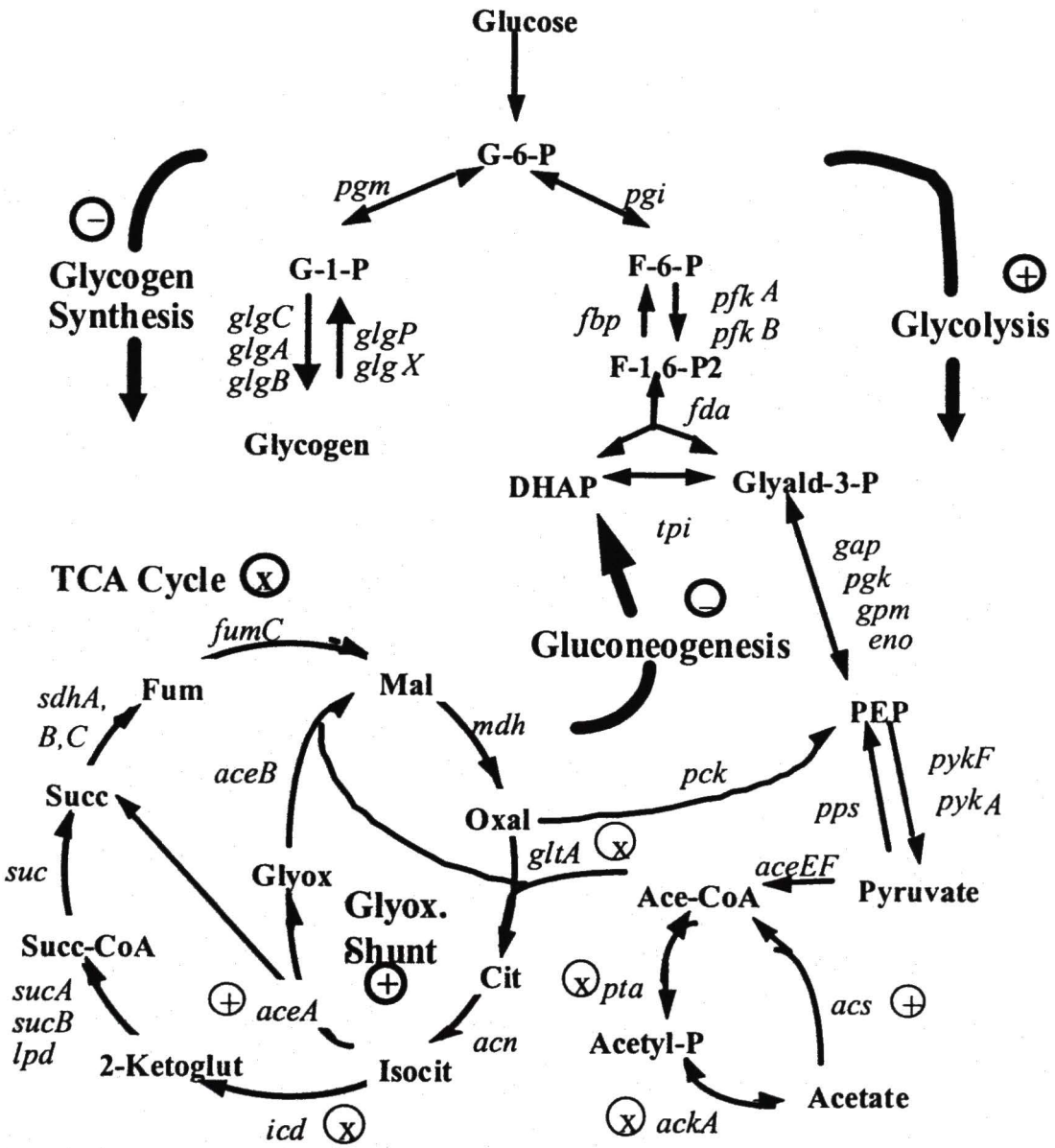

A
B
C
D
60000
40000
20000
60000
40000
20000
CPM
0
2
4
0
2
4
Time (min)

Fig.6. Effects of *csrA* on intermediary carbon metabolism in *E. coli.* A summary of previous studies on glycogen metabolism, glycolysis and gluconeogenesis and results of current studies on the reactions of acetate metabolism and the TCA cycle are shown. Pathways or reactions that are subject to positive regulation, negative regulation or little or no control are indicated by an encircled +, -, or x, respectively.


Glucose
G-6-P
pgm
pgi
Glycogen Synthesis
G-1-P
glgC
glgA
glgB
glgP
glgX
Glycogen
F-6-P
fbp
pfk A
pfk B
F-1,6-P2
fda
Glycolysis
DHAP
Glyald-3-P
tpi
gap
pgk
gpm
eno
TCA Cycle
fumC
Gluconeogenesis
sdhA, B,C
Fum
Mal
mdh
PEP
aceB
pck
pykF
pykA
pps
Succ
Oxal
gltA
suc
Glyox
aceEF
Ace-CoA
Pyruvate
Glyox. Shunt
Succ-CoA
Cit
sucA
sucB
lpd
aceA
pta
acs
acn
Acetyl-P
2-Ketoglut
Isocit
Acetate
icd
ackA

CHAPTER IV

The following manuscript was submitted to *Molecular Biology*.

POSITIVE REGULATION OF MOTILITY AND *flhDC* EXPRESSION BY THE RNA-BINDING PROTEIN CSRA OF *Escherichia coli*

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Running title: Effects of CsrA on *flhDC* expression

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Summary

Many species of bacteria devote considerable metabolic resources and genetic information to the ability to sense the environment and move toward or away from specific stimuli using flagella. In *Escherichia coli* and related species, motility is regulated by several global regulatory circuits, which converge to modulate the overall expression of the master operon for flagellum biosynthesis, *flhDC*. We now show that a mutation in the global regulatory gene *csrA* of *E. coli* K-12 causes cells to be nonmotile and lack flagella under conditions that otherwise favor flagellum biosynthesis and motility. Furthermore, a chromosomally-encoded *flhDC::lacZ* translational fusion was expressed at 3- to 4-fold higher levels in a *csrA* wild-type strain than in its isogenic mutant. Purified recombinant CsrA protein directly stimulated the coupled transcription-translation of *flhDC::lacZ* in S-30 extracts and bound specifically to the 5' non-coding segment of *flhDC* mRNA in RNA mobility shift assays. The steady state level of *flhDC* mRNA was higher and its half-life was ~3-fold greater in a *csrA* wild type versus a *csrA* mutant strain. Thus, CsrA is able to stimulate *flhDC* gene expression by a post-transcriptional mechanism that resembles its function in the repression of glycogen biosynthesis.

Introduction

Motility and chemotaxis permit bacterial cells to move away from stressful microenvironments and toward nutrients, O_2, light, or other stimuli, and are important during the infectious cycles of a number of bacterial pathogens (reviewed in Ottemann and Miller, 1997; Givaudan and Lanois, 2000). Motility is also required for biofilm formation in *Escherichia coli* and *Pseudomonas aeruginosa* under certain growth conditions (Pratt and Kolter, 1998; O'Toole and Kolter, 1998). Interestingly, the bacterial flagellum is homologous to the type III secretory pathway that is used for the transport of virulence proteins into eucaryotic host cells (Hueck, 1998), and itself may function in secreting certain virulence factors (Macnab, 1999; Young *et al.*, 1999).

The flagellum is among the most complex cellular machinery in bacteria, and requires ~2% of the total cellular energy for its biosynthesis and rotation (reviewed in Macnab, 1996; Prüß, 2000). Synthesis of the flagellum and its related components in *E. coli* involves 14 operons and over 50 genes. In order to coordinate this process, these genes are organized in a 3-tier hierarchy. At the top of this hierarchy is the master operon, *flhDC*, which encodes the subunits of a tetrameric DNA-binding protein ($FlhD_2C_2$) that recognizes second level (class II) flagellar promoters (Liu and Matsumura, 1994). The second level genes encode proteins for the basal body and the hook of the flagellum, as well as the sigma factor, FliA, which is required for the transcription of class III genes. These genes are needed for assembly of the flagellar filament, motor activity, chemotaxis, and for synthesis of the anti-sigma factor, FlgM, which accumulates upon completion of the flagellum and inhibits FliA activity (Hughes *et al.*, 1993).

The development of motility is highly responsive to environmental conditions in *E. coli* and *Salmonella typhimurium.* In addition to its well documented role in regulating motility, FlhD controls other genes (e.g. Prüß and Matsumura, 1996; Prüß *et al.*, 1997).

Accordingly, the expression of *flhDC* is affected by numerous global regulatory factors. Factors that are required for motility and full *flhDC* expression include cyclic AMP (cAMP) and cAMP receptor protein (CRP; Kutsukake, 1997; Soutourina *et al.*, 1999), histone-like nucleoid-structuring (H-NS) protein (Bertin *et al.*, 1994), the heat shock response network proteins DnaK, DnaJ, and GrpE (Shi *et al.*, 1992), Pss and Psd, which are involved in the synthesis of phosphatidylethanolamine in cell membranes (Shi *et al.*, 1993), and inorganic polyphosphate (Rashid *et al.*, 2000). In contrast, motility and *flhDC* transcription are inhibited under conditions of high acetyl phosphate levels and high osmolarity by the phosphorylated form of OmpR (Prüß and Wolfe, 1994; Shin and Park, 1995). Chemotaxis, or the detection of and movement toward or away from specific chemical stimuli, is itself controlled by distinct signal-transduction cascades (Eisenbach, 1996).

