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# Identification and Molecular Characterization of *csrA*, a Pleiotropic Gene from *Escherichia coli* That Affects Glycogen Biosynthesis, Gluconeogenesis, Cell Size, and Surface Properties

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**Current evidence suggests that a few global regulatory factors mediate many of the extensive changes in gene expression that occur as *Escherichia coli* enters the stationary phase. One of the metabolic pathways that is transcriptionally activated in the stationary phase is the pathway for biosynthesis of glycogen. To identify factors that regulate glycogen biosynthesis in *trans*, a collection of transposon mutants was generated and screened for mutations which independently increase or decrease glycogen levels and the expression of a plasmid-encoded *glgC'-'lacZ* fusion. The glycogen excess mutation TR1-5 was found to be pleiotropic. It led to increased expression of the genes *glgC* (ADPglucose pyrophosphorylase) and *glgB* (glycogen branching enzyme), which are representative of two glycogen synthesis operons, and the gluconeogenic gene *pckA* (phosphoenolpyruvate carboxykinase), and it exhibited effects on cell size and surface (adherence) properties. The mutated gene was designated *csrA* for carbon storage regulator. Its effect on glycogen biosynthesis was mediated independently of cyclic AMP (cAMP), the cAMP receptor protein, and guanosine 3'-bisphosphate 5'-bisphosphate (ppGpp), which are positive regulators of *glgC* expression. A plasmid clone of the native *csrA* gene strongly inhibited glycogen accumulation and affected the ability of cells to utilize certain carbon sources for growth. Nucleotide sequence analysis, complementation experiments, and in vitro expression studies indicated that *csrA* encodes a 61-amino-acid polypeptide that inhibits glycogen biosynthesis. Computer-assisted data base searches failed to identify genes or proteins that are homologous with *csrA* or its gene product.**

A large body of evidence shows that during the transition into stationary phase, bacteria acquire numerous new physiological properties which enhance their ability to compete and survive under suboptimal conditions (for reviews, see references 16, 25, 26, and 44). While it has become clear that in *Escherichia coli* the induction of several genes and operons in the stationary phase requires a putative sigma factor, *katF* or *rpoS* (2, 20, 25, 41), the expression of stationary-phase genes such as *mcbA* for microcin production (2) and *glgCA* for glycogen synthesis do not require *katF* (13, 38a), suggesting that additional regulators exist. We have initiated studies to identify and characterize factors that control the glycogen biosynthesis genes in *E. coli*. Our previous experiments showed that cyclic AMP (cAMP), cAMP receptor protein, and guanosine 3'-bisphosphate 5'-bisphosphate (ppGpp) stimulate the expression of the genes for the essential enzymes of the glycogen pathway, *glgC* (encoding ADPglucose pyrophosphorylase [EC 2.7.7.27]) and *glgA* (encoding glycogen synthase [EC 2.4.1.21]) (34, 38), which are apparently cotranscribed in an operon, *glgCAY*. This operon also includes the gene encoding the catabolic enzyme glycogen phosphorylase (EC 2.4.1.1), *glgY* or *glgP* (36, 53). The gene *glgB* (encoding glycogen branching enzyme [EC 2.4.1.18]) is located upstream from *glgCAY* (1), apparently in an operon, *glgBX*, that includes a gene encoding a second catabolic enzyme (36). Although the expression of the three biosynthetic genes is induced in stationary phase, *glgB* is transcribed independently of *glgCA* and it is not regulated by cAMP-cAMP receptor protein or ppGpp (32, 38). Four stationary-phase-induced transcripts have been mapped within the 0.5-kb upstream noncoding region of the *glgC* gene from *E. coli*, implying complex transcriptional regulation of *glgCA* (38).

The present paper describes the identification and molecular characterization of a gene from *E. coli* K-12, *csrA*, that negatively regulates the expression of both *glgC* and *glgB*. The cloning, nucleotide sequencing, and in vitro expression of *csrA* and some of the regulatory effects of a *csrA* mutation are presented. Genetic and physical mapping experiments which showed that *csrA* is located between the *alaS* and *serV* genes at 2,834.8 kb on the physical map of the *E. coli* K-12 genome are reported elsewhere (35). The *csrA* mutation is pleiotropic, suggesting that *csrA* encodes a global regulatory factor.

The discovery of *csrA* reemphasizes that the synthesis of glycogen in *E. coli* is controlled in response to a variety of genetic and physiological influences. This is also reflected in the array of *trans*-acting factors that regulate *glgCA* expression, in the complex transcription pattern observed for *glgC* (38), the allosteric regulation of ADPglucose pyrophosphorylase activity (for a review, see references 30 and 32), and the effects of *katF* and *glgS* on glycogen synthesis via mechanisms that are still unknown (13, 20).

## MATERIALS AND METHODS

**Chemicals and reagents.** Radioisotopically labeled $^{32}PP_i$ (2.1 mCi/μmol), α-$^{35}$S-dATP (1,200 mCi/μmol), and [$^{35}$S]methionine (1,180 mCi/μmol) were purchased from Dupont

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

| Strain, phage, or plasmid | Description | Source and/or reference |
|---|---|---|
| *E. coli* K-12 | | |
| BW3414 | Δ*lacU169* | Barry Wanner |
| CAG18642 | *zfh*-3131::Tn*10*; 57.5 min | Carol Gross (46) |
| CF1648 | [MG1655](prototrophic) | Michael Cashel (51) |
| CF1651 | Δ*relA* in CF1648 | Michael Cashel (51) |
| CF1693 | Δ*spoT* in CF1651 | Michael Cashel (51) |
| G6MD3 | Hfr *his thi* $Str^s$ Δ(*malA-asd*) | 42 |
| HG137 | *pck-13*::Mu d1 *pps* Δ*lacU169* | Hughes Goldie (9) |
| HB101 | *supE44 hsdS20*($r_B^-$ $m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | 3 |
| LE392 | *hsd R514* ($r_K^-$ $m_K^-$) *supE44 supF58 lacY galK2 galT22 metB1 trpR55*λ$^-$ | Donna Daniels and Fred Blattner |
| ML2 | *met gal hsdK$_R$ supE supF* $Km^r$ Δ*cya* | 10 |
| NK5012 | *supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21 trp tonA21* Φ80$^r$15$^r$ | Nancy Kleckner |
| SA2777 | $F^-$ *rpsL relA* Δ*crp*::$Cm^r$ | S. Garges and S. Adhya |
| TR1-5[a] | *csrA*::*kanR* | This study |
| ZK916 | W3110 Δ*lacU169 tna-2* λMAV103 (*bolA*::*lacZ*) | Roberto Kolter (2) |
| *E. coli* B | | |
| AC70R1 | *glgQ* | Jack Preiss |
| Bacteriophages | | |
| λNK1298 | Tn*10*-mini $Kan^r$ hopper | N. Kleckner |
| λ446 | Clone (*csrA*$^+$) from the Kohara collection | F. Blattner (15) |
| P1*vir* | Strictly lytic P1; forms clear plaques | Carol Gross (27) |
| Plasmids | | |
| pUC19 | Cloning vector; high copy number | 52 |
| pLG339 | Cloning vector; low copy number | 47 |
| pOP12 | Contains *asd* and *glg* gene cluster in pBR322 | 28 |
| pMLB1034 | For construction of '*lacZ* translational fusions | 45 |
| pCZ3-3 | Φ*glgC'-'lacZ* in pMLB1034 | 34 |
| pBZ1 | Φ*glgB'-'lacZ* in pMLB1034 | This study |
| pCSR10 | *csrA* gene, 0.5-kb *Dde*I from λ446 in pUC19 | This study |
| pCSR-L1 | pCSR10 deletion | This study |
| pCSR-D3 | pCSR10 deletion | This study |
| pCSR-D1-D | pCSR10 deletion | This study |
| pCSR-D1-L | pCSR10 deletion | This study |
| pTR151 | Clone of TR1-5 (*csrA*::*kanR*) in pUC19 | This study |
| pLEP2-2 | Part of *serV* operon from λ446 in pUC19 | This study |
| pPV1 | Part of *serV* operon from λ446 in pUC19 | This study |

[a] A strain designation containing the prefix TR1-5 indicates that the wild-type (*csrA*$^+$) allele has been replaced by the TR1-5 mutant allele (*csrA*::*kanR*) by P1*vir* transduction.

