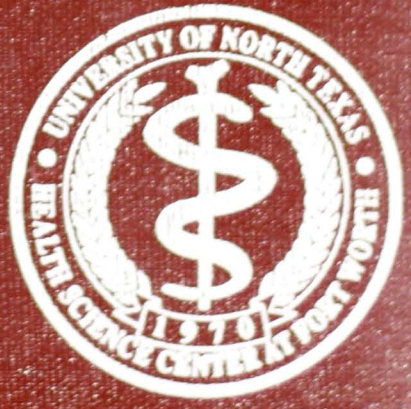
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CsrA, carbon storage regulator, is a small RNA-binding protein that acts as a global regulator and modulates specific mRNA stability in *Escherichia coli*. CsrA regulates central carbon metabolism in addition to flagella biogenesis. In this study, the phylogenetic distribution of *csrA* and its role in *Escherichia coli* biofilm development were examined. CsrA homologs were examined using Southern hybridization experiment and by analyzing existing sequencing data and was found to be widespread among eubacteria. CsrA was shown to be capable of acting as a genetic switch for biofilm formation and dispersal. A *csrA* mutant of *E. coli* was shown to increase biofilm formation and exhibit apparent pillars and channels characteristic of a mature biofilm. Over-expression of *csrA* completely inhibited biofilm formation in *E. coli* K-12 and decreased biofilm formation in related enteric pathogens. Induction of *csrA* expression from a multicopy plasmid caused dispersal of a pre-formed biofilm. Gene expression studies revealed that *csrA* expression is dynamically regulated during biofilm formation.

Several outer-membrane factors and global regulators that have been implicated in biofilm formation were examined for effects on biofilm formation in a *csrA* mutant.

Crystal violet adherence assays revealed that flagella and type I pili affect biofilm formation in a *csrA* mutant strain; however, colanic acid and curli fimbriae did not exhibit quantitative effects on biofilm formation in the mutant. OmpR, a global regulator of outer-membrane proteins, had modest effect on biofilm formation in the *csrA* mutant, but the stationary phase sigma factor, RpoS, had no quantitative effect on *csrA* mutant biofilm formation. Therefore, a *csrA* mutant will form a biofilm in the absence of each of these outer-membrane factors and global regulatory factors of biofilm formation. The effects of *csrA* on biofilm formation were found to be mediated in part through its effects on intracellular glycogen metabolism. Thus the redirection of carbon flux, in response to environmental and/or physiological cues, is important for biofilm development.

THE EFFECT OF CsrA ON BIOFILM DEVELOPMENT IN *Escherichia coli*

Debra White Jackson, B.S., M.S.

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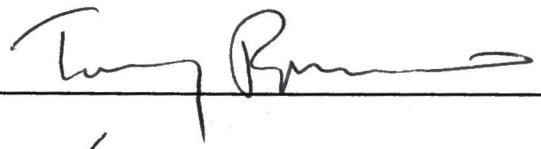

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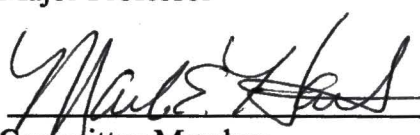

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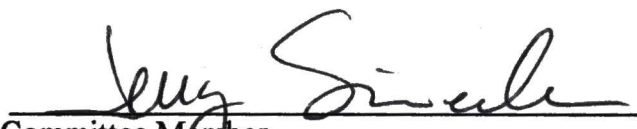

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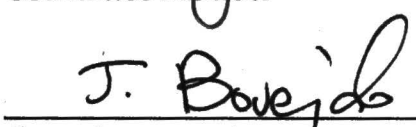

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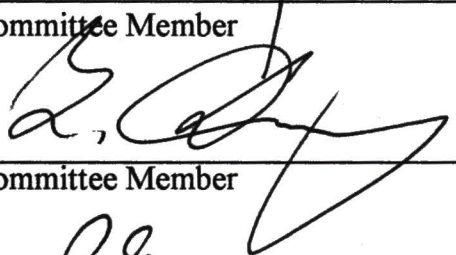

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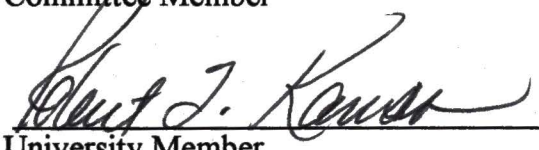

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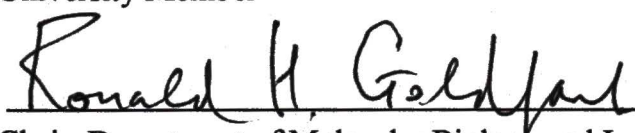

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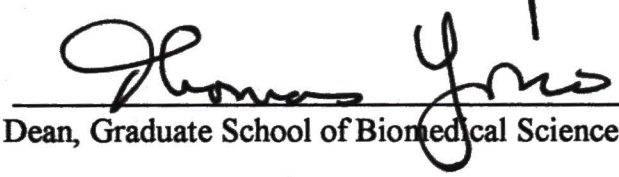

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THE EFFECT OF CsrA ON BIOFILM DEVELOPMENT IN *Escherichia coli*

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Debra White Jackson, B.S., M.S.

Fort Worth, Texas

May, 2001

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INTRODUCTION

Rationale and Specific Aims

Bacteria are very adept at surviving feast or famine conditions found in their natural environment. There are numerous bacterial characteristics that facilitate their adaptation under differing environments. When *E. coli* are faced with limited nutrient availability, they become resistant to environmental stresses such as heat shock, oxidative stress, UV and osmotic stress. This condition may also tend to alter morphology, mutability, storage capacity for nutrients, and virulence. In the laboratory, this physiological state is characterized as the stationary phase of growth. Previous studies in our laboratory identified and characterized a global regulator of *E. coli csrA* (carbon storage regulator A), which controls carbon metabolism by acting as an activator of glycolysis, and a repressor of gluconeogenesis and glycogen metabolism. CsrA was also shown to affect cell size, and surface properties in *Escherichia coli.*

CsrA is an RNA-binding protein that modulates specific mRNA stability. Homologs of *csrA* were also found in the plant pathogen *Erwinia carotovora*, *rsmA* (repressor of secondary metabolites) and in the human pathogen *Salmonella typhimurium*. RsmA represses virulence factors that cause soft-rot disease. Since *csrA* controls numerous physiological and metabolic systems in *E. coli* and virulence factors in a number of plant and animal pathogens, the phylogenetic distribution of *csrA* among eubacteria was more thoroughly examined – Aim 1.

In a previous study, it was noticed that a *csrA* mutant caused *E. coli* strains to adhere to borosilicate glass tubes suggesting that the mutant can form a biofilm. Thus, the role of *csrA* in biofilm development was examined – Aim 2. Biofilms contain cells in an extracellular matrix and exist in agricultural, industrial, and medical settings. Biofilms are the cause of many chronic and hard to treat infections and the National Institute of Health has estimated that over 60% of bacterial infections are caused by biofilms. Knowledge of factors that regulate biofilm formation and factors that ultimately cause biofilm dispersal is needed for the development of novel antimicrobial therapies.

Thus the specific aims of this project are:

1.) To assess the phylogenetic distribution of *csrA*.

2.) To examine the effects of *csrA* on *Escherichia coli* biofilm development:

a) Both biofilm formation and dispersal

b) In the presence/ absence of outer-membrane adhesion factors

c) In the presence/ absence of regulatory factors

d) In strains capable or incapable of glycogen metabolism

Background and Significance

Phylogeny of Proteobacteria. Bacteria represent a very heterogeneous group of organisms. Bacteria have been classified in two distinct kingdoms archaebacteria ("primitive") and eubacteria ("true"); however eubacteria are the most extensively studied because members of this group frequently cause disease in plants and animals (Nester, 1983). Members of the eubacteria are currently viewed as consisting of: Thermotogales, Flavobacteria, Cyanobacteria, Green non-sulfur bacteria, Firmicutes (gram-positive bacteria), other (Spirochetes, Chlamydia, Cytophaga, and Aquifex), and Proteobacteria (purple) (reviewed in Gupta, 2000). These bacteria have been classified based on sequence homology of their 16S rRNA and there are over 500 species (Woese, 1983). Recently, the Proteobacteria have been classified based on conserved signature

sequences that are unique to various Proteobacterial species or well conserved by only certain members from certain subdivisions of Proteobacteria (review Gupta, 2000).