In the past several years, we have uncovered a novel type of global regulatory system that is present in *E. coli* and numerous other bacteria, the carbon storage regulatory system (Csr). The effector of Csr is a small RNA-binding protein, CsrA. Genetic and molecular studies of CsrA have shown that it represses gluconeogenesis, glycogen biosynthesis and catabolism, and activates glycolysis and acetate metabolism (Romeo and Gong, 1993; Romeo *et al.*, 1993; Sabnis *et al.*, 1995; Yang *et al.*, 1996; Wei *et al.*, 2000). In addition, CsrA regulates biofilm formation in *E. coli* (Romeo *et al.*, 1993).

While the mechanism of positive regulation by CsrA has not been previously examined, its repression of glycogen biosynthesis has been elucidated in some detail. CsrA recognizes and binds specifically to *glgCAP* mRNA in the vicinity of the *glgC* ribosome binding site, thereby accelerating the rate of decay of *glg* transcripts. This, in turn, decreases the intracellular levels of the glycogen biosynthetic enzymes, which decreases the rate of glycogen biosynthesis (Romeo *et al.*, 1993; Liu *et al.*, 1995; Yang *et*

al., 1996; Liu and Romeo, 1997). When recombinant CsrA was purified from *E. coli*, it was found to be bound to a 360 nt non-coding RNA molecule, CsrB, in a large ribonucleoprotein complex containing ~18 CsrA protein subunits. CsrB RNA is not required for the repression of *glg* gene expression by CsrA, but instead antagonizes CsrA activity. Likewise, each of the regulatory functions of Csr that have been examined can be directly ascribed to the RNA-binding protein CsrA (reviewed in Romeo, 1998).

The broad role of CsrA in *E. coli* physiology, and the observation that *csrA* homologs of *Borrelia burgdorferi* (Fraser *et al.*, 1997) and *Bacillus subtilis* (Mirel and Chamberlin, 1989) reside within flagellar gene clusters, prompted us to examine the effects of CsrA on motility in *E. coli*. The results of this study show that a functional *csrA* gene is required for motility and flagellum biosynthesis and that the CsrA protein binds to and stabilizes the *flhDC* message. Thus, the RNA binding protein CsrA is similar to many DNA binding proteins in that it is capable of functioning directly as either an activator or a repressor of gene expression, depending upon the target nucleic acid that it recognizes.

Results

A functional csrA *gene is essential for motility and flagellum biosynthesis in* E. coli

Growth on semisolid tryptone agar revealed that the *csrA* wild type strain BW3414 was motile, while its isogenic *csrA* mutant, TR1-5BW3414, was nonmotile (Fig. 1A). A plasmid that encoded *csrA*, pCSR10, complemented the motility defect. Light microscopy also showed that BW3414 cells grown in tryptone broth to exponential phase were vigorously motile, while *csrA* mutant cells were uniformly nonmotile (data not shown). These effects of *csrA* were not strain specific, e.g. *E. coli* K-12 strain

MG1655 and its isogenic *csrA* mutant exhibited these same motility phenotypes (data not shown). The *csrA* wild type strain also was motile in LB semisolid medium (0.35% agar) containing sodium acetate (10 mM), or sodium succinate (10 mM), or in the absence of any additional carbon source, but was nonmotile in LB medium containing glucose (10 mM). The isogenic *csrA* mutant was nonmotile under each of these conditions (data not shown). Finally, an isogenic strain containing a precise chromosomal deletion of the *csrB* gene was fully motile (data not shown), indicating that while CsrA is essential for motility, CsrB RNA is not required.

The loss of motility due to the *csrA* mutation might have resulted from either the absence of flagella or from an inability to use flagella (a Mot deficient phenotype). Negative staining electron microscopy demonstrated that flagella were uniformly absent from *csrA* mutant cells. In one experiment, we observed that *csrA* wild type cells exhibited an average of one flagellum per cell and some cells contained 2 or 3 flagella, while *csrA* mutant cells grown under identical conditions completely lacked flagella (Fig. 1B, C, D).

Effects of csrA *on the expression of the* flhDC *operon*

The complete absence of flagella in *csrA* mutant suggested that flagellum biosynthesis may be regulated by *csrA*. Since FlhDC is the master regulator of flagellum biosynthesis, the expression of a chromosomally-encoded *flhDC::lacZ* translational fusion was monitored in isogenic *csrA* wild type and mutant strains. In previous studies, it was noted that *csrA* has no effect on the expression of the wild type chromosomal *lacZ* gene, validating the use of *'lacZ* reporter fusions to monitor *csrA* regulation (Yang *et al.*, 1996). Specific β-galactosidase activity from the *flhDC::lacZ* fusion in each strain was lowest early in the exponential growth phase, increased throughout the exponential phase to

reach a maximum in the late exponential phase, and thereafter it slowly declined (Fig. 2). Although the temporal pattern of expression was not altered in the *csrA* mutant, β-galactosidase activity was considerably higher in the *csrA* wild-type strain, and exhibited a maximal difference of 4-fold in the two strains during the late exponential phase.

CsrA protein stimulates coupled in vitro *transcription-translation of* flhDC

The CsrA protein only had been demonstrated to directly function as a post-transcriptional repressor (Romeo, 1998). Thus, the effects of the *csrA* mutation on *flhDC* expression might have resulted either from a direct role of CsrA as an activator or from an indirect role, e.g. through the negative regulation of a *flhDC* repressor, such as OmpR. In order to clarify this issue, the effects of a recombinant CsrA protein on coupled *in vitro* transcription-translation of *flhDC* were examined in S-30 extracts prepared from a CsrA-deficient strain. Control reactions monitoring *glg* gene expression revealed that CsrA was fully active in *glg* repression, and that cAMP and cAMP receptor protein (CRP) specifically stimulated *glg* gene expression, as previously shown (Fig. 3 A lanes 1-3; Romeo and Preiss, 1989; Liu and Romeo, 1997). Initial attempts to demonstrate the synthesis of the native FlhD and FlhC polypeptides were unsuccessful, because the molecular masses of these two proteins are similar to predominant vector-encoded gene products, which obscured the autoradiographic analyses. Therefore, the plasmid pFDCZ6 was constructed, which contained a *flhDC::lacZ* translational gene fusion capable of encoding a ~120 kDa FlhC-LacZ fusion protein, which could be resolved from vector-encoded proteins. β-galactosidase activity expressed from this gene fusion *in vivo* was activated ~3-fold by *csrA* (data not shown). The *in vitro* transcription-translation of the FlhC-LacZ fusion protein was also stimulated up to ~3-fold upon the addition of recombinant CsrA (provided as the CsrA-CsrB

ribonucleoprotein complex), the effects of which saturated at ~2.8 to 3.8 μM of monomeric CsrA protein (Fig. 3 B, C). Although the cAMP-CRP complex was active in stimulating *glg* gene expression, at best it exhibited very weak stimulatory effects on *flhDC::lacZ* expression.

The transcription-translation studies indicated that CsrA directly activates the expression of *flhDC*. In addition, a *csrA ompR* mutant remained nonmotile (data not shown), indicating that the effect of *csrA* on motility was not mediated indirectly, by the negative regulation of the only known repressor of *flhDC*, OmpR.

Effects of csrA *on* flhDC *mRNA steady state levels and stability*

Initial attempts to investigate the effects of *csrA* on *flhDC* mRNA levels *in vivo* by Northern blot analysis were unsuccessful due to the low abundance of this transcript. Therefore, RT-PCR, a more sensitive method, was chosen for the analyses. Previous studies had revealed that levels of the Krebs cycle enzyme, isocitrate dehydrogenase (encoded by *icd*), are not affected by *csrA* (Wei *et al.*, 2000). Thus, *icd* mRNA was chosen as an internal control for these analyses. Preliminary experiments revealed that *icd* mRNA was detectable after 20 cycles of PCR amplification, and exhibited a linear range of detection with respect to input RNA up to 25 cycles (data not shown). In contrast, *flhDC* mRNA was detected only after 25 cycles of amplification and exhibited a linear range of detection up to 35 cycles (data not shown). Fig. 4 shows the analysis of *flhDC* and *icd* transcripts from mid-exponential, late exponential, and early stationary growth phases of *csrA* wild type and mutant strains at 30 cycles of amplification. Under this condition, *flhDC* mRNA was not detected in either strain growing at mid-exponential stage or in the *csrA* mutant in stationary phase (Fig. 4 lanes 2, 3 and 7). At the late exponential phase, *flhDC* transcripts were detected in both strains and were ~3-fold more abundant in the *csrA* wild type strain (Fig. 4 lanes 4 and 5). In contrast, *icd* transcript

levels were not affected by *csrA*, as observed at 25 cycles of amplification for these same RT reactions (data not shown).

Previous studies revealed that the differences in the steady state levels of *glgC* transcripts in *csrA* wild type and mutant strains are due to the effects of CsrA on mRNA decay rates (Liu *et al.*, 1995). To determine whether *flhDC* transcript stability is affected by *csrA in vivo*, it was quantified by RT-PCR following the addition of rifampin to cultures (Fig. 5). The *flhDC* transcripts were almost 3-fold more stable in *csrA* wild type versus mutant cells (the chemical half-lives were ~3.5 and 1.4 min, respectively), which should account for differences in transcript levels in *csrA* wild type and mutant strains.