NEN (Wilmington, Del.). Enzymes for assaying glycogen were purchased either from Sigma Chemical Co. (St. Louis, Mo.) (hexokinase and glucose-6-phosphate dehydrogenase) or from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) (α-amylase and amyloglucosidase). Sequenase 2.0 enzyme and other reagents for DNA sequence analysis were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). Exonuclease III and mung bean nuclease were purchased from New England Biolabs (Beverly, Mass.). Protein and DNA molecular weight standards were from Bethesda Research Laboratories (Bethesda, Md.).

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

**Growth conditions.** Cultures were grown in either Kornberg medium (containing 0.5% glucose [38]), LB medium (pH 7.4 [27]), LB-glucose (LB containing 0.2% glucose), M9 medium (0.5% carbon source [27]), or supplemented morpholinepropane sulfonic acid (MOPS) medium (49). Liquid cultures were grown with rapid gyratory shaking at 37°C or as otherwise indicated. Cultures were inoculated with 1 volume of an overnight culture per 100 volumes of fresh medium or as otherwise indicated. Solid media contained 1.5% agar. Solid Kornberg medium for detection of endogenous glycogen contained 1% glucose. Anaerobic conditions for solid and liquid cultures were maintained by using a BBL GasPak system (Beckton Dickinson, Cockeysville, Md.). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 100 μg/ml; and chloramphenicol, 10 μg/ml.

**Molecular biology techniques and nucleotide sequencing.** Standard procedures were used for isolation of chromosomal DNA, plasmid DNA, and restriction fragments, restriction mapping, transformation, and molecular cloning, as previously described (36, 38). Polymerase chain reaction (PCR) was conducted by the asymmetric synthesis method (11). Deletion mutants of pCSR10 were constructed according to the method described by Henikoff (14). Nucleotide sequencing was by the dideoxynucleotide chain termination method (40). For obtaining the nucleotide sequence of *csrA*, both strands of DNA were sequenced in entirety with double-stranded plasmid DNA or single-stranded PCR products as the template. Overlap between all contiguous sequences was obtained, and the region of the TR1-5 insertion was sequenced from both the mutant and wild-type *csrA* alleles. DNA and protein sequence analyses were routinely con-

ducted by using the software DNA Strider (23). Searches for homologous genes, proteins, and motifs in the EMBL and GenBank, SwissProt, and Prosite data bases, respectively, were conducted by using the Wisconsin Genetics Computer Group software package developed by the University of Wisconsin Biotechnology Center (Madison, Wis.).

**Genetic techniques.** Transposon mutants were constructed by infecting BW3414(pCZ3-3) with λNK1298 at a multiplicity of infection of 0.025, essentially as described elsewhere (50). Transduction of the TR1-5 (*csrA*::*kanR*) mutation was conducted by using P1*vir* according to the method described by Miller (27). Transductants were isolated either by direct selection for the Kan$^r$ phenotype or by cotransduction of the closely linked Tet$^r$ marker in TR1-5CAG18642 (35).

**Electron microscopy.** A rapid method for preparing cells for transmission electron microscopy which did not require sectioning was developed. Cells were washed, and the cell density was adjusted to approximately $5 \times 10^9$ cells per ml in 0.06 M potassium phosphate buffer, pH 6.8. One drop (10 μl) of this suspension was transferred to a 150-mesh grid that had been coated with Formvar and carbon and then glow discharged. After 1 min, the excess buffer was removed and a drop of 4% glutaraldehyde in the same buffer was placed on the grid for 10 min, followed by postfixation with 2% osmium tetroxide for 10 min. The grid was rinsed three times on drops of distilled water and transferred to a drop of methylcellulose containing 0.2% uranyl acetate for 10 min. The grid was removed from this suspension with a 3.5-mm-diameter NiCr loop, excess methylcellulose was removed with filter paper, and the grid was allowed to dry for 10 min. The grid was removed from the loop with a thin needle. Micrographs of cells were taken at a magnification of ×5,000 with a Hitachi 600 transmission electron microscope.

**Enzyme, protein, and quantitative glycogen assays.** ADP glucose pyrophosphorylase activity in the pyrophosphorolysis direction, the amount of total cellular protein, and β-galactosidase activity were determined as previously described (34, 37). The β-galactosidase activity was normalized with respect to protein instead of culture turbidity, since it was found that the turbidity ($A_{600}$) of the TR1-5 mutant in liquid culture was significantly higher than that of the parent strain in the early stationary phase for equivalent levels of cellular protein. Each of the growth curve experiments was conducted at least twice to ensure that results attributed to strain differences were reproducible. Glycogen was isolated and enzymatically quantified according to the method described by Preiss et al. (31).

**S-30-coupled transcription translation.** The expression of plasmid-encoded genes in S-30 extracts and the analysis of the in vitro-synthesized proteins were conducted as previously described (38). Proteins were labeled with [$^{35}$S]methionine, separated on 10 to 22% gradient sodium dodecyl sulfate (SDS)-polyacrylamide slab gels, and detected by fluorography with sodium salicylate (5).

**Nucleotide sequence accession number.** The accession number L07596 has been assigned to the sequence shown in Fig. 10 by GenBank, which has been recorded in a data base of *E. coli* sequences compiled by Rudd et al. (39).

## RESULTS

**Isolation of a transposon mutant, TR1-5, that exhibits altered expression of the glycogen biosynthesis genes.** To identify the genes that regulate the expression of the *glg* genes, we mutagenized BW3414(pCZ3-3) with a mini-*kanR* element (50). This strain lacks a chromosomal copy of *lacZ* but contains native chromosomally encoded copies of the *glg* genes, as well as a plasmid-encoded *glgC'-'lacZ* translational fusion. The expression of this *glgC'-'lacZ* fusion is controlled by all of the factors that are known to regulate the native *glgC* gene (34). Cells were plated onto Kornberg medium containing kanamycin and ampicillin, the plates were incubated overnight at 37°C, and glycogen synthesis mutants were identified by staining with iodine vapor (36). β-Galactosidase activities in mutants which exhibited either increased or decreased iodine staining were determined. Approximately 15,000 Kan$^r$ mutants were generated, and β-galactosidase activity was determined for 47 glycogen mutants. The TR1-5 mutant exhibited intense iodine staining and five- to sixfold higher β-galactosidase activity than the parent strain, although four other glycogen excess, *glgC'-'lacZ*-overexpressing mutants which showed less dramatic effects were isolated. The transposon which was used was expected to generate stable mutations (50), and the TR1-5 mutant was found to be phenotypically stable during extensive growth experiments, indicating that second-site reversions do not present a problem in handling this strain.

TABLE 2. Glycogen and ADPglucose pyrophosphorylase levels in *E. coli* K-12 BW3414 (*csrA*$^+$) and TR1-5BW3414 (*csrA*::*kanR*)

| Strain | ADPglucose pyrophosphorylase (U/mg of protein ± SD)[a] | | Glycogen (mg/mg of protein ± SD)[b] | |
|---|---|---|---|---|
| | Exponential growth phase | Stationary growth phase | Early stationary phase | Late stationary phase |
| BW3414 | 0.08 ± 0.00 | 0.23 ± 0.01 | 0.06 ± 0.01 | 0.012 ± 0.001 |
| TR1-5BW3414 | 0.25 ± 0.01 | 2.16 ± 0.50 | 1.61 ± 0.07 | 0.346 ± 0.003 |

[a] For ADPglucose pyrophosphorylase activity, cultures were grown in Kornberg medium from a 1:400 inoculum and were harvested in late-exponential (7 h) or stationary (24 h) phase. One unit of activity is defined as 1 μmol of product formed per 10 min, in the pyrophosphorolysis direction, under maximal allosteric activation.

[b] Cultures were grown in Kornberg medium and were harvested in early (6 h) or late (24 h) stationary phase.