The Proteobacteria comprise more than 200 genera as well as the majority of the known Gram negative organisms. These organisms were previously known as "purple" bacteria because photosynthesis is dependent upon the production of purple pigments in a few of the organisms in this group (review Gupta, 2000). Within the Proteobacteria, organisms are divided into five subdivisions: α, β, γ, δ, and ε as depicted in Fig. 1. The γ-Proteobacteria bacteria are the most evolutionarily advanced based on signature sequences, and include the enteric bacteria e.g. *Escherichia coli*. Research on the Proteobacteria has provided great insight into the origin of mitochondria and eukaryotic cells (review Gupta, 2000).

Biofilm History. Much of what microbiologists know about microbial physiology has come through the study of free-swimming bacteria in pure cultures. Although these studies have led to important findings that have advanced our understanding of basic microbial physiology (reviewed Siegle, 1992), bacteria rarely exist in the natural

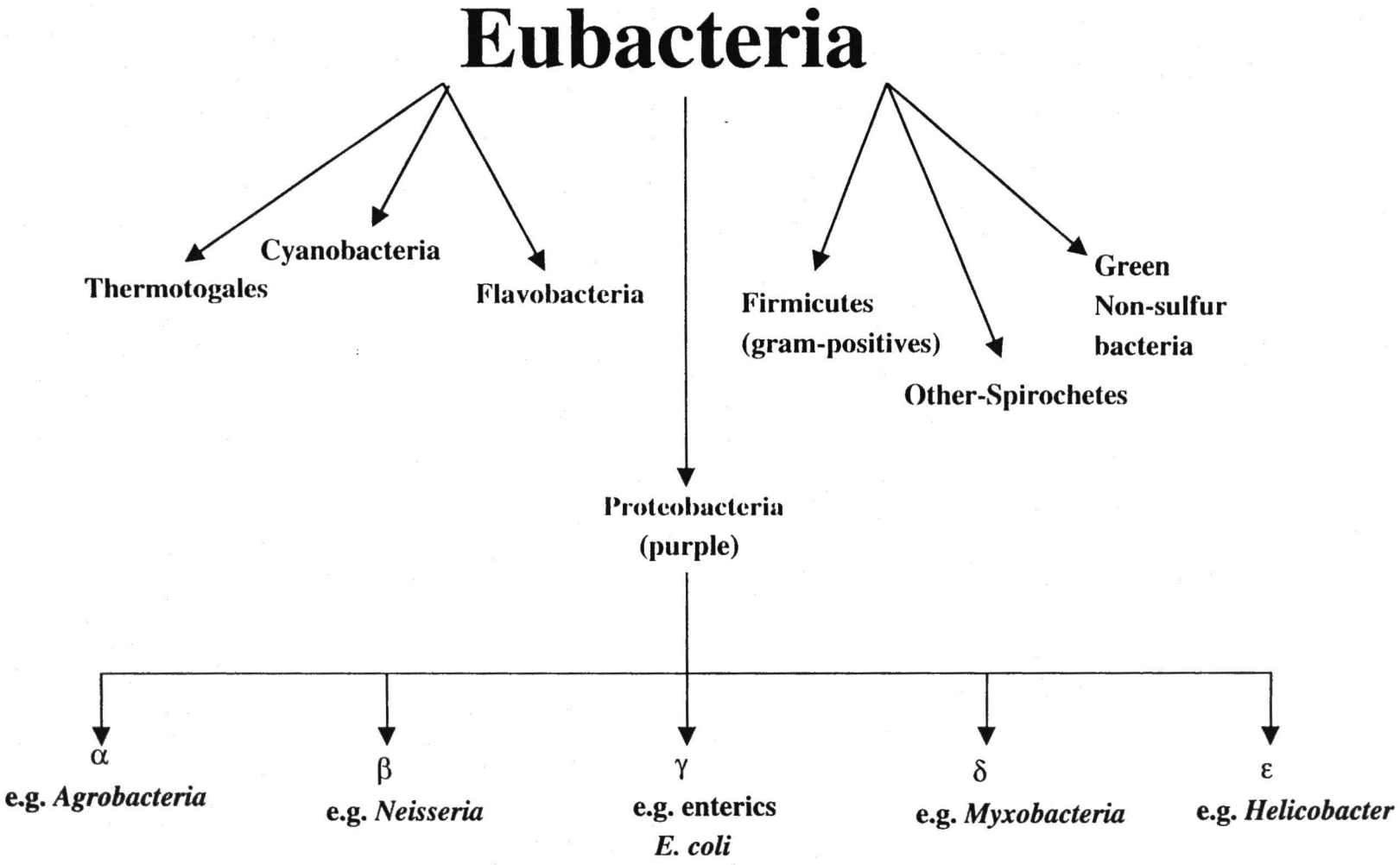


Fig. 1. Phylogenetic distribution of eubacteria and the subdivisions of the Proteobacteria (adapted from Gupta, 2000).

environment as free-swimming cells (Costerton *et al.*, 1995). The major mode of growth of bacteria is within biofilms (reviewed Costerton, 1995). Analyses of biofilms have revealed their presence in environmental, industrial, and medical settings (Pratt, 1999). Antoni van Leeuwenhoek in the 17th century scraped "animaculi", which were various forms of bacteria, from his teeth and observed them in the first primitive microscope. This is believed to be the first record of a biofilm (Slavkin, 1997). Prior to 1978, biofilms had been described in aquatic systems (Marshall *et al.* 1971). However, the term biofilm was not coined until 1978 (Costerton, 1978). Biofilms have been defined as a matrix-enclosed microbial population adherent to each other and/or to surfaces (Costerton *et al.*, 1995). Scientist then began extensive physical and chemical analysis of biofilms (Costerton *et al.*, 1995). In the mid-1980s the transmission electron microscope allowed scientists to view the presence of biofilms in the lungs of cystic fibrosis patients (reviewed in Potera, 1999). Presently, the study of biofilms focuses on the genes that are involved in biofilm development (Pratt, 1999).

Biofilm Structure. In the basic biofilm model, biofilm formation starts with the interaction between bacteria and surfaces (Costerton *et al.*, 1995). Gram negative bacteria are thought to initiate biofilm formation by using flagella and/or type IV pili to

overcome repulsive forces between the bacteria and the surface (O'Toole, 1998; Pratt, 1998). Once the bacteria have reached the surface, they use specific outer-membrane adhesins, such as antigen 43, type I pili, curli fimbriae, type IV pili, and mannose sensitive hemagglutinin pilus (MSHA) for stable attachment (Pratt, 1998; O'Toole, 1998; Watnick, 1999). Finally, in a mature biofilm, there is production of exopolysaccharide that stabilizes the three-dimensional structure of the biofilm as shown in Figure 2 (Boyd, 1995; Danese, 2000). Intracellular communication has also been shown to be important for biofilm formation (Davies *et al.*, 1998). The capacity to behave collectively is a necessity for biofilm growth (Davies *et al.*, 1998). Intracellular communication or quorum sensing is based on the accumulation of molecules collectively called autoinducers. Using autoinducers, bacteria are able to regulate their behavior according to population density. When in high enough concentrations, autoinducers bind to and activate a transcriptional regulator that induces expression of target genes (reviewed in Bassler, 1999). Quorum sensing signals have been found in aquatic biofilms on submerged stones, in biofilms on urethral catheters, and in cystic fibrosis patients (Greenberg, 2000; McLean,1997; Stickler,1998).

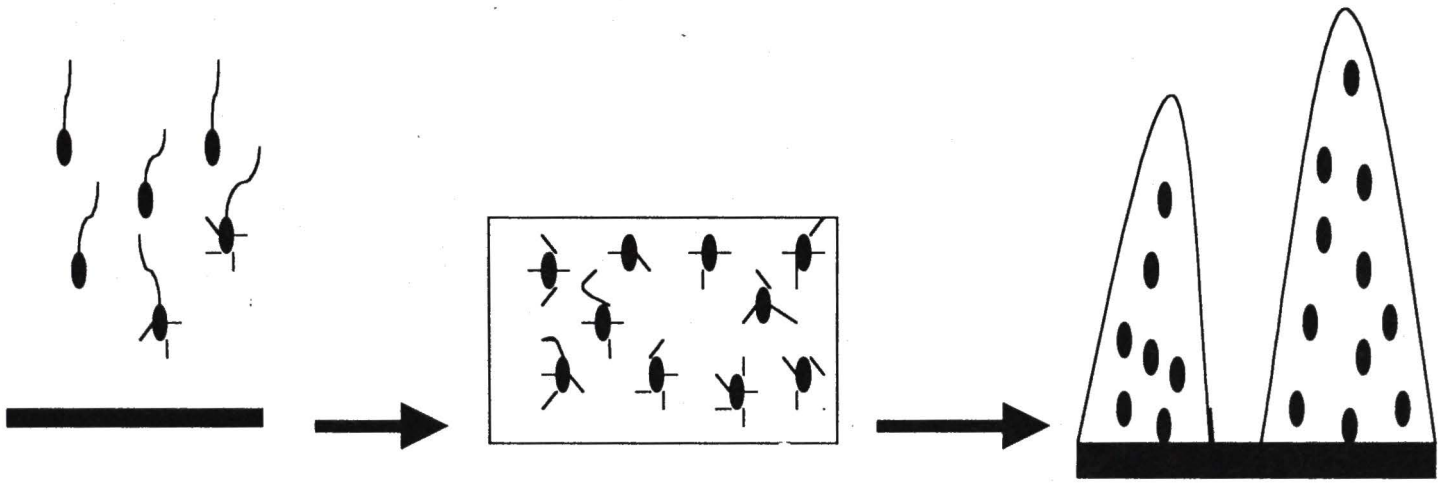


Fig.2. Basic biofilm model for Gram-negative bacteria (adapted from Pratt, 1998 and O'Toole, 1998).