Mapping of the flhDC *transcript initiation site*

Previous studies to identify the 5' end of *flhDC* mRNA were conducted on *flhDC*-transcripts expressed *in vivo* from plasmid clones (Shin and Park, 1995; Soutourina *et al.*, 1999). The first study identified one major and two minor transcripts, none of which contained an apparent *flhDC* promoter sequence. The second study revealed a single major transcript. In order to map the 5' end of the chromosomally-encoded transcript, primer extension analysis was conducted with RNA isolated from plasmid-free *csrA* wild type and mutant strains, using two different primers (flh1 and flh2; Table 2). Both primers revealed the same results. Only a single major product was identified, which was detectable only in the *csrA* wild type strain (Fig. 6A). The 5' end of this transcript was mapped to a G residue, 197 bp upstream from the *flhD* initiation codon (Fig. 6A and B), which was the same site found by Soutourina *et al.* (1999). The apparent –10 and –35 promoter boxes for this transcript exhibit 67 and 50% identity with σ^{70} consensus sequences, respectively. These hexamers and the transcript initiation region exhibit ~90% identity to those of the *Proteus mirabilis flhD* gene (Furness *et al.*, 1997; Fig. 6B).

CsrA binds specifically to flhDC *transcripts*

RNA gel mobility shift assays were used to determine whether CsrA protein recognizes and specifically binds to *flhDC* transcripts. Three ^{32}P radiolabeled runoff transcripts, A, B, and C, were prepared and isolated (Fig. 7). Transcript A included only upstream non-coding RNA, extending from +1 to +146 nt, relative to the start of transcription. Transcript B extended from +147 to +276, and included the remaining upstream non-coding RNA segment and 79 nt of the coding region. Transcript C extended from +1 to +276 nt. Each of the transcripts generated retarded complexes in the presence of the purified recombinant CsrA-CsrB complex. The relative mobility shifts observed at the lowest concentrations of CsrA were modest, and could only be explained by transfer of CsrA protein from CsrA-CsrB complex to the labeled transcripts. As increasing amounts of CsrA-CsrB were added to the binding reactions, additional shifted complexes with decreasing mobilities were observed for transcripts A and C, but not for transcript B, which apparently formed only a single binding complex under these conditions. Unlabeled *flhDC* transcript competed for CsrA binding in each case, while RNA from *Saccharomyces cerevisiae* did not compete for binding with any of the labeled transcripts. In the absence of CsrA-CsrB complex (lanes 1 of panels A, B, and C), there was only one major band, representing the transcript of interest. However, preparations of transcripts B and C each contained a minor contaminating transcript with slightly lower mobility than the expected product. CsrA did not appear to bind to these minor transcripts.

Discussion

Considerable evidence indicates that the decision to become motile in *E. coli* and related species is determined by the expression level of the master operon, *flhDC*. Genes encoding global regulatory factors generally exhibit abundant regulatory features, and this

is true for *flhDC*. The present study reveals that in addition to transcriptional repression by phosphorylated OmpR (Shin and Park, 1995) and activation by cAMP-CRP and H-NS (Soutourina *et al.*, 1999), *flhDC* is regulated post-transcriptionally by CsrA-mediated stabilization. Interestingly, recent studies by Claret and Hughes (2000) reveal that the turnover of the FlhD and FlhC polypeptides of *Proteus mirabilis* is rapid, and is controlled by a mechanism that involves Lon protease. A short half-life for FlhD (~2 min) is consistent with its regulatory role in cell cycle control (Prüß and Matsumura, 1997). Finally, the heat shock proteins DnaJ, DnaK, and GrpE appear to affect *flhDC* expression, as well as the assembly of the mature FlhDC protein, presumably via their chaperon functions (Shi *et al.*, 1992). Thus, there is currently evidence that *flhDC* expression is regulated at the levels of transcription, message stability, and possibly translation (discussed below), and that the assembly and turnover of the FlhDC protein are also strictly controlled.

The series of experiments described in the present study reveals that the global regulator CsrA post-transcriptionally activates motility and flagellum biosynthesis in *E. coli* and that the *flhDC* transcript is the direct target of regulation. First, *csrA::kanR* mutant cells were nonmotile due to the absence of flagella, and this phenotype was complemented by a plasmid encoding the *csrA* gene. Second, the expression of a chromosomally-encoded *flhDC::lacZ* translational fusion was decreased by ~4-fold in the *csrA* mutant. This effect on *flhDC* expression should be sufficient to account for the total loss of flagella in mutant cells (Kutsukake, 1997). Third, *flhDC* message levels were ~3-fold higher and were more stable in a *csrA* wild type strain relative to its isogenic mutant. Fourth, a recombinant CsrA protein activated the *in vitro* transcription-translation of *flhDC::lacZ* translational fusion and bound specifically to three different runoff transcripts that contained *flhDC* upstream noncoding RNA. During the final stages of

this study, both *csrA* wild type and mutant strains were found to be motile in CFA medium (data not shown). Interestingly, *flhDC::lacZ* expression in cells grown in CFA medium was still positively regulated by CsrA, and was 2- to 3-fold higher in both wild type and *csrA* mutant cells grown in CFA versus LB or tryptone broth (data not shown). The increased basal expression of *flhDC* apparently was sufficient for flagellum biosynthesis in the mutant. While the means by which growth in CFA medium increases *flhDC* expression is unknown, these observations reinforce the concept that the absolute level of *flhDC* expression is critical for the development of motility.

We previously hypothesized that CsrA is part of an adaptive response pathway (Romeo *et al.*, 1993), which carries the implicit assumption that CsrA is regulated in response to environmental or physiological conditions. While the specific factors that regulate *csrA* expression remain to be defined, it is interesting to note that both CsrA protein levels and *csrA::lacZ* expression increase during the growth curve, and reach a maximum at the late exponential phase / transition to stationary phase (S. Gudapaty, K. Suzuki, X. Wang, P. Babitzke and T. Romeo, unpublished data). Although *flhDC* transcript levels and *flhDC::lacZ* expression were decreased in a *csrA* mutant, they otherwise retained normal temporal control, and were optimal at late exponential phase of the growth curve in both *csrA* wild type and mutant strains. Thus, in the cell, the CsrA protein appears to be well poised to bind to and stabilize *flhDC* transcripts as they accumulate in the late exponential phase.

The precise mechanism by which CsrA binding stabilizes *flhDC* mRNA remains to be defined. Studies on the regulation of protein synthesis have shown that the RNA secondary structural features present in the 5' untranslated segment can dramatically influence both mRNA stability and translation initiation. Furthermore, the rate-limiting step in mRNA decay is often an initial endonucleolytic cleavage at the 5' extremity

(reviewed in Kushner, 1996; Grunberg-Manago, 1999). Both the long 5' untranslated segment (197 nucleotides) of *flhDC* mRNA and the complex mobility shift patterns observed herein suggest that the molecular details of this regulation are complex. Previous genetic and molecular studies revealed that the segment of the *glgC* message in the vicinity of the ribosome binding site is involved in negative control (Liu *et al.*, 1995; Liu and Romeo, 1997). These observations are consistent with a potential role for CsrA as a translational repressor, which subsequently destabilizes the *glgC* transcript (reviewed in Romeo, 1998). Likewise, it is plausible that CsrA binding activates *flhDC* translation, although the available data could be explained by a mechanism involving direct protection of *flhDC* mRNA by CsrA against endonucleolytic attack. The relatively poor Shine-Dalgarno sequence for *flhD* (Soutourina *et al.*, 1999) is also consistent with the idea that ribosome binding at *flhD* might be favored by an accessory factor.

It is becoming increasingly clear that CsrA homologues perform central regulatory roles in host-microbe interactions. Certainly, the effect of CsrA on motility has implications for a variety of motility-dependent host interactions (reviewed in Ottmann and Miller, 1997; Young *et al.*, 1999). The *E. coli csrA* gene has strong effects on adherence and biofilm formation (Romeo *et al.*, 1993). It is now recognized that the formation of adherent, matrix-enclosed, and protected population of bacterial cells, i.e. biofilm, within the human host presents a serious problem in numerous bacterial infections (e.g. Costerton *et al.*, 1999). Studies by Arun Chatterjee and his coworkers have documented important roles for the highly conserved CsrA homologue, RsmA, in the virulence mechanisms of the plant pathogen *Ervinia carotovara*, a close relative of *E. coli* (Chatterjee *et al.*, 1995; Cui *et al.*, 1995). This bacterium utilizes RsmA as a repressor of genes encoding a battery of secreted lytic enzymes that are largely responsible for soft rot disease. The genes repressed by RsmA are expressed during the

stationary phase of growth, similar to those that are repressed by CsrA in *E. coli.* In contrast to the role of CsrA in motility, the overexpression of *rsmA* inhibited motility of *Erwinia* species (Mukherjee *et al.*, 1996). This would seem to suggest distinct physiological roles for motility in these two bacteria. However, caution is warranted in the later case, since a mutation in *rsmA* was not tested and the mechanistic basis for the effects of *rsmA* overexpression on motility have not yet been examined (discussed in Romeo, 1998). More recently, *Salmonella typhimurium* has been reported to utilize CsrA and CsrB to regulate genes involved in invasion of the intestinal mucosa, including *hilA, invF, prgH*, and *sspC* (Altier *et al.*, 2000). Finally, FlhD or FlhDC regulate properties in addition to motility, including the production of host-damaging enzymes such as lipase and hemolysin in *Xenorhabdus nematophilus* (Givaudan and Lanois, 2000), and genes involved in cell division (Prüß *et al.*, 1997) and respiration (B. Prüß, W. Hendrickson, X. Liu and P. Matsumura, submitted for publication) in *E. coli.* Based on the results of the present study, CsrA may be predicted to indirectly control a variety of processes that are regulated by FlhD or FlhDC.