**The TR1-5 mutation alters glycogen biosynthesis via effects on the expression of two glycogen biosynthetic operons.** The extremely intense staining of colonies of the TR1-5 mutant indicated that glycogen levels were much higher than in the parent strain. The TR1-5 mutation was transduced into BW3414 to generate a mutant strain that did not carry the pCZ3-3 plasmid and to provide a genetic background which had not been subjected to transposon mutagenesis. Glycogen levels were quantitatively determined in the mutant TR1-5BW3414 and wild-type BW3414 strains and were compared (Table 2). TR1-5BW3414 accumulated 20- to 30-fold more glycogen than the parent strain, consistent with the qualitative results of iodine staining.

Levels of ADPglucose pyrophosphorylase were found to be significantly higher in the TR1-5 mutant than in an isogenic wild-type strain, in both the exponential and stationary phases of growth, with the maximal difference of almost 10-fold occurring in the stationary phase (Table 2). The specific activity of β-galactosidase expressed from the *glgC'-'lacZ* fusion was monitored throughout the exponential and stationary phases of wild-type and TR1-5 mutant strains. The TR1-5 mutant overexpressed this gene fusion in both the exponential and stationary phases (Fig. 1). It was observed that specific β-galactosidase activity was sevenfold higher in stationary phase (24 h) versus mid-log phase in both mutant and wild-type strains and that a change of approxi-

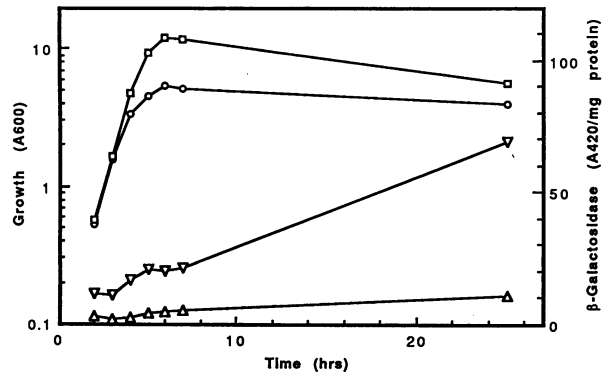


FIG. 1. Expression of a *glgC'-'lacZ* translational fusion in BW3414 and TR1-5BW3414. Strains containing plasmid pCZ3-3 were grown in Kornberg medium, and assays were conducted as described in Materials and Methods. Sample identities are as follows: circles, and squares, respectively, growth ($A_{600}$) of cultures for BW3414 and TR1-5BW3414; triangles, β-galactosidase activity, with apices facing up or down for BW3414 or TR1-5BW3414, respectively.

mately two- to threefold in activity occurred after the cultures had already entered stationary phase.

In addition to the *glgCA* genes, glycogen biosynthesis depends on *glgB* expression to provide the glycogen branching enzyme, which catalyzes the formation of the α-1,6 branches of glycogen. A *glgB'-'lacZ* translational fusion was constructed to allow *glgB* expression to be readily determined. A 0.34-kb *Bam*HI-*Hin*cII fragment of pOP12 that spans the region from +53 bp of the *glgB* coding region (1) upstream through the 275-bp *glgB-asd* intervening region and 8 bp into the coding region of *asd* (12) was cloned into the *Bam*HI-*Sma*I cloning site of pMLB1034 (45). The resulting plasmid, pBZ1, contained an in-frame translational fusion of codon 18 of *glgB* with codon 7 of *lacZ*. The specific β-galactosidase activity that was expressed from this *glgB'-'lacZ* fusion was two- to threefold higher in the TR1-5 mutant than in the isogenic wild-type strain (Fig. 2). The induction of the *glgB'-'lacZ*-encoded β-galactosidase activity in the mutant occurred at the same time in the growth curve and with a similar degree of stationary-phase activation, approximately 3.5- to 4-fold, as observed in the wild-type strain, i.e., the shapes of the induction curves were similar.

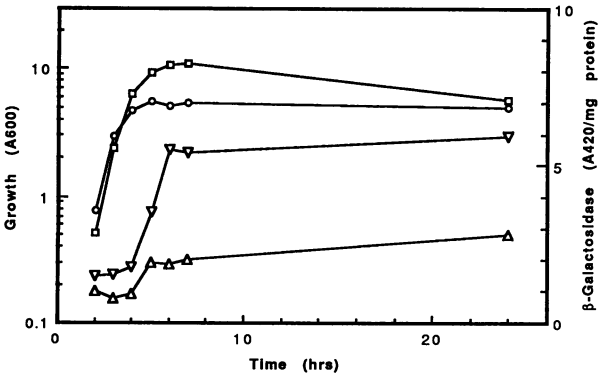


FIG. 2. Expression of a *glgB'-'lacZ* fusion in BW3414 and TR1-5BW3414. Cultures were grown in Kornberg medium. Sample identities are as described in the legend to Fig. 1, except that the strains contained plasmid pBZ1 instead of pCZ3-3.

**The TR1-5 mutation affects glycogen biosynthesis independently of cAMP and ppGpp.** The observation that *glgB* expression was elevated in the TR1-5 mutant suggested that its effect on glycogen levels was not mediated indirectly via cAMP or ppGpp, which have been shown not to regulate *glgB* (34, 38). To directly test this idea, the TR1-5 mutation was transduced into several strains that were deficient in one or more of the genes needed for cAMP or ppGpp control. The TR1-5 mutation resulted in enhanced glycogen levels in cAMP-deficient (Δ*cya*) and in cAMP receptor protein-deficient (*crp*) strains (Fig. 3) and in strains that accumulate low levels of ppGpp (Δ*relA*) or are completely deficient in ppGpp (Δ*relA*Δ*spoT*; Fig. 4). These results showed that the TR1-5 mutation affects glycogen biosynthesis independently of the factors that were previously known to regulate the expression of *glgCA*.

**Expression of a *pckA-lacZ* transcriptional fusion is enhanced in TR1-5.** To test whether the TR1-5 mutation affects the expression of genes in other metabolic systems, the pathway for gluconeogenesis was examined. Gluconeogenesis is functionally related to glycogen synthesis, since it can provide glucose-1-phosphate for glycogen biosynthesis and for other biosynthetic processes when exogenous glucose is not available for cell growth. The expression of *pckA*, the gene encoding a key regulatory enzyme for gluconeogenesis, phosphoenolpyruvate carboxykinase (EC 4.1.1.49), has been shown to be subject to growth phase control and to positive regulation by cAMP (9). The TR1-5 mutation was transduced into HG137, a strain containing a chromosomally encoded *pckA-lacZ* operon fusion (9). The specific β-galactosidase activity from this gene fusion was twofold higher in the TR1-5 mutant than in the isogenic wild-type parent. The induction curve in the mutant paralleled that of the wild-type strain (Fig. 5).

**Effects of the TR1-5 mutation on cell morphology.** It was observed that the maximum turbidity of liquid cultures of strains containing the TR1-5 mutation was higher than that of isogenic wild-type strains in Kornberg medium (Fig. 1, 2, and 8). Although it is possible that the high level of glycogen present in the mutant cells may affect turbidity, the size of individual cells was considered to be another parameter that might account for this result. The sizes of mutant and wild-type cells were compared both by direct measurements of cell length taken from electron micrographs (Fig. 6) and by calculation of viable counts per milligram of protein (Fig. 7). The ratio of CFU per milligram of protein for the wild type versus the isogenic TR1-5 mutant indicated that the mutant strain was 1.74-, 2.55-, 3.36-, 4.05-, 4.12-, and 1.42-fold larger at 2, 3, 4, 6, 7, and 24 h, respectively. Therefore, the maximal difference in size occurred in the early stationary phase, and this was apparently because cells of the mutant strain did not undergo a dramatic decrease in size during entry into the stationary phase.

The gene *bolA* has been shown to directly influence the change in cell size which occurs during entry into stationary phase (reference 2 and references therein). Comparison of *bolA* expression in TR1-5 and wild-type strains revealed that the TR1-5 mutation does not significantly alter the level of *bolA* expression (Fig. 8). Therefore, the significant difference in size between the two strains appears not to be mediated via regulatory effects on the expression of *bolA* and perhaps is simply an indirect effect of *csrA* on endogenous glycogen.