Ubiquity of bacterial biofilms. In the industrial setting, bacterial biofilms are involved in the production and degradation of organic matter, degradation of environmental pollutants, and the cycling of nitrogen, sulfur, and some metals (reviewed in O'Toole, 2001). Studies have shown that biofilms are involved in the removal of sewage in ground water contamination (Massol-Deya, 1995). Biofilms also form in extreme environments such as acid mine drainage where they contribute to the cycling of sulfur (Edwards, 2000). Biofilm formation can cause flow modification in linear systems and plugs in porous media (Bryers, 1981). In waste water treatment, biofilms spread to large diameters and block conduits (Costerton, 1995). In the natural environment, cyanobacterial biofilms have been studied in thermal springs (Ramsing, 2000 and Ward, 1998) and these biofilms have been found to conduct a variety of biological processes including photosynthesis, nitrogen fixation, and fermentation.

Biofilms can have both deleterious effects and they can also be beneficial. Soil is a nutrient poor environment (Williman, 1985), where soil organic matter varies from 0.8 to 2.0%. The rhizosphere (root surface and the region immediately surrounding a root) is an ecological niche where nutrients are more readily available. Certain bacteria have developed mechanisms to take advantage of this niche. Rhizodeposition (release of organic material from the roots as they grow through the soil) enhances microbial growth

and drives the structuring of the microbial biofilms in the rhizosphere (Cambell, 1990). The rhizodeposition consists of : (a) amino acids, simple sugars and organic acids that are passively released from the roots, (b) actively released carbohydrates and enzymes, (c) sloughed off cells and cell lysates, and (d) carbon dioxide and ethylene (Weller, 1994). Numerous bacteria are attracted to the rhizosphere and colonize this location (Weller, 1994). Biofilms that form on roots may attach by the use of outer-membrane proteins, polysaccharides, lipopolysaccharides (LPS), and/or cell surface aggulutinins (Michiels 1991). Exopolysaccharides are produced by the bacteria in the rhizosphere, and this not only provides protection for the biofilm but also enhances soil aggregation that stabilizes water a process which is critical to the survival of the plant (Amellal, 1998).

In the medical setting, biofilms develop preferentially on dead tissue or bone, and medical devices (Lambe, *et al.* 1991). Clinical biofilm infections are marked by symptoms that typically reoccur even after repeated treatment with antibiotics. Bacterial biofilms on prosthetic valves are the leading cause of endocarditis in heart valve replacement patients (reviewed in O'Toole, 2001). The mortality rate of these patients is as high as 70% (Hyde 1998). Catheters (e.g. central lines, intravenous and urinary catheters) serve as potential surfaces for biofilm formation. Biofilms can also form on contact lenses and cause keratitis (McLaughlin-Borlace, *et al.* 1998). Up to 80% of all

nosocomial infections are due to biofilms according to the National Institute of Health. Biofilms grow slowly to produce overt symptoms (Ward, 1992). Sessile or adherent bacterial cells release antigens and stimulate production of antibodies, but the antibodies are not effective in killing bacteria within the biofilm and may cause immune complex damage to surrounding tissues (Cochrane,1988). Biofilm infections are rarely resolved by the host defense system (Khoury, 1992). Antibiotic therapy typically resolves symptoms caused by released planktonic cells but fail to kill the biofilm (Marrie, 1987). Bacterial cells within a biofilms can be up to 1500 times more resistant to antibiotics than planktonic or free-swimming cells (reviewed in Costerton, 1999). A consequence of the failure to kill cells within a biofilm is that biofilm infections show recurring symptoms after cycles of antibiotic therapy, often until the sessile population is surgically removed (Costerton *et al.*, 1995). Biofilms can also act as a focal point for the spread of acute infection if the host defenses can not eliminate planktonic cells (Read, 1989).

Ecological Advantage of a biofilm. Biofilms offer much in the way of protection for the bacteria. Bacteria have shelter and homeostasis when in a biofilm (O'Toole, 2001) due to the surrounding matrix composed of exopolysaccharide, protein, nucleic acids, and/or other substances. The exopolysaccharide matrix can also physically prevent access of

certain antimicrobial agents into the biofilm by acting as an ion exchanger, to restrict diffusion of compounds from the surrounding environment (Gilbert ,1997). The negatively charged extracellular polysaccharide (EPS) e.g. colanic acid or alginate matrix of certain bacteria particularly inhibits aminoglycoside antibiotics that are hydrophobic and positively charged (Nickel, 1989). EPS matrix has also been reported to sequester metals, cations, and toxins (Decho 1990, and Flemming 1993). Slow growth of bacteria within a biofilm may also lead to increased resistance to antibiotics (Brown 1999). This resistant phenotype might be induced by nutrient limitation, certain types of stress, high cell density or a combination of factors (Mah, 2001).

The biofilm is not a uniform structure, but exhibits water channels dispersed through the biofilm which allow exchange of nutrients and metabolites. This enhances nutrient availability as well as removal of toxic metabolites (Costerton *et al.*, 1995). The mushroom shaped colonies of biofilms provide a stable internal environment and promote metabolic cooperativity of bacteria within the biofilm (Costerton *et al.*, 1995).

The transfer of genes to acquire new genetic traits has undoubtedly played an important role for the evolution and genetic diversity in the microbial communities (reviewed in O'Toole, 2000). Because of the close proximity of cells within a biofilm, it is thought that conjugation may be the primary mechanism by which bacteria in biofilms

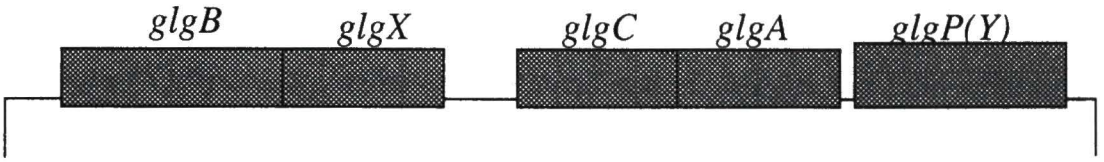
transfer genes within or between populations (LeBaron, 1997; Roberts, 1999). A recent study demonstrated that *Bacillus subtilis*, harboring a tetracycline resistant conjugative transposon could transfer that transposon to a *Streptococcus spp.* within a biofilm (Roberts *et al*, 1999). It is commonly believed that the transfer of genes between microorganisms within a biofilm may have produced the emergence of many multi-drug resistant bacteria, since conjugation can cross phylogenetic barriers (Davies, 1994).

Escherichia coli biofilm formation. Escherichia coli is a gram negative bacterium that can form á biofilm under a variety of nutrient conditions (Pratt, 1999). *E. coli* biofilms cause biliary tract infections. bacterial prostatitis, urinary catheter cystitis and may be found on endotracheal tubes, causing pneumonia in those patients (Costerton, 1999). *E. coli* initiates biofilm formation by the use of flagella to approach the surface (Pratt 98). In *E. coli* strains that are deficient for flagella, curli fimbriae are believed to be used to initiate biofilm attachment (Vidal. 1998). *E. coli* also uses type I pili and the outer membrane adhesin, antigen 43 to attach to an abiotic surface (Danese,2000; and Pratt, 1998). The exopolysaccharide colanic acid is required for normal development of mushroom shaped colonies in *E. coli* (Danese, 2000). Global regulatory genes such as *rpoS* (stationary phase sigma factor) and *ompR* (outer membrane protein) have been

proposed to play a role in *E. coli* biofilm formation (Adams, 1999; and Vidal, 1998). *E. coli* cells lacking *rpoS* have low cell density within the biofilm and other differences in biofilm structure (Adams, 1999). It has been suggested that *rpoS* has a role in biofilm resistance to oxidative biocides (Cochran, 2000). OmpR regulates the production of curli fimbriae that are used for initial biofilm attachment in *E. coli* (Vidal, 1998). At present, the genes involved in *E. coli* biofilm dispersal are unknown.