Experimental procedures

Bacterial stains, plasmids, media, and growth conditions

Table 1 lists the strains and plasmids that were used in this study, their sources, and relevant genotypes. Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.4]) supplemented with 0.2% of glucose was used for routine laboratory cultures. For motility studies, LB medium was prepared without glucose. Tryptone broth contained 1% tryptone and 0.5% NaCl, pH 7.4. Colonization factor antigen (CFA) medium contained 1% casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, and 0.0005%

$MnCl_2$, pH 7.4 (Evans *et al.*, 1977). Media were supplemented with the following compounds as required: kanamycin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; and ampicillin, 100 μg ml^{-1}. Cultures were inoculated with 1 volume of overnight culture per 500 volumes of freshly prepared medium and were grown at 30°C on a gyratory shaker at 250 rpm.

Motility Assays

The assay was initiated by stabbing a colony from an overnight culture into a semisolid agar plate (tryptone broth solidified with 0.35% agar). The plates were kept in a humidified incubator at 30°C for 18 hours. Swimming motility was confirmed by light microscopy of liquid cultures.

Electron microscopy

Bacterial cells were grown in tryptone broth to late exponential phase, negatively stained using 1% (v v^{-1}) phosphotungstic acid, pH 7.2, and mounted on Formvar- and carbon-coated 200-mesh nickel grids (Mukherjee *et al.*, 1996). Samples were visualized using a Zeiss EM 910 transmission electron microscope.

Total protein and β-galactosidase assays

Total cell protein was measured by the bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Smith *et al.*, 1985). β-galactosidase specific activity was assayed and calculated as described previously (Romeo *et al.*, 1990).

Molecular biology techniques and nucleotide sequencing

Standard procedures were used for isolation of plasmid DNA and restriction fragments, restriction mapping, transformation, P1 transduction, molecular cloning, and polymerase

chain reaction (PCR) amplification as described previously (Romeo and Preiss, 1989; Sambrook *et al.*, 1989; Ausubel *et al.*, 1999). Dideoxynucleotide sequencing reactions (Sanger *et al.*, 1977) were conducted using Sequenase™ version 2.0 under the conditions described by the manufacturer (U.S. Biochemical Corp.).

Preparation of a flhDC'-'lacZ *translational fusion*

Plasmid pFDCZ6 contained a *flhDC-lacZ* translational fusion in which codon 14 of *flhC'* was fused in frame to codon 11 of *'lacZ*. It was constructed by subcloning a 1.8-kb *Pvu*II fragment from pPM61 (Bartlett *et al.*, 1988), which included the complete upstream regulatory region of the *flhDC* operon, an intact *flhD* coding region, and the N-terminus of *flhC*, into the *Sma*I site of pMLB1034 (Silhavy *et al.*, 1984).

S-30 coupled transcription-translation reactions

Effects of a recombinant CsrA protein containing a carboxy-terminal his tag (Liu *et al.*, 1997) on the expression of flagellum biosynthesis genes were conducted in S-30 extracts prepared from TR1-5BW3414 (*csrA::kanR*), as previously described (Romeo and Preiss, 1989; Liu and Romeo, 1997). Proteins were labeled by incorporation of [^{35}S] methionine (1,175 Ci mmol^{-1}; NEN™ Life Science Products, Inc.), denatured, and equal volumes of each reaction were subjected to electrophoresis on 9.5% sodium dodecyl sulfate-polyacrylamide slab gels. Radiolabeled proteins were detected by fluorography using sodium salicylate (Chamberlain, 1979).

RNA isolation and analysis

RNA was purified using MasterPure™ RNA Purification Kits, according to the manufacturer's instructions (Epicentre Technologies, Madison, WI; Watson *et al.*, 1998).

RNA was quantified by absorbance at 260 nm and 280 nm. For molecular mass estimation, purified RNA was subjected to denaturing electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde (Sambrook *et al.*, 1989).

RT-PCR analysis of flhDC mRNA.

Total RNA was isolated from cells grown at 30°C in LB medium (lacking glucose) to mid-exponential, late exponential or stationary growth phase. Subsequently, 0.5 μg of RNA was reverse-transcribed using 15 U of ThermoScript RT (GIBCOL BRL) in a total reaction volume of 20 μl. Each reaction was incubated at 55°C for 50 min and terminated by incubation at 85 °C for 5 min. Oneμl of RNase H (2 units μl^{-1}) was added to the reaction mixture and incubated at 37°C for 20 min. Two μl of reverse-transcription products (cDNA) were amplified by PCR using 2.5 U of HotStarTaq DNA polymerase (Qiagen) and 25 pmol each of *flhDC* forward and reverse primers and *icd* forward and reverse primers (flh-RT1, flh–RT2, icd-RT1 and icd-RT2, respectively; Table 2) in a total volume of 100 μl. Each PCR program started with an initial heat activation step at 95°C for 15 min. A typical PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (59°C, 1 min), and an elongation step (72°C, 1 min). Typical reactions included several full cycles (20 to 35), followed by an additional extension step (72°C, 10 min). The PCR products were 202 bp and 100 bp for *flhDC* and *icd*, respectively. PCR products were separated by electrophoresis on 2% agarose gels containing 0.5 μg/ml of ethidium bromide. They were subsequently visualized and photographed under UV light, and quantified by densitometry using an AlphaImager™ 2000 Documentation & Analysis System (AlphaInnotech Corporation, San Leandro, CA).

mRNA stability determination

Bacterial cultures were grown at 30 °C in LB medium (lacking glucose) to late exponential phase and treated with rifampin at a final concentration of 200 μg ml^{-1} to inhibit transcription. Samples were collected at 0, 2, 4, 6, 8, and 12 min after rifampin treatment. The cells were harvested at 13,000 rpm in a microcentrifuge and frozen in dry ice, allowing no more than two min to elapse between sampling and freezing. Total RNA was extracted and *flhDC* and *icd* mRNA levels were determined by RT-PCR analysis.

Primer extension mapping

Total RNA was prepared from cultures grown at 30°C in LB (lacking glucose) to the late exponential phase of growth. Two oligonucleotide primers were utilized. Primer flh1 was complementary to +175 to +194; primer flh2 was complementary to +89 to +110 of *flhDC* mRNA and (Table 2). The primers were end labeled using T4 polynucleotide kinase and [^{32}P] ATP (3,000 Ci $mmol^{-1}$, NEN™ Life Science Products, Inc.) according to standard procedures (Ausubel *et al.*, 1999). Approximately 0.5 ng of labeled primer was annealed to 20 μg of total RNA. cDNA was synthesized using 15 U of ThermoScript RT (GIBCOL BRL) in a total volume of 20 μl and the reaction was conducted at 55°C for 50 min and terminated by incubating at 85°C for 5 min. One μl of RNase H (2 units μl^{-1}) was added to the reaction mixture and incubated at 37°C for 20 min. The same two primers were used to prepare DNA sequencing ladders that served as standards for the corresponding reverse transcriptase signals, which were analyzed on urea-containing sequencing gels (e.g. Romeo and Preiss, 1989).

RNA gel mobility shift assay

Three runoff *flhDC* transcripts, A (146 nt), B (130 nt), and C (276 nt), respectively, were

synthesized from PCR products using T7 RNA polymerase. The primers for synthesis of these PCR products are shown in Table 2. The resulting PCR products each contained a T7 promoter, followed by: 146-bp (+1 to +146 relative to *flhDC* transcription start site using flh-MS1 and flh-MS3), 130-bp (+147 to +276, using flh-MS2 and flh-MS4) or 276-bp (+1 to +276, using primers flh-MS1 and flh-MS4) for transcripts A, B, and C, respectively. The PCR products were gel purified and used as templates for *in vitro* transcription. ^{32}P-labeled transcripts were prepared according to the method described previously (Liu and Romeo, 1997). The RNA binding reaction mixtures (20 µl) included CsrA-CsrB complex at various concentrations, labeled transcript (0.5 nM), 10 mM Tris-acetate (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl, 10 mM dithiothreitol, and 5% glycerol (Alifano *et al.*, 1992). The reactions were incubated at room temperature for 30 min, mixed with 1 µl loading dye (97% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol), immediately loaded and separated on 5% polyacrylamide vertical slab gels (Liu and Romeo, 1997). The resulting gels were dried onto filter paper and subjected to autoradiography.

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Table 1. Bacterial strains and plasmids used in this study.