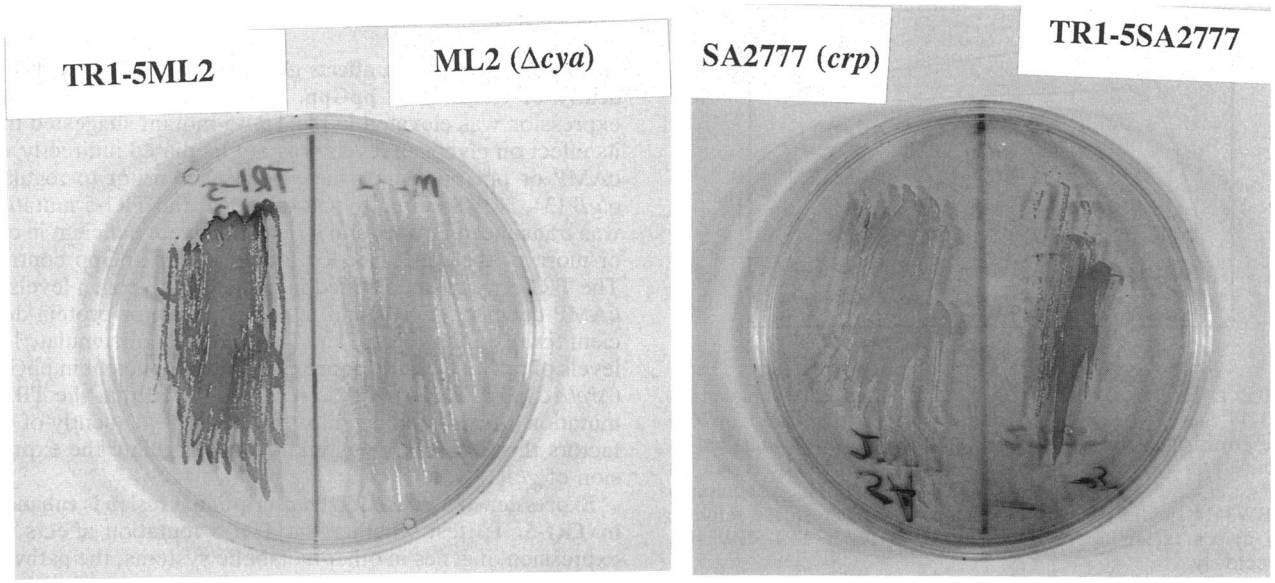


FIG. 3. Effects of the TR1-5 mutation on glycogen biosynthesis in cAMP (Δ*cya*) or cAMP receptor protein (*crp*)-deficient strains. The TR1-5 mutation was introduced by P1*vir* transduction into strains that lack cAMP (Δ*cya*) or a functional cAMP receptor protein (*crp*). Cultures were incubated overnight on Kornberg medium and stained for endogenous glycogen with iodine vapor.

**Molecular cloning and nucleotide sequence of the native and TR1-5 mutant alleles of *csrA*.** The TR1-5 mutant allele of *csrA* was cloned by ligating restriction fragments from a partial *Sau*3A1 digest of chromosomal DNA from a secondary P1*vir* transductant of the TR1-5 mutation (TR1-5BW3414) into the *Bam*HI site of pUC19 and selecting for Kan$^r$ Amp$^r$ transformants of HB101. Five plasmid clones containing the TR1-5 mutation were isolated, including pTR151.

The nucleotide sequence determined from pTR151 (Fig. 9 and 10) and subclones derived from this plasmid was used to search the EMBL and GenBank data bases for homologous genes. This search showed that the Kan$^r$ marker had been inserted downstream from *alaS*, which encodes alanine tRNA synthetase (33). None of the Kan$^r$ plasmid clones that were isolated contained *csrA* DNA from the side of the marker opposite *alaS*. Attempts to subclone the entire region of the native *csrA* gene directly from a bacteriophage λ clone (λ446 of the Kohara miniset [15]) into pUC19 or pLG339 were unsuccessful, although numerous plasmid clones containing restriction fragments that originated near this region were isolated. Two of these clones, pPV1 and pLEP2-2 (Fig. 9), were partially sequenced and were found

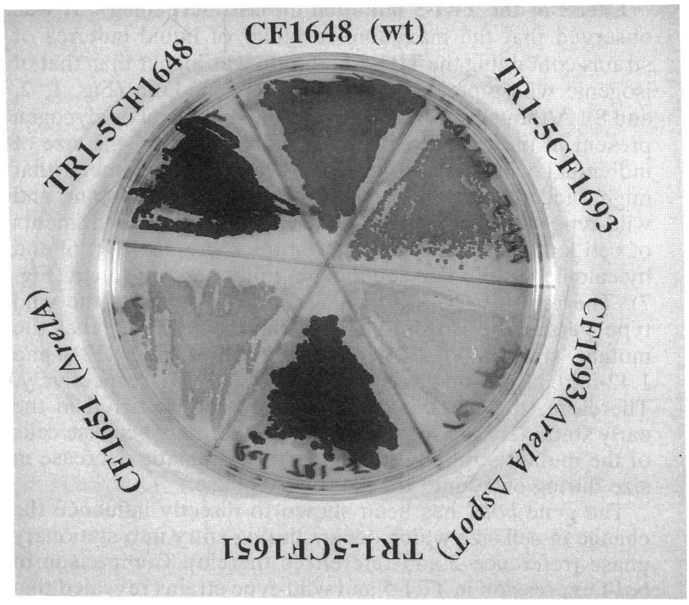


FIG. 4. Effects of the TR1-5 mutation on glycogen synthesis in strains deficient in ppGpp. The TR1-5 mutation was introduced by P1 transduction into strains that are either prototrophic, deficient in ppGpp (Δ*relA*), or completely lack ppGpp (Δ*relA* Δ*spoT*). Cultures were incubated overnight and stained for endogenous glycogen with iodine vapor.

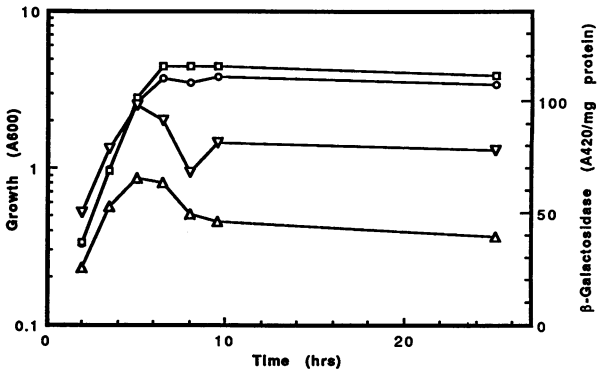


FIG. 5. Expression of a chromosomally encoded *pckA*::*lacZ* transcriptional fusion in HG137 and TR1-5HG137. Cultures were grown in LB medium without added glucose, at 32°C. Sample identities are as indicated in the legend to Fig. 1, except that the genetic background was HG137 instead of BW3414.