Csr System. Bacterial survival in the natural environment depends on bacteria evolving sophisticated mechanisms for sensing and responding to the natural environment (Romeo, 1998). In the laboratory, stationary phase probably best approximates the predominant physiological state in the environment (Siegle *et al.*, 1992). Upon entering stationary phase, bacteria become more resistant to environmental stresses such as heat shock, oxidative stress, UV, and osmotic stress (Siegle *et al.*, 1992). In addition, bacteria exhibit altered morphology and increased mutability (Siegle *et al.*, 1992). One of the ways in which bacteria regulate their responses is through global regulatory systems. Global regulatory genes control processes needed to respond to ever changing environmental and physiological demands. A novel global regulatory gene, *csrA* (carbon storage regulator), was discovered several years ago in our laboratory (Romeo *et al.*,

1993). CsrA is a 61 amino acid protein that has sequence homology to several other RNA-binding proteins (Liu *et al.* 1995). CsrA facilitates the decay of the *glgCAP* mRNA transcripts, that encode two glycogen biosynthetic enzymes, *glgC* and *glgA*, as well as the catabolic enzyme, *glgP*, by binding near the ribosome binding site (Liu and Romeo, 1997). During the transition from exponential to stationary phase, one of the metabolic pathways induced is the glycogen metabolism pathway (Table 1). The biological role of glycogen in microbial physiology has not been rigorously elucidated, but it has been postulated that glycogen provides an energy reserve during starvation conditions (reviewed by Preiss and Romeo 1989 and 1994). Glycogen synthesis is regulated by several global pathways and factors including catabolite repression - cAMP/CRP (cAMP Repressor Protein), stringent response-ppGpp (guanosine tetraphosphate) (Romeo Preiss 1989 and Romeo et al 1990), and RpoS, stationary phase sigma factor, (Hengge-Aronis and Fisher 1992, Yang *et al*, 1996). In a *csrA* mutant strain of *E. coli*, glycogen levels increased >20-fold. It has also been shown that *csrA* activates flagella biogenesis and controls the master operon *flhDC* (Wei *et al*, 2001). The molecular mechanism by which *csrA* activates *flhDC* is not precisely defined, but data show that it involves stabilization of the *flhDC* message (Wei *et al.*, 2001). Disruption of the *csrA* gene in *E. coli* has been



Structural Genes	Enzymes
Glycogen Biosynthesis	
glgC	ADP glucos pyrophosphorylase
glgA	Glycogen synthase
glgB	Glycogen branching enzyme
Glycogen Catabolsim	
glgP	Glycogen phosphorylase
glgX	Glycogen debranching enzyme

Table 1. The glycogen operon with the structural genes and enzymes required for glycogen metabolism.

shown to cause cells to adhere to borosilicate glass tubes; thereby, forming a biofilm (Romeo, 1993).

The second component of the Csr system is the noncoding RNA, CsrB. The transcript of CsrB is approximately 360 nucleotide in length and binds multiple CsrA protein subunits (Liu *et al.*, 1997). CsrB acts as an antagonist of CsrA *in vivo* (Liu *et al*, 1997). CsrB is a unique RNA in that it contains 18 imperfect repeats of 5'-CAGGA(U,C,A)-G-3' (Liu *et al.*, 1997). These repeats are found predominately in the loops of predicted hairpins and are closed by the 5'-C:G-3' base pair of the consensus sequence (Romeo, 1998). Theses haripin loops are distributed throughout the RNA molecule and in single-stranded segments between the hairpins (Liu, 1997). Homologs of CsrB have been found in *Erwinia carotovora* and *Salmonella typhimurium* (Cui, 1995; Altier, 2000). A proposed model for the interaction of the CsrA/CsrB system involves decay of the *glgCAP* transcript by the binding of CsrA (Liu *et al*, 1997). This may result from direct endonucleolytic attack upon the CsrA-mRNA complex. Alternatively, it may happen by the binding of CsrA to transcripts close to the ribosome binding site inhibiting translation and resulting in ribosome clearance and mRNA decay as depicted in Fig. 3 (Romeo, 1998).

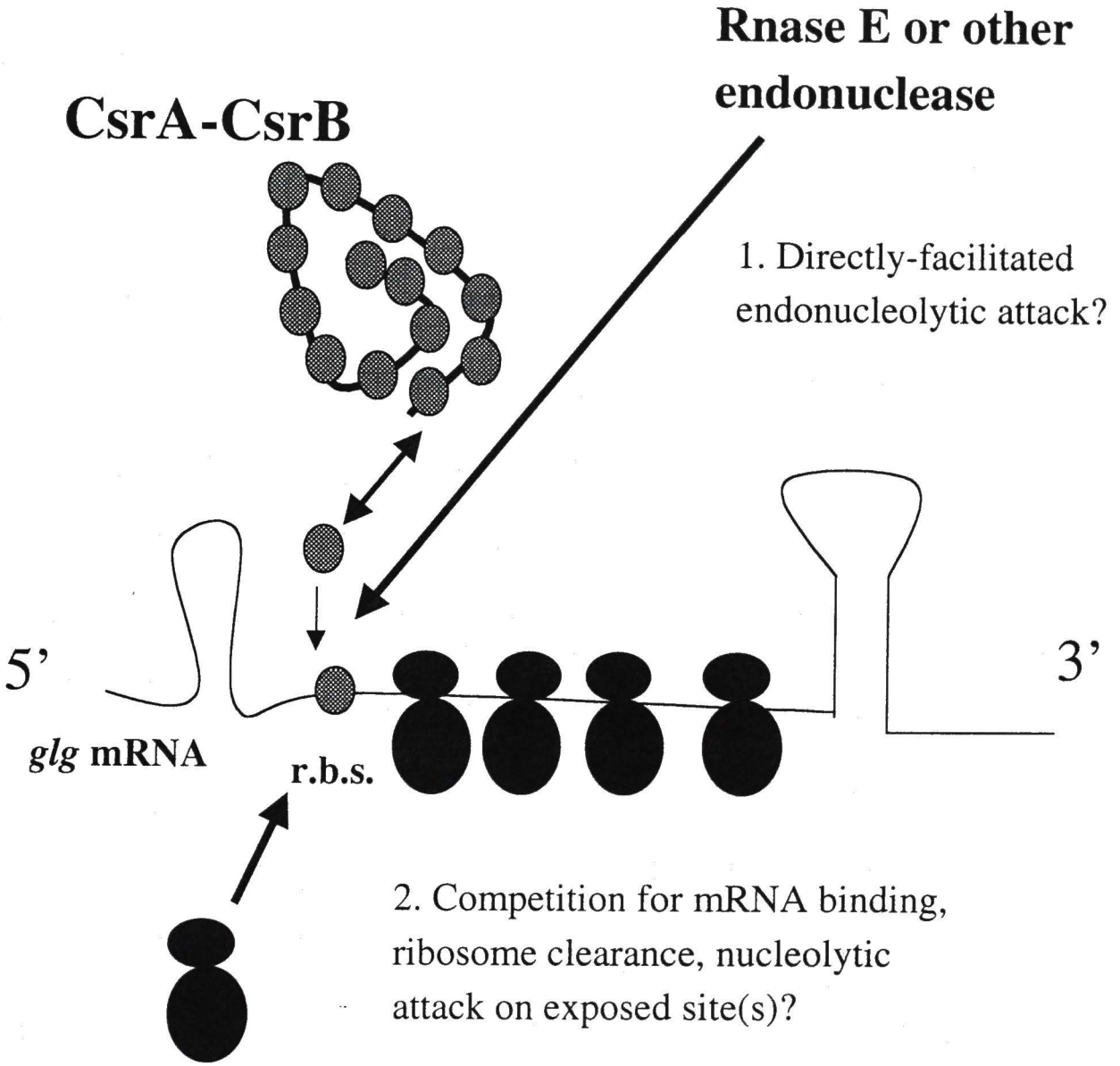


Fig. 3 A model for post-transcriptional regulation for gene expression by CsrA and CsrB (Adapted from Romeo, 1998).

Csr in Bacterial Virulence. The plant pathogen, *Erwinia carotovora* uses a CsrA homolog, RsmA (repressor of secondary metabolite), to repress motility and to regulate virulence factors that cause soft rot disease. RsmA represses production of extracellular enzymes (pectate lyase, cellulase and protease), tissue maceration, secondary metabolites, antibiotic activity, and pigment production in a number of *Erwinia spp.* (Cui, 1995; Chaterjee, 1995). Overexpression of *rsmA* was reported to repress the production of the quorum sensing factor N-(3-oxo hexanoyl)-L-homoserine lactone (HSL), but a mutation in *rsmA* did not significantly affect HSL production (Ciu, 95). Interestingly, *rsmA* represses $hrpN_{ECC}$, which induces the plant hypersensitive response (HR). This is a rapid local response by the plant to invasion by non-host bacteria (Mukherjee *et al.* 1996).