Strain, plasmid	Description	Source or reference
E. coli Strains		
BW3414	*ΔlacU169 rpoS*(Am)	Barry Wanner
MG1655	Prototrophic	Michael Cashel
MC4100	F⁻ *Δ(argF-lac)U169 rpsL relA flhD deoC ptsF rbsR*	Shin and Park, 1995
CP992	*flhDC::lacZ*	Shin and Park, 1995
TK821	MC4100 *ompR331::Tn10*	Garrett *et al.*, 1983
YKBW3414	BW3414 *flhDC::lacZ*	This study
TKBW3414	BW3414 *ompR331::Tn10*	This study
TR1-5	*csrA::kanR*	Romeo *et al.* 1993
Plasmids		
pUC19	Cloning vector; Amp^R	Yanisch-Perron *et al.*, 1985
pCSR10	*csrA* in pUC19	Romeo *et al.*, 1993
pPM61	contains *flhDC*; Kan^R	Bartlett *et al.*, 1988
pMBL1034	'*lacZ* fusion vector; Amp^R	Silhavy *et al.*, 1984
pFDCZ6	*flhDC::lacZ* in pMBL1034	This study
pOP12	*glg* gene cluster (*asd-glgP* '); Tet^R	Okita *et al.*, 1981

Table 2. Oligonucleotide primers used in this study[a].

Name	Sequence (5' to 3')
flh1	TCCCACCCAGAATAACCAAC
flh2	GCATTAGAATAGTTGCGATAAG
flh-MS1 (F)	GTAATACGACTCACTATAGATTTAGGAAAAATCTTAGATA
flh-MS2 (F)	GTAATACGACTCACTATAGGGGTGCGGTGAAACC
flh-MS3 (R)	GTGATGTCGCCGGCAAGC
flh-MS4 (R)	GAACAATCAAACGCTGTGCAAG
flh-RT1 (F)	GTGTAAAGACCCATTTCTATTTGTAAGGAC
flh-RT2 (R)	TGTGTTTCAGCAACTCGGAGGTATG
icd-RT1 (F)	GGAATCGGTGTAGATGTAACCCC
icd-RT2 (R)	CGTCCTGACCATAAACCTGTGTGG

[a] All primers were purchased from Integrated DNA Technologies, Inc., Coralville, IA.

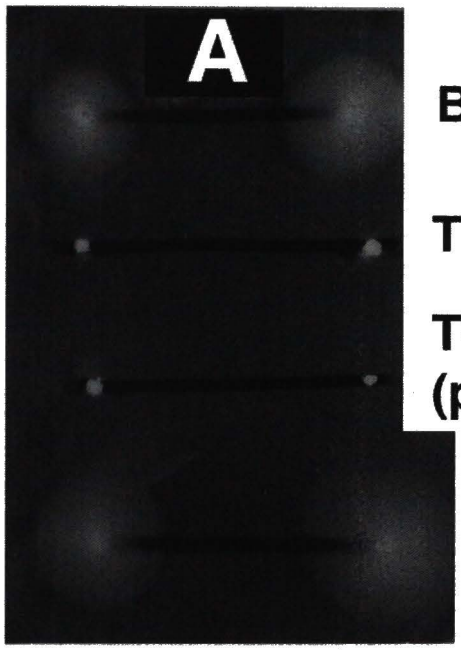
Fig. 1. Motility and electron microscopy (EM) of *csrA* wild type and mutant strains.

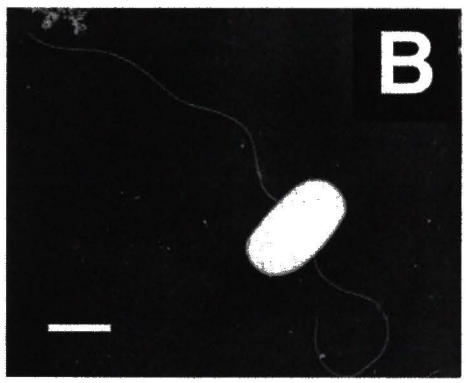
A. Motility of the *csrA* wild-type (BW3414), mutant (TR1-5BW3414), and the *csrA* over-expressing strain (TR1-5BW3414[pCSR10]) of *E. coli* on semisolid tryptone-agar (0.35%) after 18 hours of growth at 30 °C.

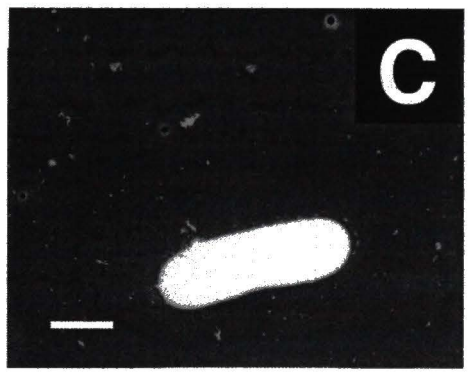
B. Negative staining EM of BW3414 grown to late exponential phase in tryptone broth.

C. EM of TR1-5BW3414 (as in B). Note the absence of flagella in this strain.

D. Flagella were counted under direct EM examination.







D

	Flagella / cell			
	0	1	2	3
Strain	**Cell count**			
BW3414	12	5	7	1
TR1-5BW3414	53	0	0	0

Fig. 2. Effects of *csrA* on the expression of a chromosomal *flhDC::lacZ* translational fusion. Cultures were grown in LB medium (minus glucose) at 30°C on a gyratory shaker (250 rpm) and ß-galactosidase specific activities were determined. Turbidity readings of cultures (A_{600}) of BW3414 and TR1-5BW3414 (*csrA::kanR*) are indicated by open and solid circles, respectively. ß-galactosidase activities of these two strains are shown as open and solid squares, respectively. This experiment was repeated twice with similar results.

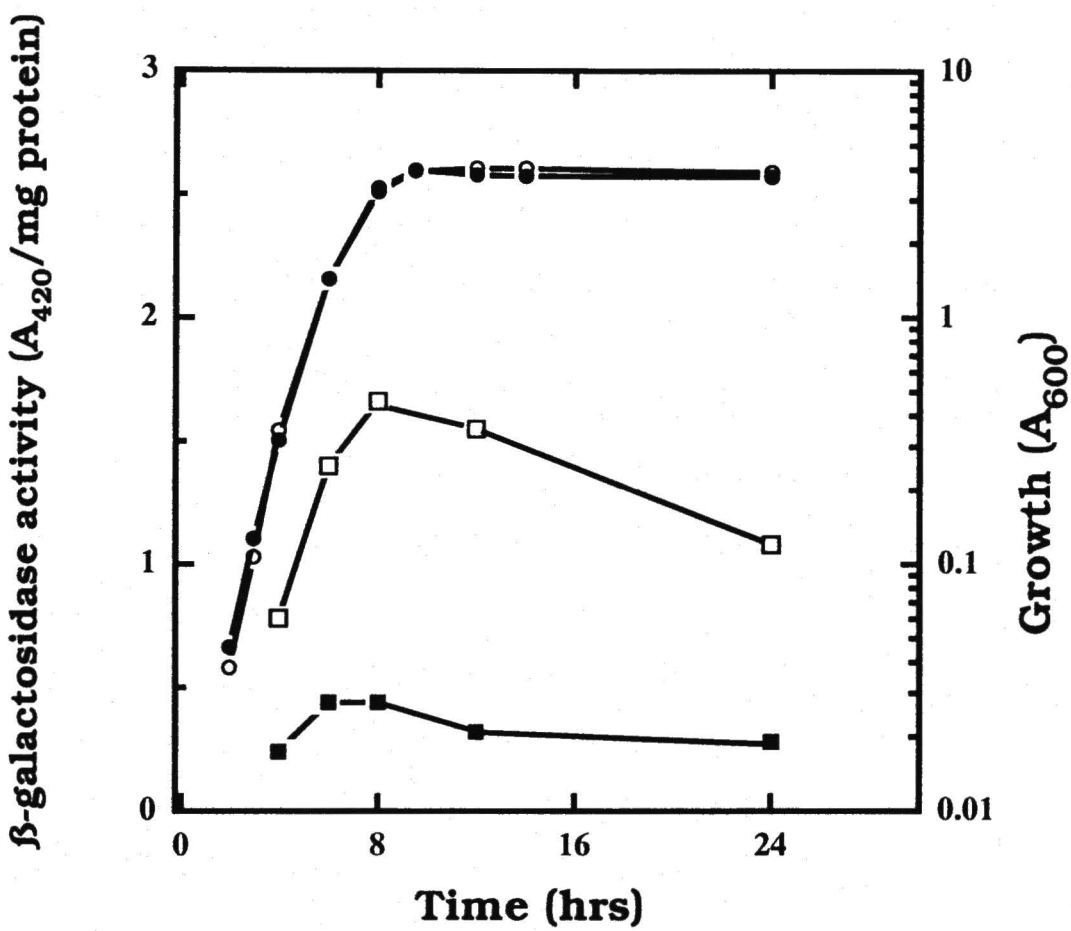

ß-galactosidase activity (A_{420}/mg protein)
Growth (A_{600})
Time (hrs)
0
1
2
3
0.01
0.1
1
10
0
8
16
24

Fig. 3. Effects of purified CsrA-CsrB complex on the coupled transcription-translation of the *flhDC::lacZ* translational fusion encoded by pFDCZ6. Reactions (35 μl) contained 2 μg of plasmid DNA and were conducted in an S-30 extract from TR1-5BW3414 (*csrA::kanR)* . Reactions conducted in the presence of cAMP (100 μM) also contained cAMP receptor protein (CRP; 1 μg or 0.61 μM).

A. Control reactions containing pOP12 plasmid DNA are shown in lanes 1-3. Reactions containing pFDZ6 DNA, carrying a *flhDC::lacZ* fusion, are shown in lanes 4-7. One _g of CsrA protein (3.8 μM monomer or 0.21 μM CsrA-CsrB complex), was present in each of the reactions that contained CsrA-CsrB complex.