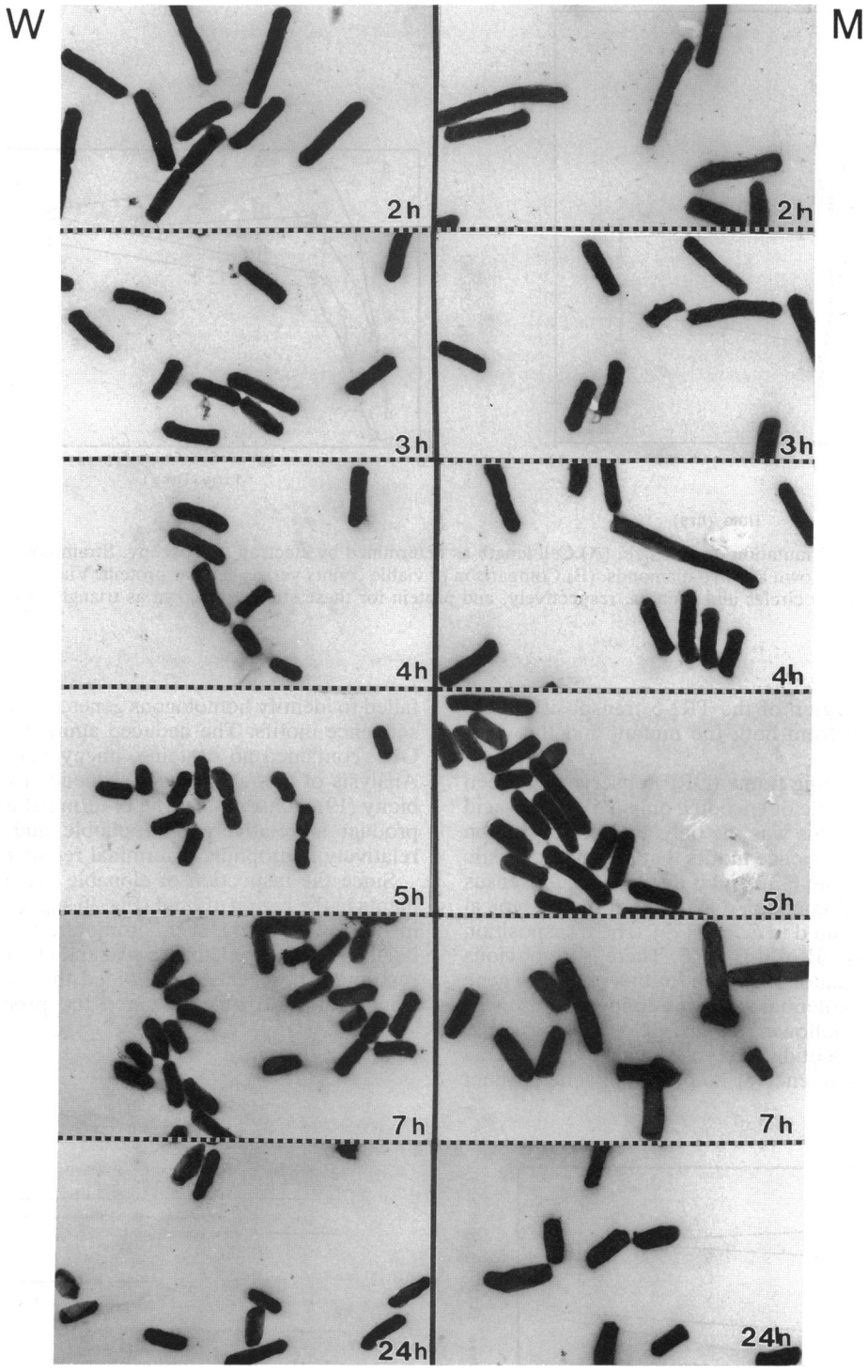


FIG. 6. Electron microscopy of BW3414 versus TR1-5BW3414. Cultures were sampled throughout the growth curve at the indicated times. BW3414 is indicated by W (wild type); TR1-5BW is indicated by M (mutant).

to contain part of the *serV* operon of tRNA genes (17). These sequence data and the restriction map of *E. coli* K-12 (39) indicated that approximately 0.2 kb of DNA sequence remained undetermined on the counterclockwise side of the Kan$^r$ marker, i.e., between *serV* and the *kanR* gene. Each strand of DNA in this region was individually amplified from λ446 DNA by asymmetric PCR (11) and was sequenced directly from the PCR products. Thus, the nucleotide sequence of both strands of the 0.7-kb gap separating the *alaS* and *serV* genes on the *E. coli* K-12 chromosome was

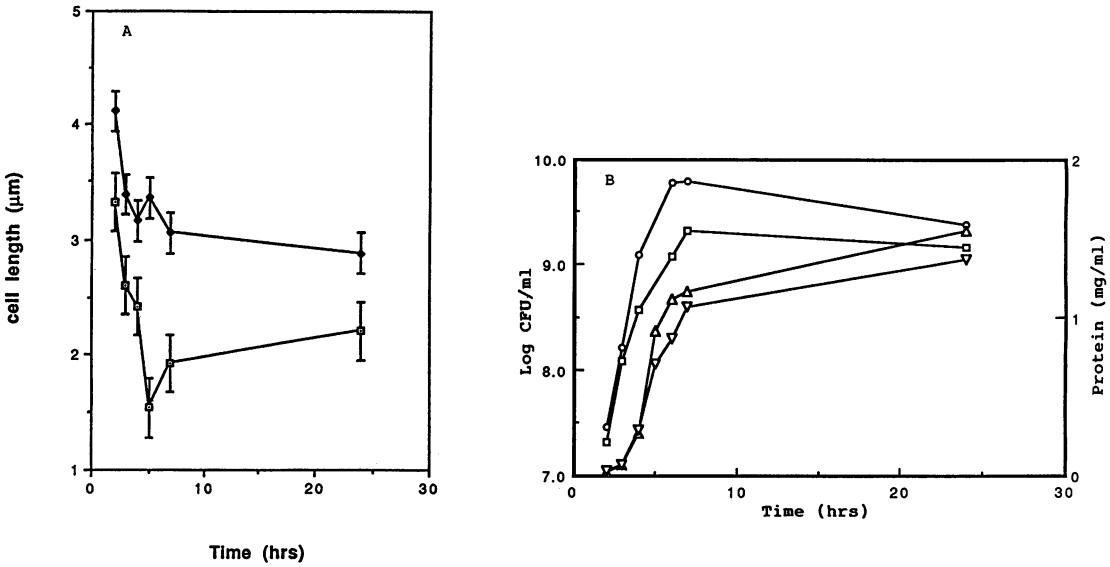


FIG. 7. Effects of TR1-5 mutation on cell size. (A) Cell length as determined by electron microscopy. Strain BW3414 is shown as open squares; TR1-5BW3414 is shown as filled diamonds. (B) Comparison of viable counts versus cellular protein. Viable counts for BW3414 and TR1-5BW3414 are shown as circles and squares, respectively, and protein for these strains is shown as triangles with apices facing up or down, respectively.

determined, and the region of the TR1-5 transposition mutation was sequenced from both the mutant and the wild-type *csrA* alleles.

The largest open reading frame (ORF) observed between *alaS* and *serV* was capable of encoding only a 61-amino-acid polypeptide (Fig. 10). This was the only ORF in this region that was preceded by sequence motifs that are characteristic of a ribosome-binding site (43), and it displayed a consensus sequence in the initiation codon-distal region that is typical of genes that are translated in *E. coli* (29). The transposition site is located at codon 51 of this ORF. There is no obvious rho-independent terminator sequence for this proposed gene (Fig. 10), although a potential stem and loop structure was found immediately following *alaS*. Computer-assisted searches with the nucleotide sequence of this ORF or the deduced amino acid sequence of its proposed gene product failed to identify homologous genes, proteins, or procaryotic sequence motifs. The deduced amino acid sequence of the ORF contained no cysteine, phenylalanine, or tryptophan. Analysis of this amino acid sequence to predict hydrophobicity (19) indicated that the N-terminal half of the proposed product is relatively hydrophobic and is followed by a relatively hydrophilic C-terminal region (data not shown).

Since the inspection of clonable versus unclonable fragments in the region of *csrA* (Fig. 9) suggested that an element in the region of the *serV* promoter, perhaps the promoter itself, was not amenable to plasmid cloning, a 0.53-kb *Dde*I restriction fragment that avoided the intact *serV* promoter (Fig. 9 and 10) but contained the proposed *csrA* coding

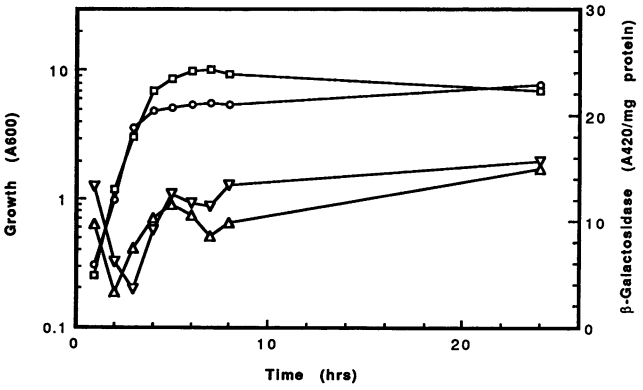


FIG. 8. Effect of the TR1-5 mutation on the expression of a *bolA*::*lacZ* gene fusion. Cultures were grown in Kornberg medium. Sample identities are as described in the legend to Fig. 1, except that the genetic background of the strains was ZK916, which contains a chromosomally encoded *bolA-lacZ* fusion.