Pseudomonas fluorescens colonizes the roots of some plants and thereby protect them from fungal infections (Laville *et al.*, 1992)). The two component system *GacS/GacA* activate the production of extracellular protease (AprA) and secondary metabolites including hydrogen cyanide that protect the plant roots from fungal pathogens (Blumer *et al*, 1999). Overexpression of the *Pseudomonas rsmA* homolog was found to repress production of these extracellular factors (Blumer, 1999). RsmA functions to control a number of genes needed for successful colonization of some plants by *Pseudomonas fluorescens* (Blumer, 1999).

In the human pathogen *Salmonella typhimurium*, the *csrA* homolog regulates the genes *hilA, invF, sipC,* and *prgH* that are located on *Salmonella* pathogenicity island 1 (SPI1) and are needed for invasion of intestinal epithelial cells (Alteir et al, 2000). A mutation in *csrA* or overexpression of *csrA* renders *S. typhimurium* unable to efficiently invade cultured epithelial cells (Altier *et al,* 2000). This suggests that *csrA* levels must be rigorously maintained to permit bacterial invasion (Altier, 2000).

Significance. CsrA plays a central role in directing carbon flux and other physiological functions in *E. coli*. Homologs of *csrA* regulate virulence properties in other related species. This dissertation will establish the widespread occurrence of *csrA* as well as establish the role of *csrA* in biofilm development. Biofilms in the natural environment are usually composed of a multi-species consortium and there is a need to find common regulatory genes that govern biofilm development. Biofilms are considered the major mode of bacterial growth in the natural environment. Global regulatory genes such as *csrA* may offer a selective advantage in the natural environment. CsrA is the first known repressor of biofilm formation. Therefore, an understanding of the phylogenetic distribution of *csrA* among bacteria that can potentially form biofilms may help in the development of novel antimicrobial therapies. By finding genes that are common to a

multitude of species that can prevent biofilm formation or activate biofilm dispersal, mimics of this gene may be used in the treatment of biofilm infections.

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Chapter II

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PHLYOGENETIC DISTRIBUTION OF THE CARBON STORAGE REGULATOR GENE, *csrA*, AMONG EUBACTERIA

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Abstract

The gene *csrA* encodes a unique kind of global regulator, CsrA, which modulates glycolysis, gluconeogenesis, glycogen biosynthesis and glycogen catabolism in *Escherichia coli*. Southern hybridization and nucleotide sequencing data have revealed apparent *csrA* homologs within several families of the α and γ subdivisions of the proteobacteria (purple bacteria) and in the Gram-positive bacterium *Bacillus subtilis*. Thus, the CsrA regulatory system appears widely distributed among eubacteria.

KEY WORDS: chemiluminescent detection, nucleotide sequence, southern hybridization, purple bacteria, gram-positive bacteria, deduced amino acid sequence

The carbon storage regulator gene, *csrA*, encodes a 61 amino acid protein which negatively regulates glycogen biosynthesis, glycogen catabolism and gluconeogenesis, which are induced in the early stationary phase of growth, and is a positive regulator of glycolysis in *Escherichia coli* (Romeo *et al.*, 1993; Sabnis *et al.* 1995; Yang *et al.*, 1996). The *Erwinia caratovora* homolog of *csrA*, *rsmA*, represses the expression of several virulence factors involved in soft-rot disease of plants (Cui *et al.*, 1995), providing further evidence for an important role of *csrA* in the control of bacterial physiology and metabolism in these enteric species. The regulatory mechanism of the CsrA protein is unique, and has been shown to involve the destabilization of mRNA encoding the *E. coli* glycogen biosynthesis enzymes. Its deduced amino acid sequence contains a KH motif, which is characteristic of a diverse group of RNA-binding proteins (Liu *et al.*, 1995). The central role of CsrA in modulating carbon metabolism and virulence in these species prompted us to ask whether this regulatory system is present in other groups of bacteria.

Genomic DNA was collected from species representing four of the major branches of the domain Bacteria, the purple bacteria (proteobacteria), flavobacteria, cyanobacteria, and Gram-positive bacteria (Woese, 1987; Woese *et al.* 1990), and probed with a PCR-product consisting of the *E. coli csrA* coding region. Apparent *csrA* homologs were detected in *Agrobacterium tumefaciens* A348 [pTiA6NC] (from Eugene

Nester), *E. coli* B (laboratory strain), *Erwinia chrysanthemi* Ecp1 (from James F. Preston), *Vibrio vulnificus* O (from James F. Preston and Gary Rodrick), *Vibrio furnissii* ATCC 35016 (from James F. Preston), *Salmonella typhimurium* LT2 (laboratory strain), and *Pseudomonas aeruginosa* PAO1 (laboratory strain). These species are members of four different families of the α and γ purple bacteria (Fig.1). Genomic DNA from *Flavobacterium meningosepticum* (ATCC 13253), the Cynobacteria *Anabaena sp.* PCC7120 (from James Golden) and *Synechococcus sp* PCC7942 (from Susan Golden), *Zymomonas mobilis* CP4 (from Christine K. Eddy), *Pasteurella multocida* P-1591 (laboratory strain), *Staphylococcus auerus* 8325-4 (laboratory strain) and *Enterococcus faecalis* JH2-2 laboratory strain did not specifically hybridize to the probe (data not shown). DNA sequence data have further indicated that *csrA* homologs are present in *E. coli*, *Erwinia carotovora*, *Haemophilus influenzae* and in the Gram-positive species *Bacillus subtilis* (Fig. 2). Nucleotide sequences of the homologs from *E. coli* and *B. subtilis* are 51% identical (data not shown), indicating there has been sufficient divergence to prevent hybridization of the *B subtilis* homolog with the *E. coli* probe. Thus, only postive results in the hybridization experiments are meaningful for phylogenetic interpretations, and species which did not show hybridization may also contain *csrA* homologs. While these results collectively point to a possible universal

distribution of *csrA* among eubacteria, the genomic sequence of the degenerate species *Mycoplasma genitalium* did not contain a *csrA* homolog and also lacked virtually all other regulatory genes (Fraser *et. al.*, 1995). The rapidly accumulating data from the systematic sequencing of additional genomes promise to reveal a more complete picture of the pylogenetic distribution of this unique regulatory system in the near future.

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Fig. 1. Hybridization of the *Escherichia coli* K-12 *csrA* coding region to *Eco*RI-digested genomic DNA (7.5ug) from *Agrobacterium tumefaciens*, *Escherichia coli* B, *Erwinia carotovora*, *Vibrio vulnificus* O, *Vibrio furnissi*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* (lanes 1-7 respectively). Genomic DNA was digested to completion with *Eco*RI, separated by agarose gel electrophoresis (0.8%), blotted onto Magna Graph neutral nylon membrane (Micron Separations Inc. Westboro, MA, USA), and detected by Southern hybridization. A probe was synthesized by PCR- amplification of the *csrA* coding region from pCSR10 (Romeo *et al.* 1993) and labeled by random-primed DNA synthesis using the manufacturer's recommendation for digoxigenin UTP incorporation (Boehringer Mannheim, Indianapolis, IN, USA). Hybridization was conducted at 48°C under previously described conditions (Smeltzer *et al.* 1993). Stringent washes of the blot were carried out as follows: twice at room temperature in 2xSCC, twice at 48°C in 2xSSC-1% sodium dodecyl sulfate and twice at room temperature in 0.1 x SCC. Chemiluminescent detection of specific hybrids was accomplished using alkaline phosphatase-conjugated anti-digoxigenin antibody and the substrate Lumingen PPD (Boehringer Mannheim. Indianapolis, IN, USA).