B. The effects of CsrA-CsrB on the transcription-translation of the *flhDC::lacZ* fusion of pFDCZ6 were monitored in the presence or absence of cAMP-CRP.

C. The incorporation of ^{35}S methionine into the FlhC-LacZ hybrid protein during transcription-translation was determined by liquid scintillation counting, as previously described (Romeo and Preiss, 1989). Reactions performed in the presence or absence of cAMP-CRP are depicted by open triangles or open squares, respectively. The basal reaction, to which no CsrA-CsrB or cAMP-CRP were added, synthesized 14 fmol of the FlhC-LacZ fusion protein.

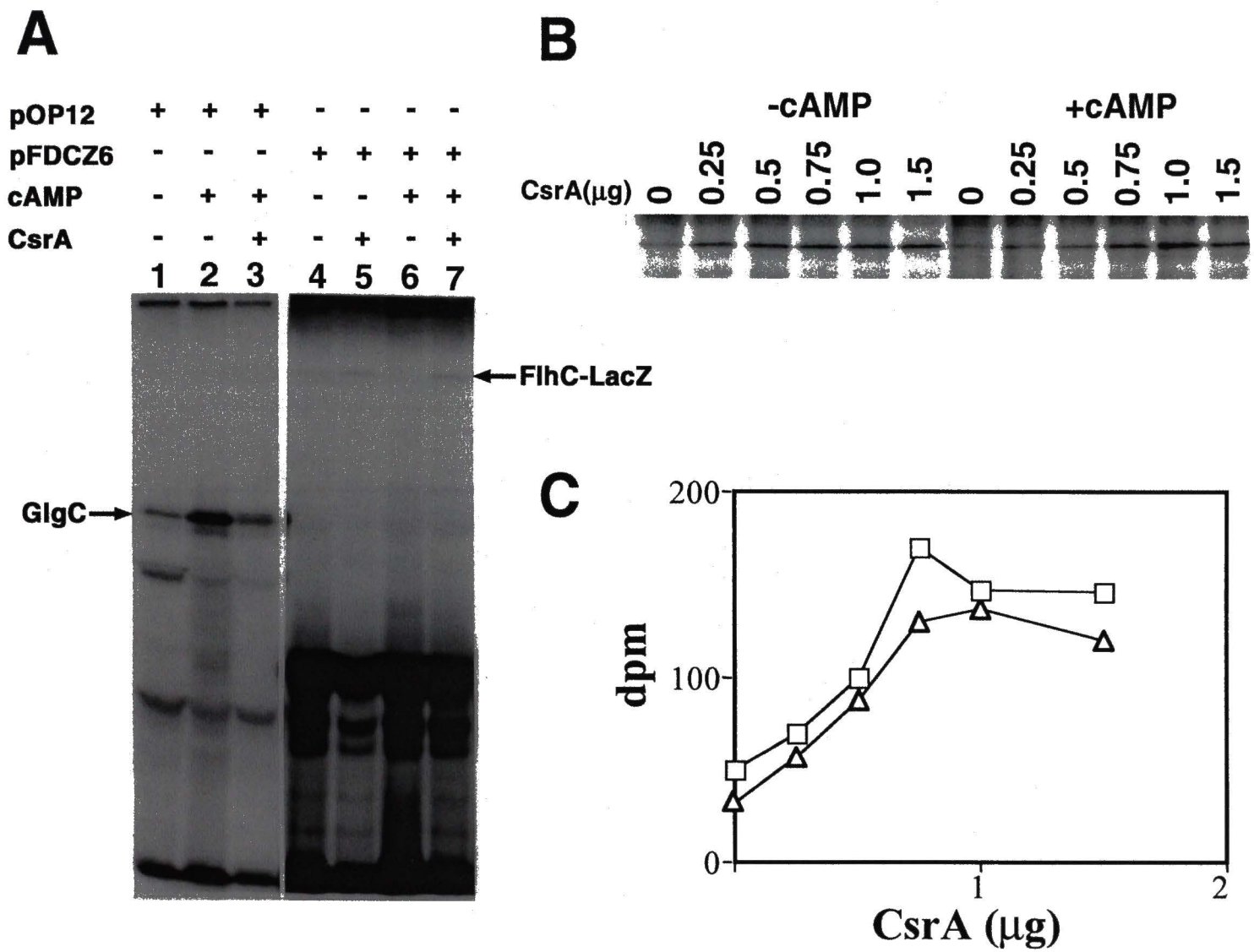

A
pOP12 + + + - - - -
pFDCZ6 - - - + + + +
cAMP - + + - - + +
CsrA - - + - + - +
1 2 3 4 5 6 7
FlhC-LacZ
GlgC
B
-cAMP
+cAMP
CsrA(μg) 0 0.25 0.5 0.75 1.0 1.5 0 0.25 0.5 0.75 1.0 1.5
C
200
100
0
dpm
1
2
CsrA (μg)

Fig. 4. Detection of *flhDC* mRNA by RT-PCR.

A. Total RNA was isolated from mid-exponential (lanes 2 & 3), late exponential (lanes 4 & 5), or stationary growth phase (lanes 6 & 7), 0.5 μg RNA was reverse transcribed, and the resulting cDNA was amplified by PCR for 30 cycles. Lanes 2, 4, & 6 show results with RNA from BW3414; lanes 3, 5, & 7 show reactions with RNA from TR1-5BW3414(*csrA::kanR*). Lane 1 shows the direct PCR amplification of *flhDC* from the plasmid pPM61, using the same primers as for RT-PCR analysis. Lane 8 contains RNA size standards.

B. Densitometric analysis of the *flhDC* data depicted in Panel A.

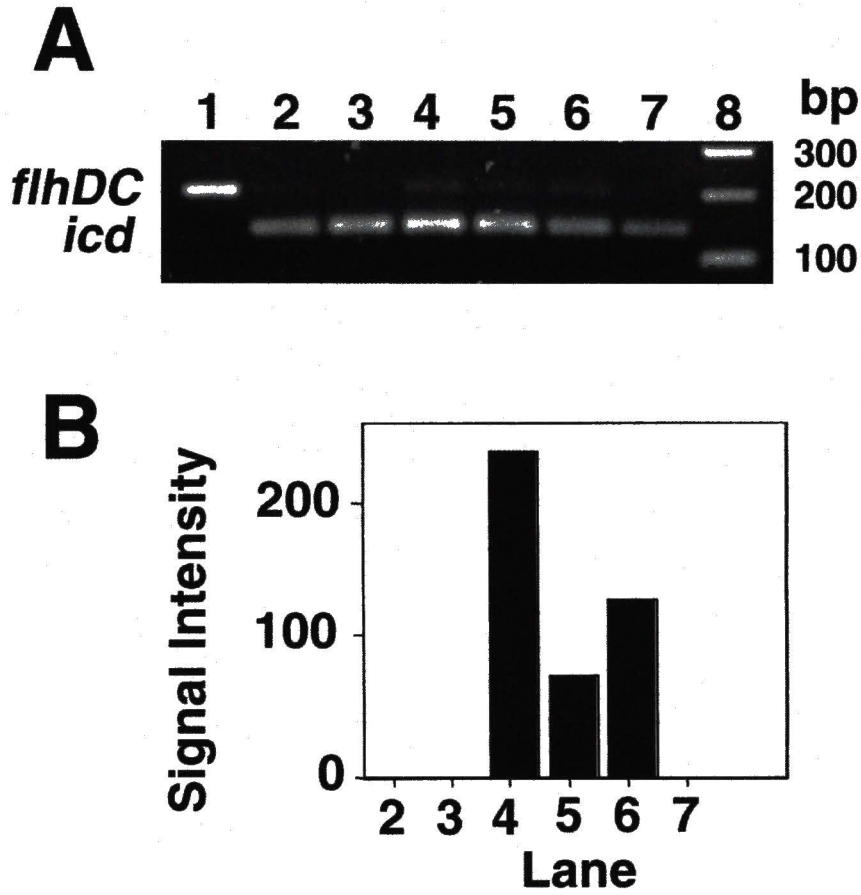

A
1 2 3 4 5 6 7 8
bp
300
200
100
flhDC
icd
B
Signal Intensity
200
100
0
2 3 4 5 6 7
Lane

Fig. 5. Analysis of *flhDC* mRNA stability in *csrA* wild type and mutant strains by RT-PCR.

A. Cultures of BW3414 and TR1-5BW3414 were grown to late exponential phase, treated with rifampin, and harvested at several time points thereafter. Total RNA was isolated and 0.5 μg RNA was used for cDNA synthesis in 20 μl reactions. Two μl of each reaction was subjected to PCR amplification for either 30 or 31 cycles, for cDNA from BW3414 or TR1-5BW3414 (*csrA::kanR*), respectively.

B. The *flhDC* PCR products were quantified by densitometry. Mean values derived from two independent experiments are shown.