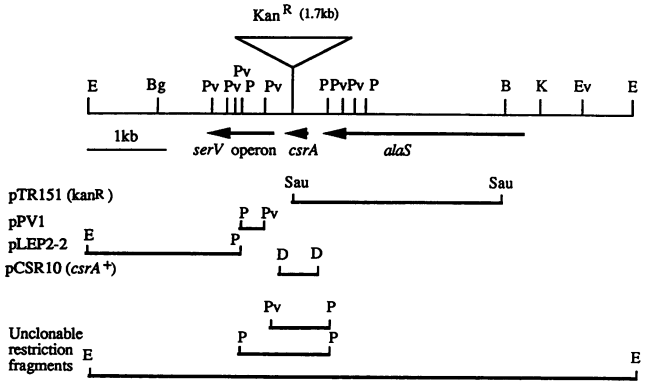


FIG. 9. Plasmid clones isolated from the region of *csrA*. Restriction fragments from a *Sau*3A1 digest of chromosomal DNA from TR1-5BW3414 were cloned into the vector pUC19 to generate pTR151. The plasmid clones pPV1, pLEP2-2, and pCSR10 were generated by subcloning the indicated restriction fragments from λ446 into pUC19. Restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*I; D, *Dde*I; E, *Eco*RI; Ev, *Eco*RV; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; Sau, *Sau*3A1.

```
                870 (alaS)
AGTGTGAAAGGCTGGGTCAGCGCGAAATTGCAATAATATAAGCGTCAGGCAATGCCGTGG   60
 S  V  K  G  W  V  S  A  K  L  Q OCH

ACTCGCTTCACGGCATTCGCATTAACGCTATCGACAACGATAAAGTCAGGTTGAAGTTGT  120

                                                  DdeI
GTATATCGGCTAAACTTAGGTTTAACAGAATGTAATGCCATGACTGCTTAGATGTAATGT  180

GTTTGTCATTGCTTACTTTTTGGCGTTATATGATGGATAATGCCGGGATACAGAGAGACC  240

                     S.D.       1 (csrA)                  10
CGACTCTTTTAATCTTTCAAGGAGCAAAGAATGCTGATTCTGACTCGTCGAGTTGGTGAG  300
                                M  L  I  L  T  R  R  V  G  E

                               20                          30
ACCCTCATGATTGGGGATGAGGTCACCGTGACAGTTTTAGGGGTAAAGGGCAACCAGGTA  360
 T  L  M  I  G  D  E  V  T  V  T  V  L  G  V  K  G  N  Q  V

                               40                          50
CGTATTGGCGTAAATGCCCCGAAGGAAGTTTCTGTTCACCGTGAAGAGATCTACCAGCGT  420
 R  I  G  V  N  A  P  K  E  V  S  V  H  R  E  E  I  Y  Q  R

↓                              60
ATCCAGGCTGAAAAATCCCAGCAGTCCAGTTACTAATCTTTCCGCGTCTCATCTTTATCG  480
 I  Q  A  E  K  S  Q  Q  S  S  Y OCH

GTGAGACGCACCCTCAAAATTTCTCCTTCACTCTATAGTCTTTTCGCTTTACTCCCGTTC  540

ATTCAACTTAAGTCTCCATTTTTTTGCATTACTACTATCTGTCAGACCTCCATTCTTCTG  600

TTGATAAAACACTCTTTTTGACGTTTTTACAGACTAATTGAACGTGAAGTGTGCAAACGA  660

                  -35  (serV)  DdeI   -10
TAAAAGTGTAGGAAAAATTGTTTGACTTATAAGTCTCAGAAAGTAATAT  709
```

FIG. 10. Nucleotide sequence of the *csrA* gene and the deduced amino acid sequence of its ORF. The nucleotide sequence of the region between the *alaS* and *serV* genes on the *E. coli* K-12 genome is shown. Three bases are shown in boldface type in the *alaS* region to indicate discrepancies with the previously published sequence (33). The proposed ribosome-binding region (Shine-Dalgarno [S.D.]; 43) of *csrA*, the −10 and −35 regions of *serV* (17), and an inverted repeat sequence immediately downstream from *alaS* are underlined. The nucleotides 283 to 286 are complementary to nucleotides near the 5′ terminus of *E. coli* 16S RNA and fulfill the criteria described by Petersen et al. (29) for sequences which are expected to be found within the initiation codon-distal region of expressed genes. An arrow marks the site of the TR1-5 insertion mutation, which is between 421 and 422 bp. The *Dde*I sites used in subcloning *csrA* into pUC19 to generate pCSR10 are located at 168 and 698 bp.

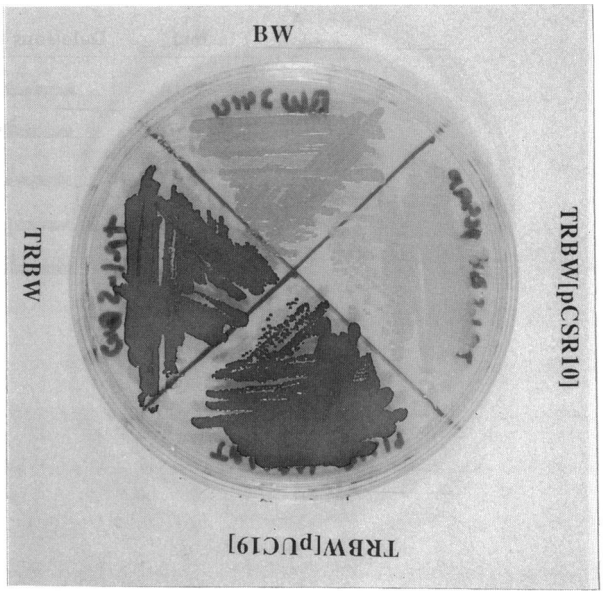


FIG. 11. pCSR10 strongly inhibits glycogen biosynthesis in vivo. Cultures were streaked onto Kornberg medium and incubated overnight at 37°C before staining with iodine. BW, BW3414; TRBW, TR1-5BW3414.

region was isolated from λ446, made blunt ended with the Klenow fragment, and subcloned into the *Sma*I site of pUC19 to generate pCSR10. Transformation of the glycogen-overproducing mutant TR1-5BW3414 with pCSR10 has a dramatic effect on glycogen accumulation (Fig. 11). A variety of *E. coli* strains have been transformed with the plasmid pCSR10, and in all cases the transformants have been found to be deficient in glycogen. These results suggest that pCSR10 contains the functional *csrA* gene.

**Complementation and in vitro expression studies of *csrA*: evidence that *csrA* encodes a 61-amino-acid polypeptide that inhibits glycogen biosynthesis.** Several deletion derivatives of pCSR10 were constructed and sequenced to allow analysis of the proposed *csrA* coding region (Fig. 12). A 57-bp deletion that is positioned 94 bp downstream from the coding region did not affect the ability of plasmid pCSR-L1 to complement the TR1-5 mutation. A deletion that removed 8 amino acids of the coding region and resulted in the addition of 24 heterologous amino acids had a slight effect on the ability of plasmid pCSR-D1-L to alter the glycogen synthesis phenotype. Finally, two deletions that remove 21 or 16 amino acids from the proposed *csrA* coding region severely disrupted the ability of plasmids pCSR-D3 and pCSR-D1-D, respectively, to complement the TR1-5 mutation. These analyses show that disruption of the 3′ end of the ORF that was proposed to encode *csrA* interferes with the effect of these plasmids on the glycogen phenotype. The observation that removing 8 amino acids of the coding region only partially inactivates *csrA* in a multicopy plasmid allows that a partially active product could also be produced from the TR1-5 mutant allele (an insertion at codon 51). In the latter case, the negative effect of the mutant gene product on glycogen synthesis may be minimal or negligible, since it should be expressed at a much lower level.