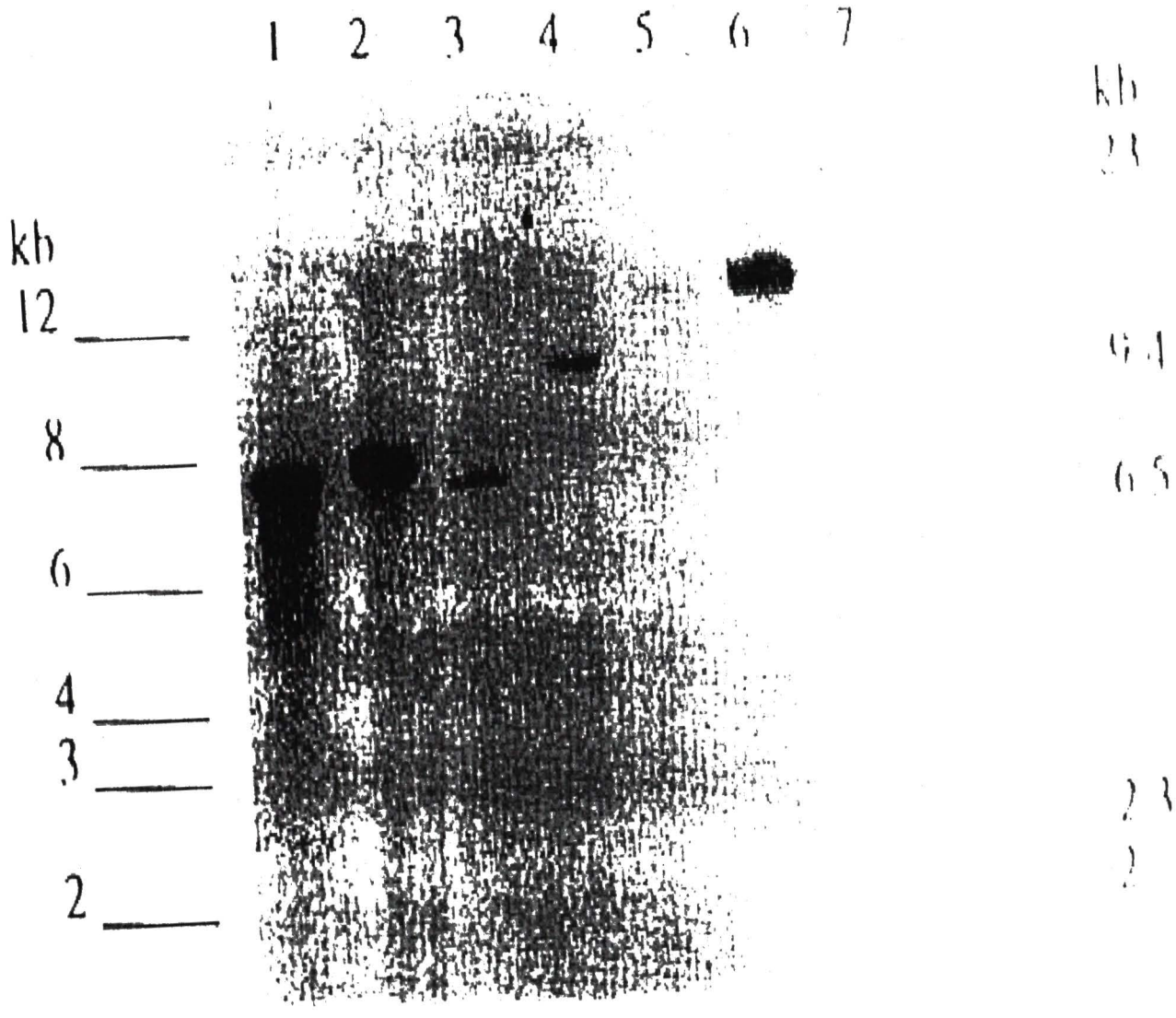
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Fig. 2. Alignment of the deduced amino acid sequences of apparent *csrA* homologs. *Escherichia coli* K-12, *E.c.*K (Romeo *et al.* 1993); *Erwinia carotovora*, *E.c.* (Cui *et. al.*, 1995); *Haemophilus influenzae*, *H.i.* (Fleischmann *et al.*, 1995); *Bacillus subtilis*, *B.s.* (DNA sequenced by Mirel and Chamberlin, 1989; identified by K. Robison, Swissprot accession no P33911), respectively. Astericks indicate residues identical to those of *E. coli* K-12.

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E.c K-12  MLILTRRVGETLMIGDEVTVTVLGVKGNQVRIGVNAPKEVSVHREEIYQRIQAEDSQQSSY
          ********** ********** ********** ********** ********** ** **
E. c.     MLILTRRVGETL I I GDEVTVTVLGVKGNQVRIGVNAPKEVSVHREEIYQRIQAEKSQPTSY
          * *** ** *** *** * *** * *** * ** ********** **********
H. i.     MLILTRKVGESVLI GDD IS I TVLSVRGNQVKLGVEAPKEVSVHREEIYQRIKQTKDEPYLGSS
          * * * * * * ** * * * * * ** * * ** ** ** *** ** *
B. s.  MLVLSRKINEAIQIGADIEVKVIAVEGDQVKLGIDAPKHIDIHRKEIYLTIQEENNRAAALSSDVISALSSQKK
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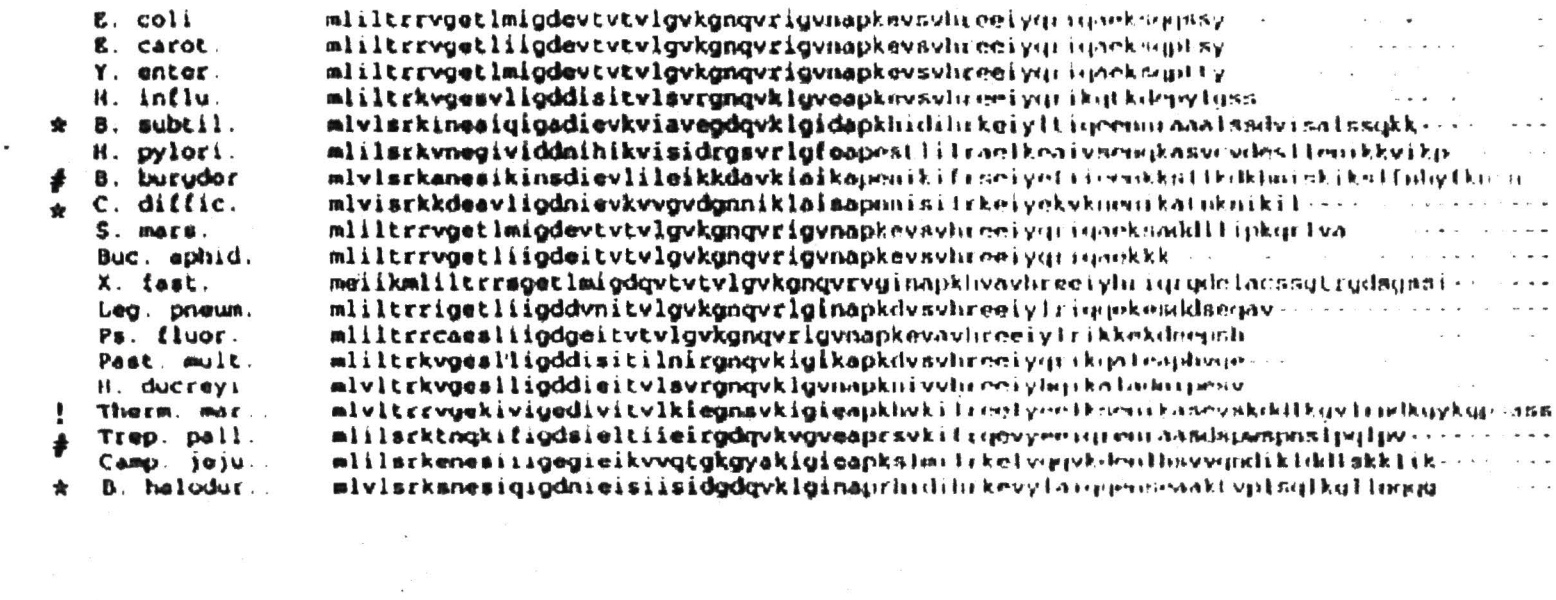
Summary of Chapter II

Since the time this paper was published, numerous genomic sequencing projects have revealed *csrA* homologs in other bacteria (Fig. 3). Many of the homologs of *csrA* were found in ε and γ Proteobacteria. It has been reported (reviewed in Gupta, 2000) that the Proteobacteria represent the most abundant division of the eubacteria, because they have evolved to exist in numerous environments. However, sequencing data revealed *csrA* homologs in additional gram-positives, Spirochaetes, and in *Thermotoga maritima.* Within the major subdivisions of eubacteria, *csrA* homologs have not been found in Cyanobacteria (*Synechococcus spp.*), α and β Proteobacteria (*Agrobacterium tumefaciens* and *Neisseria meningitidis* respectively), some gram positive bacteria (*Mycobacterium leprae*) and some other bacteria such as *Deinococcus radiodurans*, *Lactococcus lactis* (gram positive rod), and *Mycoplasma genitalium.*

It is interesting to note that in the Firmicutes (gram-positive bacteria) homologs of *csrA* have only been found, thus far, in bacilli and none in the cocci. Since the Firmicutes (gram-positives), in particular the cocci, have been hypothesized to be the closest to the eubacterial progenitor cell (reviewed Gupta, 2000), this suggests that *csrA* evolved in the Firmicutes (gram positives) early in the eubacterial lineage.