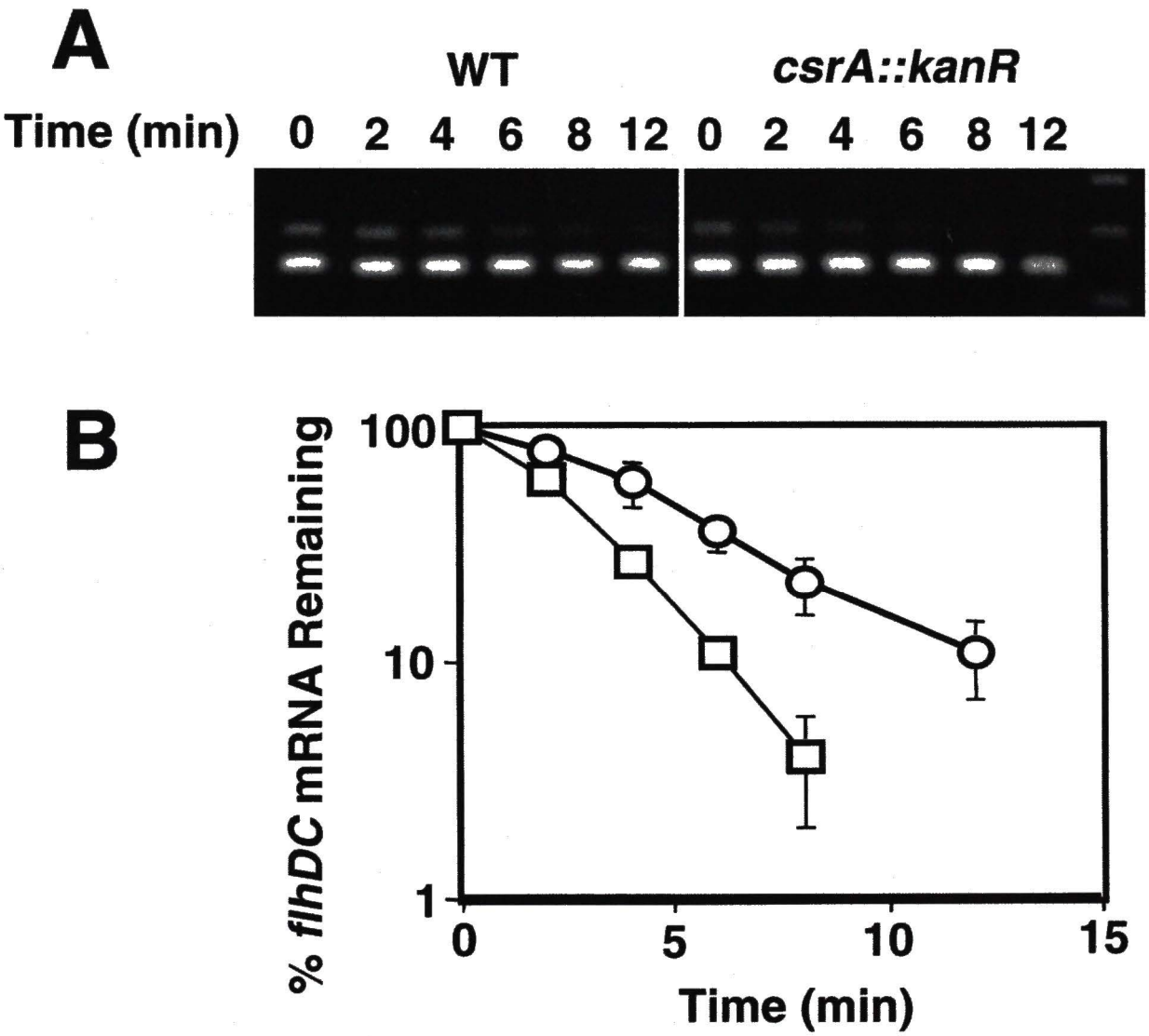
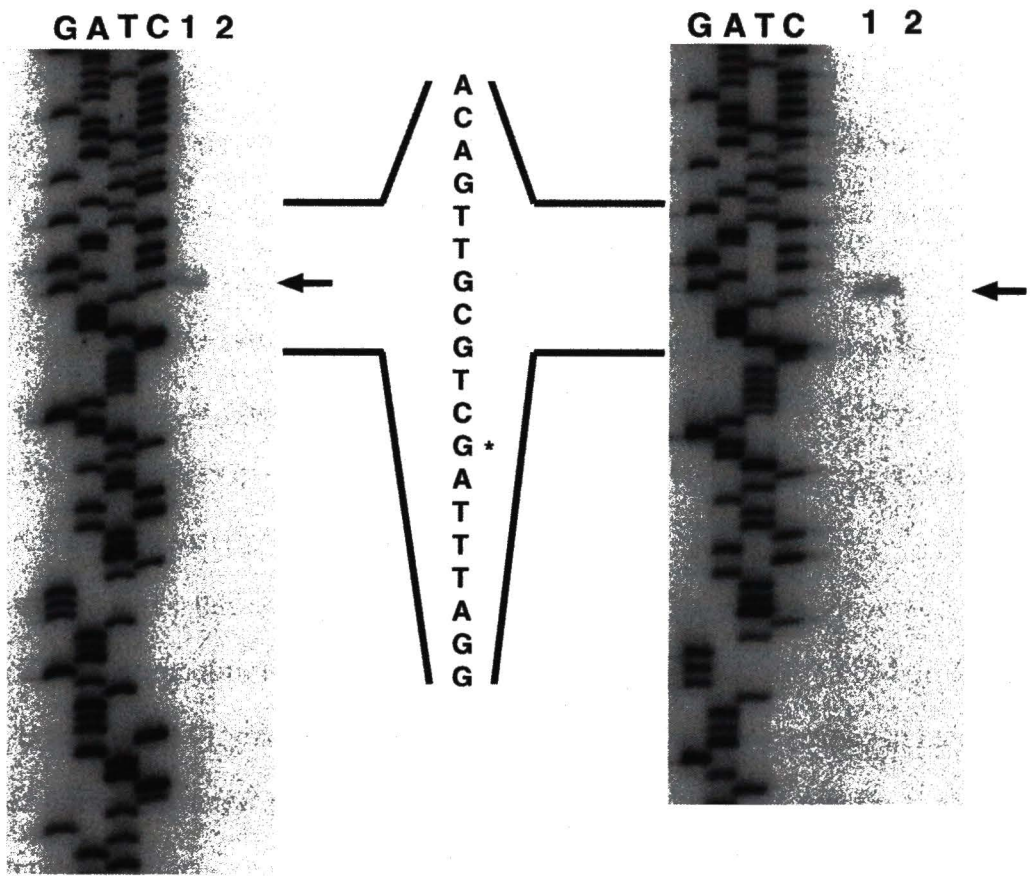

A
WT
csrA::kanR
Time (min) 0 2 4 6 8 12 0 2 4 6 8 12
B
% flhDC mRNA Remaining
100
10
1
0
5
10
15
Time (min)

Fig. 6. Primer extension analysis of *flhDC* mRNA.

A. Results of transcript mapping experiments using primers flh1 or flh2 are shown in the left or right side of Panel A, respectively. Lanes 1 and 2 of each experiment used RNA from strain BW3414 or TR1-5BW3414 (*csrA::kanR)*, respectively. The DNA sequencing reactions were conducted with the corresponding primers and pPM61 as the plasmid template. The sequences shown are those of the coding stand.

B. Alignment of the *E. coli* K-12 *flhDC* promoter sequence with that of *Proteus mirabilis*.

A



B

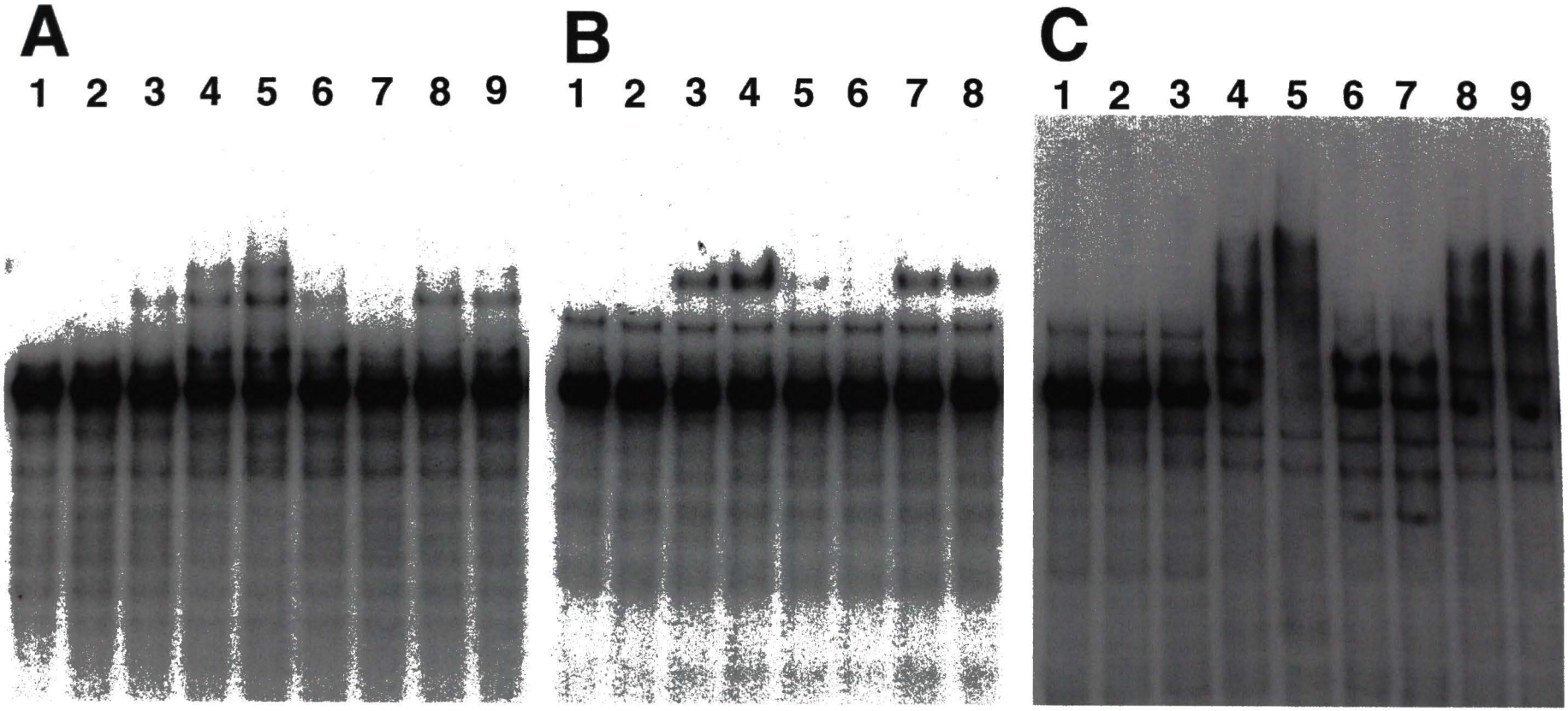
	-35	-10
	TTGACA	TATAAT
E. c.	TCCGTTGTATGTGCGTGTAGTGA	CGAGTACAGTTGCGTCGATTTAGGAA
	******* ** * * *	***** **** *** ******
P. m.	TCCGTTGAATAACTTAATCATTAAGGAGTAAAGTTATCTCG	TTAGGAT