The proteins encoded by pUC19, pCSR10, and two of the deletion derivatives that alter the proposed coding region of *csrA* were analyzed in vitro in S-30-coupled transcription translation reactions (Fig. 12C). pCSR10 encoded only a single polypeptide that was not observed in the reaction with pUC19 as a template (Fig. 12C and data not shown). This polypeptide was strongly expressed in vitro and exhibited a mobility on SDS-polyacrylamide gel electrophoresis that was consistent with the molecular mass of the proposed *csrA* gene product, 6.8 kDa. One of the deletions (present in pCSR-D1-L) resulted in a net change of +16 amino acids in the coding region, and the second (pCSR-D1-D) resulted in a net change of −12 amino acids. The pUC19-independent (insert-specific) proteins that were expressed from these deletion derivatives were detected in significantly lower levels than the insert-specific protein expressed from pCSR10, making interpretations about these deletions less than definitive. However, insert-specific proteins consistent with an increase (pCSR-D1-L) or a decrease (pCSR-D1-D) in gene product size, predicted by ORF analysis, were weakly detectable, suggesting that the observed 183-bp ORF encodes the native *csrA* gene product. The poor expression of the two deletion derivatives could be due to effects of the deletions on transcript or protein stability. Alternatively, one or more regulatory sites for *csrA* expression may be located distal to the *csrA*-coding region.

Taken together, these experiments provided both structural and functional evidence indicating that the proposed 183-bp ORF represents the *csrA* coding region and that the regulatory effects of *csrA* on glycogen biosynthesis are mediated via its 61-amino-acid gene product.

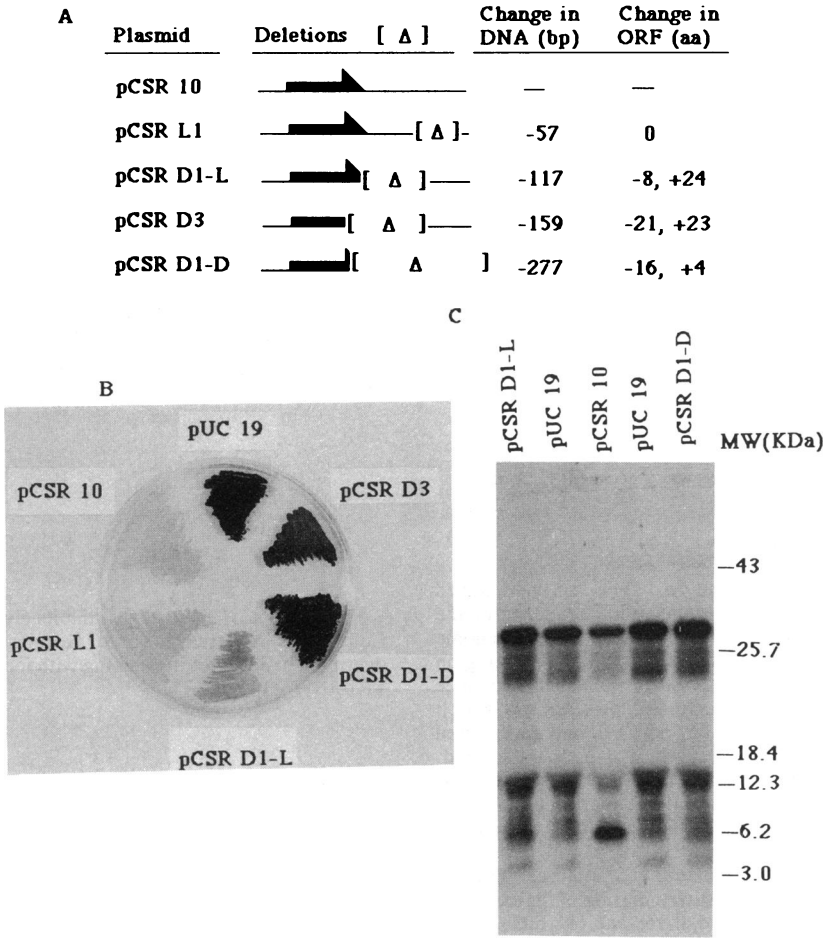


FIG. 12. In vitro expression and complementation analysis of *csrA*: evidence that *csrA* encodes a 61-amino-acid polypeptide that inhibits glycogen biosynthesis. A series of deletions from the plasmid pCSR10 was constructed and characterized by nucleotide sequencing. The extent of each of the deletions with respect to the nucleotide sequence shown in Fig. 10 is as follows: pCSR-L1, 549 to 604; pCSR-D1-L, 431 to 547; pCSR-D3, 389 to 547; and pCSR-D1-D, from 405 through the *Kpn*I site of the pUC19 vector. (A) The extent of each deletion, relative to the proposed ORF for *csrA* (indicated by an arrow), is shown schematically. The effect of each deletion on the size of the ORF is also shown. (B) pCSR10, pUC19, and the deletion derivatives were transformed into TR1-5BW3414 (*csrA*::*kanR*) and were tested for endogenous glycogen by iodine staining. (C) Analysis of proteins synthesized in S-30-coupled transcription translation reactions with pUC19, pCSR10, pCSR-D1-L, and pCSR-D1-D as genetic templates. Protein standards utilized as molecular mass markers were ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), cytochrome *c* (12.3 kDa), bovine trypsin inhibitor (6.2 kDa), and insulin (A and B chains [3 kDa]). aa, amino acids.

**Effects of *csrA* on cultures under varied growth conditions: anaerobiosis and sole carbon sources.** The TR1-5 mutant, isogenic *csrA*$^+$ (BW3414), and pCSR10-containing strains were grown on solid media under a variety of conditions and were stained with iodine vapor to observe the effect of the *csrA* gene on glycogen biosynthesis. Strains grown anaerobically on Kornberg medium revealed iodine-staining properties that were at least as distinct as (or probably more so than those of colonies grown aerobically, i.e., the mutant stained dark brown within seconds, whereas the wild-type and pCSR10-containing strains only stained yellow over 2 or more min of exposure to iodine vapor (data not shown). This suggested that *csrA*-mediated regulation of glycogen synthesis is important under both aerobic and anaerobic conditions.

Under aerobic conditions on a rich defined medium, supplemented MOPS medium, the TR1-5 mutation (in the BW3414 genetic background) resulted in enhanced iodine staining on glucose, fructose, and glycerol. Glycogen levels were negligible, as indicated by yellow staining with iodine, when the TR1-5 mutant and *csrA*$^+$ strains were grown on acetate or succinate (data not shown).

Surprisingly, the pCSR10-containing strain grew as pinpoint colonies on MOPS medium when sodium succinate was added as the major carbon source, although it grew well on all other carbon sources tested, including sodium acetate. The wild-type (BW3414), TR1-5 mutant, and pUC19-containing strains grew well on sodium succinate (data not shown). This suggests that overexpression of *csrA* results in a specific defect in the ability of cells to utilize succinate as a carbon source.