Lastly, to date no *csrA* homologs have been found in the archaebacteria or in eukaryotes. This would suggest that *csrA* is conserved only within the eubacteria. However, it remains possible that *csrA* homologs have diverged beyond detection in archaebacteria and eukaryotes.

Figure 3. Comparison of deduced amino acid sequences of apparent CsrA homologs. The homologs were aligned using MACAW; Schuler et al., 1991. Homologs were found in *Escherichia coli* (*E. coli*) [Romeo *et al.*, 1993]; *Erwinia carotovora* (*E. carot.*) [Cui *et al.*, 1995]; *Yersinia enterocolitica* (*Y. enter.*) [Romeo, 1996; originally sequenced by J. P. Throup, G.S.A.B. Stewart and P. Williams]; *Haemophilus influenzae* (*H. influ.*) [Fleischmann *et al.*, 1995]; *Bacillus subtilis* (*B. subtil.*) [DNA sequenced by Mirel and Chamberlin, 1989; gene identified in 1993 by K. Robison SWISSPROT accession no. P33911]; *Helicobacter pylori* (*H. pylori*) [Tomb *et al.*, 1997]; *Borrelia burgdorferi* (*B. burgdor.*) [Fraser *et al.*, 1997]; *Serratia marscens* (*S. mars.*) [Ang S. *et al.*, 2001]; *Treponema pallidum* (*Trep. pall.*) [Fraser *et al.*, 1998]; and *Campylobacter jejuni* (*Camp. jeju.*) [Parkhill *et al.*, 2000]. Several unpublished genomic sequences, available through NCBI (http://www/ncbi.nlm.nih.gov/) also revealed CsrA homologs in *Clostridium difficile* (*C. diffic.*), *Buchnera aphidicola* (*Buc. aphid.*), *Xylella fastidiosa* (*X. fast.*), *Pseudomonas fluorescens* (*Ps. fluor.*), *Pasteurella mutocida* (*Past. mult.*), *Haemophilus ducreyi* (*H. ducreyi*), *Thermotoga maritima* (*Therm. mar.*), *Legionella pneumophila* (*Leg. pneum.*), and *Bacillus halodurans* (*B. halodur.*).



Legend

* Firmicutes (gram-positives)
! Thermotogales
Spirochaetes

Those with no symbol are Proteobacteria

PREFACE TO CHAPTER III

The results of the previous investigation demonstrated the widespread distribution of *csrA* among many eubacterial plant and animal pathogens. It has been postulated that, in the natural environment, bacteria mostly exist in complex communities called biofilms. Many of the bacteria with *csrA* homologs have been shown to form biofilms. This study will investigate the role of CsrA in *Escherichia coli* biofilm formation and/or dispersal. The question of whether *csrA* influences biofilm formation through effects on known extracellular factors and regulators will be addressed. Finally the role of intracellular glycogen in biofilm formation will be addressed.

Chapter III

The following manuscript will be submitted to Journal of Bacteriology

Biofilm Formation and Dispersal Under the Influence of the Global Regulator CsrA in *Escherichia coli*

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Running title: Regulation of biofilm formation and dispersal

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Abstract

The predominant pattern of growth of bacteria in the environment is within sessile, matrix-enclosed communities known as biofilms. Biofilms complicate the majority of chronic and difficult to treat bacterial infections by protecting bacteria from the immune system, decreasing antibiotic efficacy, and dispersing planktonic cells to distant body sites. While the biology of bacterial biofilms has become a major focus of microbial research, the regulatory mechanisms of biofilm development remain poorly defined, and those of dispersal are unknown. Here, we establish the role of the RNA-binding global regulatory protein CsrA (carbon storage regulator) of *Escherichia coli* K-12, as both a repressor of biofilm formation and an activator of dispersal. CsrA also regulates biofilm formation in related enteric pathogens. A *csrA* mutant is capable of forming a biofilm in the absence of each previously reported surface/extracellular factors and regulators including type I pili, curli fimbriae, colanic acid, motility, OmpR, or RpoS. The effects of CsrA on biofilm formation are mediated largely through the regulation of intracellular glycogen biosynthesis and catabolism. We propose that the global redirection of carbon flux, in response to environmental and/or physiological cues, is important for biofilm development.

Introduction

Bacteria have evolved elaborate mechanisms for adhering to, and colonizing, solid surfaces, thereby establishing complex communities known as biofilms (11). It has become increasingly clear that biofilms represent a distinct lifestyle for bacteria, which provides protection from deleterious conditions. In medicine, biofilms complicate the majority of chronic, and difficult to treat, bacterial infections, including prostatitis, biliary tract infections, and urinary catheter cystitis caused by *Escherichia coli* (12). Thus, the biology of bacterial biofilms has become a major focus of microbial research.

Studies of the biophysical, structural, and chemical properties of biofilms have culminated in our present concept of the mature biofilm as a complex community exhibiting pillars and channels that may facilitate nutrient exchange and waste removal (11). A biofilm is initiated by the attachment of individual cells to a surface, followed by migration and replication to form microcolonies, which will eventually produce a mature biofilm (28,30). A variety of extracellular molecules and surface organelles participate in biofilm development. In *E. coli*, flagella and type I pili are needed for initiation of biofilm formation (30). Moreover, curli fimbriae are likewise involved in the attachment of *E. coli* strains to abiotic surfaces (41). In *Pseudomonas*, flagella and type IV pili have been implicated in biofilm formation (28). The extracellular polysaccharide

of *E. coli*, colanic acid is needed for the proper construction of the three-dimensional structure of *E. coli* biofilm (15). Similarly, the extracellular polyuronide alginate is essential for mature biofilm formation in *Pseudomonas aeruginosa* (30).

The regulatory mechanisms that guide biofilm development have also come under recent scrutiny. The social behavior known as quorum sensing, whereby the concentration of a diffusible autoinducer provides a regulatory signal in response to population density is essential for the construction of a mature biofilm in *P. aeruginosa* (17). The Crc protein (catabolite repression control), is also required, in part, because it activates the synthesis of Type IV pili (29). Intracellular polyphosphate has pleiotropic effects on a variety of extracellular and regulatory functions in *P. aeruginosa*, which ultimately play a significant role in biofilm formation (32,33). In *E. coli*, OmpR (outer membrane protein regulator) and σ^s (a sigma factor needed for the transcription of stationary phase genes) are apparently involved (1, 41). Nutritional cues, e.g. carbon and iron availability in *Pseudomonas fluorescens* and *P. aeruginosa*, respectively, also significantly affect biofilm formation (8,30). Nevertheless, the regulation of biofilm development is apparently highly complex, and remains poorly understood in any species.

The dispersal or shedding of planktonic cells from a biofilm may be essential to permit bacteria to escape the confines of the biofilm in order to colonize new locations (11). Direct microscopic observation has revealed that physiological and environmental signals influence the dispersal process (5). Biofilm dispersal in *P. aeruginosa* and *P. fluorescens* has been postulated to involve the degradation of alginate (2, 10). Furthermore, it has been suggested that dispersal of cells from a biofilm in *E. coli* may be cell-cycle mediated (3). Finally, the differential expression of chitinase genes among a subpopulation of marine bacteria may promote detachment of cells from biofilm (7). In summary, the regulatory factors and pathways that facilitate biofilm dispersal have not been defined in any species.

We previously observed, under a single growth condition, that a *csrA* disruption caused *E. coli* to adhere to culture tubes, forming a coating suggestive of a biofilm (35). The *csrA* gene encodes a global regulatory protein, carbon storage regulator (CsrA), which represses several metabolic pathways that are induced in the stationary phase of growth, including glycogen biosynthesis and catabolism and gluconeogenesis (38). Conversely, CsrA activates glycolysis, acetate metabolism, and motility (42,43,44). CsrA is an RNA binding protein that regulates gene expression post-transcriptionally, by binding to mRNA transcripts of regulated genes and either decreasing or increasing their

decay rates (25,42). Furthermore, CsrA binds in multiple copies to a noncoding RNA molecule, CsrB, which antagonizes its activity both in vivo and in vitro (24). A highly repeated sequence element found in the loop segments of the stem-loops of CsrB has been proposed to mediate this binding (24,34). Homologues of *csrA* are widely distributed among eubacteria, including numerous human pathogens, but are not apparent in eucaryotes (34). The *csrA* gene of *Salmonella typhimurium* represses genes that are involved in the invasion of mammalian gastrointestinal mucosa (4), and the *csrA* homolog, *rsmA*, of plant pathogenic *Erwinia* species represses the production of proteins involved in both plant disease and host response (13,14). The goal of present study was to investigate the regulatory role of *CsrA* in biofilm development.