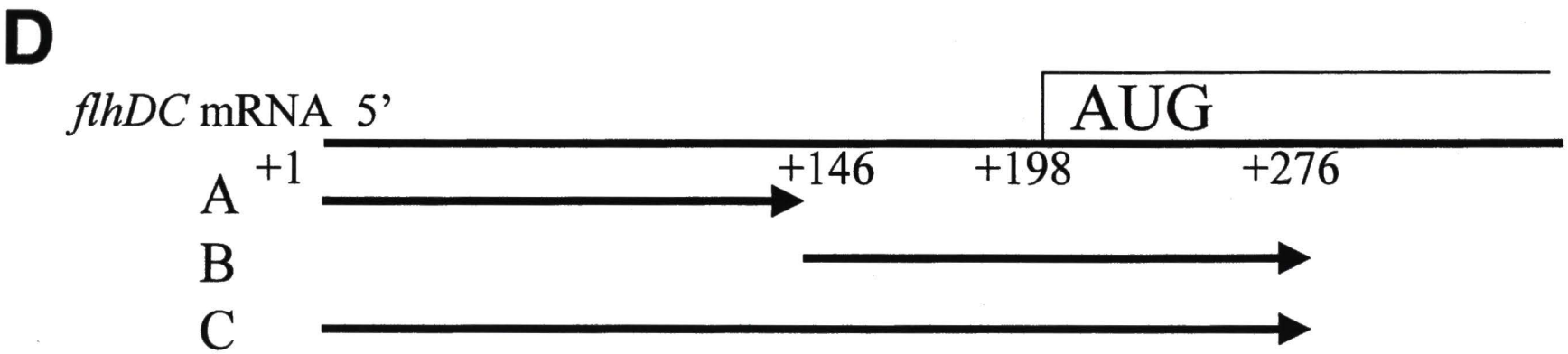
Fig. 7. RNA mobility shift assay with *flhDC* runoff transcripts. A radiolabeled *flhDC* runoff transcript (0.5 nM), A, B, or C as depicted in panel D, was incubated in the presence or absence of CsrA-CsrB complex at various concentrations and analyzed on a 5% native polyacrylamide gel.

A. Reactions in lanes 1 through 5 contained 0, 1.0, 2.5, 5.0, or 10 μM CsrA monomer, respectively. Lanes 6 through 9 depict competition experiments conducted using 0.5 nM *flhDC* transcript A and 5.0 μM CsrA monomer (lane 4 conditions). The reactions shown in lanes 6 and 7 were incubated in the presence of the unlabeled runoff transcript A at 100- and 400-fold excess relative to the labeled transcript, respectively. Lanes 8 and 9 contained *Saccharomyces cerevisiae* total RNA at 100- and 400-fold excess relative to the mass of the labeled transcript.

B. Reactions in lanes 1 through 4 contained 0, 1.0, 5.0, or 10 μM CsrA monomer. Lanes 5 through 8 show competition experiments using reactions containing 0.5 nM of *flhDC* transcript B and 5.0 μM CsrA monomer (lane 3 conditions). The reactions in lanes 5 and 6 contained unlabeled runoff transcript B at 100- and 400-fold excess relative to the labeled transcript, respectively. Lanes 7 and 8 contained *Saccharomyces cerevisiae* total RNA at 100- and 400-fold mass excess.

C. Reactions in lanes 1 through 5 contained 0, 0.1, 1.0, 5.0, or 10 μM CsrA monomer, respectively. Lanes 6 through 9 depict competition experiments using reactions containing 0.5 nM of *flhDC* transcript C and 5.0 μM CsrA monomer (lane 4 conditions). The reaction mixtures in lanes 6 and 7 contained unlabeled runoff transcript C at 200- and 500-fold excess relative to the mass of the labeled transcript, respectively. Lanes 8 and 9 contained *Saccharomyces cerevisiae* total RNA at 200- and 500-fold excess.


A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8 9


D
flhDC mRNA 5'
AUG
+1
+146
+198
+276
A
B
C

CHAPTER V

CONCLUSIONS

This study revealed that the *csrA* gene positively regulated the Acs pathway of acetate metabolism and the glyoxylate shunt. Although the effect on these two pathways was not dramatic in each case (~ 2- to 3-fold inhibition), the successive inhibition of two related pathways essential for utilization of acetate in part explains the growth defect of the *csrA::kanR* mutant on acetate. We did not determine whether the regulation of these genes was through a direct or an indirect mechanism. However, the expression of the *acs::lacZ* transcriptional fusion, but not the *aceB::lacZ* transcriptional fusion, was modulated by the *csrA* gene, suggesting that the regulatory mechanism for these two genes may be different.

The growth of *csrA rpoS* mutant strains was very poor on acetate as a sole carbon source. Surprisingly, growth also was inhibited specifically by the addition of modest amounts of acetate to rich media, which resulted in the rapid appearance of suppressor mutations that disrupt glycogen biosynthesis. This study indicated that the major metabolic problem caused by adding acetate to *csrA rpoS* stains was depletion of the TCA cycle, apparently resulting from a combination of central carbon flux imbalance, inhibition of amino acid uptake, and a deficiency in acetate metabolism. We conclude that this endogenous stress caused by defective regulation of central carbon metabolism can provide very strong selective pressure for adaptation. An intriguing question is whether the *csrA* gene is involved in *E. coli* genome stability. Further investigation would address this possibility.

Motility is one of the major mechanisms that bacteria use in response to environmental stress. Although several global regulatory systems have been involved in the control of motility and flagellum synthesis, this study provided evidence that a functional *csrA* gene is essential for *E. coli* cells to be motile. CsrA post-transcriptionally regulates flagellum biosynthesis and the expression of flagellar master operon, *flhDC*, both in *vivo* and in *vitro*. Apparently, the *csrA* gene functions primarily through a mechanism distinct from other known systems, such as cAMP-CAP, H-NS, or OmpR.

CsrA has been shown to bind specifically to *glgC* mRNA and facilitates the message turnover, thus repressing the overall expression of *glgC*. In contrast, CsrA stabilized *flhDC* mRNA in this study. Therefore, the RNA binding protein CsrA is similar to many DNA binding proteins, which may function as either activators or repressors of gene expression, depending upon the target nucleic acids that are recognized. The precise mechanism by which CsrA binding modulates mRNA stability is not yet known. Studies on *glgC* mRNA suggest that CsrA binds in the vicinity of ribosome binding site of the transcript and thus completes with 30S ribosome, resulting in the inhibition of translation. The mechanism by which CsrA positively regulates *flhDC* might involve the activation of *flhDC* translation upon the binding of CsrA. Consistent with this hypothesis is the presence of a relatively poor Shine-Dalgarno sequence for *flhDC*, whose function might be favored by an accessory factor. In addition, the binding of CsrA to the 5'-UTR of *flhDC* transcript could result in direct protection of *flhDC* mRNA against endonucleolytic attack. In conclusion, the complex mobility shift patterns observed in this study, and the unusually long 5' untranslated segment (197 nucleotides) of *flhDC* mRNA suggest the molecular details of this regulation are complex.

Motility and flagellum synthesis are connected to acetate metabolism by the universal phosphor donor, AcP. However, this study revealed that the function of the

csrA gene in motility is direct, and is not mediated through OmpR, suggesting that AcP may not be involved in this regulation. In fact, the present investigation on the regulatory role of *csrA* in acetate metabolism also disputed that AcP is responsible for the different motility phenomenons observed between *csrA* wild type and mutant strains. The *csrA* gene does not affect AckA-Pta pathway of acetate metabolism, thus the cellular level of AcP in these two strains seemed not to be altered.