When wild-type, TR1-5, and pCSR10- or pUC19-containing strains (in the CF1648 genetic background) were streaked onto M9 minimal medium, it was found that the pCSR10-containing strain was unable to grow on any gluconeogenic substrates that were tested, including succinate, glycerol,

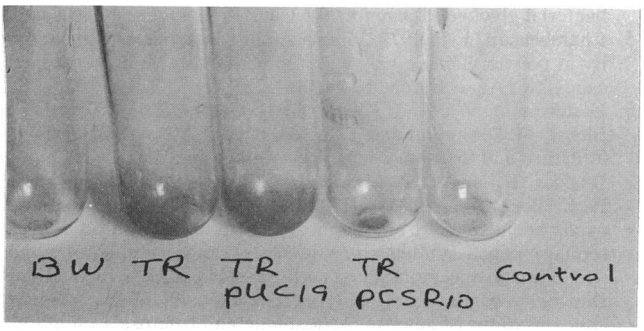


FIG. 13. *csrA* cells adhere tightly to borosilicate glass culture tubes. Strains BW3414 (BW), TR1-5BW3414 (TR), TR1-5BW3414 [pUC19] (TR pUC19), or TR1-5BW3414[pCSR10] (TR pCSR10) were grown overnight anaerobically, without shaking, in supplemented MOPS medium (49). Culture tubes were treated with Gram's safranin to stain adherent cells and were rinsed three times with deionized water. The control tube contained uninoculated medium.

pyruvate, and L-lactate, although it grew well on glucose and fructose, and although the wild-type and TR1-5 mutant strains grew on all of these substrates (data not shown).

**The *csrA* gene affects cell surface properties.** The TR1-5 mutant grown under anaerobic conditions in liquid MOPS medium was found to be strongly adherent to glass culture tubes (Fig. 13). The isogenic wild-type parent was nonadherent, and the adherent phenotype of TR1-5 was abolished by pCSR10, confirming that the *csrA* mutation caused this phenotype.

## DISCUSSION

This paper describes the identification, molecular cloning, and characterization of the gene *csrA*, which exhibits pleiotropic effects on gene expression and on some general microbiological properties of *E. coli*. Evidence indicates that *csrA* encodes a 61-amino-acid polypeptide which can be expressed in a biologically active form from a multicopy plasmid. The *csrA* gene affects the expression of structural genes in both the glycogen biosynthesis and gluconeogenesis pathways (hence its designation, carbon storage regulator). An even wider physiological role for *csrA* than regulation of carbon storage is suggested by the present study, although the full scope of its effects remains undetermined. Our working hypothesis for the biological function of *csrA* is that it encodes a critical regulatory factor within an adaptive response pathway which is yet to be defined.

The enhancement in the rate of glycogen accumulation which occurs as cultures enter stationary phase is attributable to an increase in biosynthesis, since the enzymes for catabolism of glycogen are found at extremely low levels (for reviews, see references 18, 30, and 32). Evidence suggests that the glycogen synthesis pathway allows endogenous storage of available carbon and energy for survival under conditions of nutrient stress. Thus sequestered, glycogen can be metabolized over an extended time via intracellular catabolic enzymes. The regulation of glycogen synthesis involves a complex assemblage of factors which adjust the rate of synthesis according to the physiological status of the cell. These factors have been rigorously shown to affect glycogen synthesis on at least two levels, allosteric regulation of the committed step of the pathway, catalyzed by ADPglucose pyrophosphorylase, and genetic regulation of the expression of the structural genes *glgC*, *glgA*, and *glgB*, encoding ADPglucose pyrophosphorylase, glycogen synthase, and glycogen branching enzyme, respectively. Evidence for a third level of control is suggested by the isolation of a class of genes that enhance endogenous glycogen but do not affect *glgC* expression (37) and by the identification of *glgS*, a gene that is stimulatory for glycogen accumulation and is transcribed via the putative sigma factor *katF* or *rpoS* but whose biochemical mechanism remains unknown (13). The discovery of *glgS* partially accounts for the positive effect of *katF* on glycogen accumulation, since *katF* is not required for *glgCA* expression (13, 20). The present study provides evidence that glycogen synthesis is negatively controlled via the effects of the gene product of *csrA* (CsrA) on the expression of the operons *glgCAY(P)* and *glgBX*. A *csrA*::*kanR* transposon insertion increased the expression of *glgC* and *glgB*; however, it did not temporally alter the induction or change the shape of the induction curves for these genes, suggesting that *csrA* functions in addition to the factors that mediate the growth phase response.

The discovery of *csrA* identifies a third system for the regulation of glycogen biosynthesis via *glgCA* expression. It contrasts with the other two systems (cAMP-cAMP receptor protein and ppGpp) which are positive regulators of *glgCA* (34, 38) and of glycogen synthesis (4, 7, 8, 21, 22, 48) and which do not affect *glgB* expression. The physiological role provided by these systems may be to establish an intrinsic metabolic capacity for glycogen synthesis in response to nutritional status. The effects of other regulatory factors, such as the allosteric effectors fructose 1,6-bisphosphate and AMP, may be viewed as being superimposed upon this intrinsic metabolic capacity. It should be noted that three regulatory systems have been found not to be involved in *glgCA* expression, the nitrogen starvation system, mediated by NtrC and NtrA or $\sigma^{54}$ (32, 38); heat shock, mediated by $\sigma^{32}$ (32); and the *katF*-dependent system (13, 38a), implying that the effects of *csrA* are not mediated indirectly via these global systems.

Evidence for a negative factor that regulates glycogen synthesis was previously presented, on the basis of the properties of an *E. coli* B mutation, *glgQ* (for a review, see references 30 and 32). The fact that *glgQ* was identified in *E. coli* B has hampered its genetic analysis, and *glgQ* has been neither mapped nor cloned. However, we showed that *glgQ* regulates the transcription of *glgCA* and presented evidence suggesting that its effects were independent of cAMP-cAMP receptor protein and ppGpp (34, 38). The magnitude of the effects of *glgQ* and *csrA* on *glgC* expression are similar, and *csrA* and *glgQ* both affect the expression of *glgB*. Unlike *csrA*, the *glgQ* mutation does not result in an adherent phenotype, and a rigorous understanding of the relationship of *csrA* to *glgQ* awaits further studies.

Two kinds of evidence suggest that *csrA* is involved in the regulation of gluconeogenesis. The TR1-5 mutation caused twofold overexpression of a *pckA-lacZ* transcriptional fusion. The *csrA* mutation did not alter the shape of the *pckA-lacZ* induction curve, a response that was similar to that of the *glg* genes. The plasmid pCSR10, which overexpresses the *csrA* gene product, as indicated by in vitro expression studies and by its strong inhibitory effect on glycogen synthesis, prevented growth on gluconeogenic substrates in M9 minimal medium. Since *pckA* was overexpressed in the TR1-5 mutant, the overexpression of *csrA* in cells containing pCSR10 could be predicted to cause excessive repression of *pckA* and possibly other gluconeogenic genes, resulting in the failure of pCSR10-containing cells to grow with gluconeogenic substrates on M9 medium. The

poor growth of pCSR10-containing cells specifically on succinate as a major carbon source in MOPS-supplemented medium suggests that *csrA* may affect succinate uptake. These hypotheses will be tested in future studies.

It was surprising to find that *csrA* affects cell surface properties. Glycogen is an endogenous polymer, and the effect of *csrA* on adherence is not due to its effect on glycogen synthesis, since transduction of the TR1-5 allele into the glycogen-deficient strain G6MD3 also caused this strain to become adherent (data not shown). However, the surface molecule(s) that promotes adherence in the TR1-5 mutant remains undefined. The adherence to and colonization of surfaces by bacteria with the formation of a biofilm, as observed for TR1-5, is affected by nutrient availability and other environmental conditions and is an alternative strategy for cell survival (for reviews, see references 6 and 24). The physiology of cells within a biofilm is different from that of suspended or pelagic cells, but the physiological significance of the effect of *csrA* on adherence awaits further study.

The failure to identify homologous genes, proteins, or procaryotic sequence motifs via computer-assisted research presents the intriguing possibility that CsrA is a member of a new class of genetic regulatory factors. The limited regions of *glgC* and *glgB* DNAs that were used in the construction of the *'lacZ* translational fusions in this study should contain *cis*-acting sites for CsrA-mediated regulation of *glgC* and *glgB*. Preliminary S1 nuclease mapping experiments suggest that *csrA* affects *glgC* transcription, and experiments are in progress to rigorously characterize the effects of *csrA* on transcripts from both *glgC* and *glgB* and to identify the required *cis*-acting regions. We hope to eventually elucidate the mechanism of genetic regulation via this system. The environmental stimulus for and physiological role of *csrA* in the control of glycogen biosynthesis also remain as future chapters to be written in this interestingly complex story.

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