MATERIALS AND METHODS

Bacterial Strains and Media. The strains, phage, and plasmids used in these experiments are listed in Table 1. Bacteria were routinely cultured at 37°C and were cultured for biofilm formation at 26°C. Mutant derivatives of the strains listed in Table 1 are isogenic except for the mutant alleles indicated in their strain names. Biofilm assays were typically carried out in CFA (colony forming antigen medium) [per liter; 10 g

casamino acids (Difco, Detroit, MI), 1.5 g yeast extract (Difco, Detroit, MI), 50 mg $MgSO_4$ and 5 mg $MnCl_2$, pH 7.4] (18) or in artificial urine medium [per liter; 0.65 g $CaCl_2$ H_20, 0.65 g $MgCl_2$ $6H_20$, 4.6 g $NaCl_2$, 2.3 g Na_2SO_4, 0.65 g sodium citrate, 0.20 g sodium oxalate, 2.8 g KH_2PO_4, 1.6 g KCl, 2.0 g NH_4Cl, 12 g urea, 1.1 g creatinine, 1% tryptic soy broth (BBL, Cockeyville, MD)] (27). Glycogen biosynthesis was assessed using Kornberg agar medium (per liter; 11 g K_2HPO_4, 8.5 g KH2PO_4, 6 g yeast extract, 15 g agar, and 10 g glucose, pH 6.8) (25). M63 salts solution was [per liter; 13.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.5 mg $FeSO_4$ $7H_20$, 1 ml of 1M $MgSO_4$, pH 7.0]. Antibiotics were used as required for biofilm studies at the following concentrations: ampicillin, 200 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 100 μg/ml; and tetracycline, 10 μg/ml. Ampicillin and kanamycin were used at 50 μg/ml and 40 μg/ml, respectively, during the construction of the *csrA'-'lacZ* fusion.

Quantitative Biofilm Assay. Overnight cultures were inoculated 1:100 in fresh medium. In the microtiter plate assay, inoculated cultures were grown in a 96-well polystyrene microtiter plate. Growth of planktonic cells was determined by absorbance at 600 nm or total protein assay. Biofilm was measured by discarding the medium, rinsing the wells with water (3X) and staining bound cells with crystal violet (BBL,

Cockeyville, MD) (28). The dye was solubilized with 33% acetic acid (EM Science, Gibbstown, NJ) and absorbance at 630 nm was determined using a microtiter plate reader (DynaTech, Chantilly, VA). All comparative analyses were conducted between strains that were incubated within the same microtiter plate to minimize variability. In order to confirm that effects on biofilm formation were not surface-specific, cultures were grown and tested simultaneously in new borosilicate glass test tubes (18 mm). Each experiment was performed at least in triplicate, and the data were analyzed by Tukey Multigroup Analysis (StatView-SAS Institute Inc., Cary, NC).

Molecular and Genetic Techniques. Transduction or cotransduction of resistance markers, subcloning, and molecular genetic techniques were performed by standard procedures, as previously described (26). The construction of a chromosomal *csrA'-'lacZ* translational fusion first involved the preparation of a plasmid containing an in frame *csrA'-'lacZ* fusion, pCAZ1, by subcloning a 0.4 Kb blunt ended *Eco*RI-*Bgl*II restriction fragment from pTR151P1 into the *Sma*I site of pMLB1034. This fusion was transferred to λInCh1, the recombinant phage was moved into the chromosome, and most of the λ DNA was eliminated to generate a stable chromosomal fusion, which was

confirmed by PCR, as previously described (9). The fusion was transduced into CF7789 for expression studies.

β-galactosidase, protein and glycogen assays. For β-galactosidase assays, static cultures were grown in borosilicate test tubes at 26°C and planktonic cells were separated from adherent cells. The biofilm was disrupted in CFA medium with pipetting to disrupt cell aggregates. β-galactosidase activity was determined as previously described (38). Cell protein was precipitated with ice cold 10% TCA and quantified using the bicinchoninic acid method with bovine serum albumin as the standard protein (40). Glycogen phenotypes were determined by staining colonies with iodine vapor (28).

Microscopy. Sterile microscope cover slips were aseptically placed into sterile petri dishes along with 10 ml of a freshly inoculated culture. The cover slips were removed at various times, gently rinsed with phosphate buffer (per liter 11 g K_2HPO_4 and 8.5 g KH_2PO_4, adjusted to pH 7.4 with NaOH), stained with acridine orange 30 μg/ml (K&K Laboratories, Inc., Plainville, NY), rinsed again, and mounted to the microscope slide with Immun-Mount (Shandon, Pittsburgh, PA).

A Nikon Microphot-FXA microscope with a 40X Plan Apo objective lens was used for Nomarski interference microscopy. All images were collected with a Roper Sensys cold-slow scan CCD camera (Roper Scientific Co., Phoeniz, AZ) and the imaging

program Image-Pro Lab software for MacIntosh (Scanalytics, Fairfax, VA). A Zeiss LSM 410 Confocal Microscope with a 40X 1.2 N.A. C-Apochromat objective lens was used to generate a topographical image and cross-sections through the *csrA* mutant biofilm. Optical sections were collected at 2 μm steps through a sample depth of 20 μm using 488 nm and 510-525 nm excitation and emission wave lengths, respectively.

Results

A *csrA* mutant forms a prolific biofilm. The previous report of *csrA* mutant forming a biofilm was based upon a qualitative observation, conducted under a single growth condition (35). To assess more thoroughly the role of CsrA in biofilm development, a quantitative assay was used to monitor biofilm formation over time. The wild type *E. coli* strain MG1655 and its isogenic *csrA* mutant exhibited similar growth in liquid CFA medium (Fig. 1A). In each strain, biofilm development was somewhat delayed until the stationary phase of growth, and thereafter biofilm accumulated much more rapidly and extensively in the *csrA* mutant. Nomarski interference microscopy showed that biofilm formation was accelerated in the *csrA* mutant (Fig. 1B). In order to determine whether the *csrA* mutant formed a mature biofilm containing the characteristic pillars and channels, scanning confocal laser microscopy was utilized. From a

topographical image, it was determined that the biofilm generated by the *csrA* mutant was 20 μm thick. Furthermore, a cross section of this biofilm revealed pillars and channels that are characteristic of a mature biofilm (11) (Fig. 1C). The strong stimulatory effect of a *csrA* mutation on biofilm has been observed in every growth medium that has been examined, including MOPS (28), CFA +/- 0.2% glucose or glycerol, Kornberg with 1% glucose, LB with 0.2% glucose, or M63 medium with 0.2% glucose (data not shown). Biofilm formation also occurs under anaerobic conditions (35).

Overexpression of *csrA* represses biofilm formation. Since a disruption in *csrA* increases biofilm formation, the effects of *csrA* overexpression on biofilm formation were examined. Overexpression of *csrA* from a plasmid clone totally inhibited biofilm formation in the wild type *E. coli* strain while the plasmid vector exhibited little to no inhibitory effects (Fig 2A). When overexpressing *csrA* from a plasmid, biofilm formation in a *csrA* mutant strain is inhibited and the plasmid vector had no effect as well (Fig. 2A). Because biofilms are ubiquitous in nature, we examined whether *csrA* could inhibit biofilm formation in bacterial pathogens as well as a laboratory strain of *E. coli*. Urinary tract cystitis is a major health problem for over 100,000 people each year use indwelling catheters (21). Two clinical strains that were isolated from colonized urinary catheters, *E. coli* P18 and *Citrobacter freundii* P5, were tested for biofilm formation in

artificial urine medium to mimic their host environment (Fig.2B). Similar to the previous data, overexpressing *csrA* in these strains was still inhibitory to biofilm formation. Biofilm formation was modestly inhibited in the food-borne pathogens *E. coli* 0157:H7 strain EF302, an enterhemorrhagic isolate, and *Salmonella typhimurium* ATCC14028, a diarrheal pathogen. *Salmonella typhimurium* was overexpressing its *csrA* homolog that was cloned and sequenced in this laboratory (GenBank Accession No. 276860).

Biofilm Dispersal. Biofilm can act as a nucleus from which infection can spread or disperse to other sites in the body (6). To examine the potential role of *CsrA* in biofilm dispersal, a strain was constructed in which *csrA* expression could be induced with IPTG (isopropyl-β-D-thiogalactopyranoside), TR1-5JM101[pCSRH6-19]. The *csrA*-inducible phenotype of this strain was confirmed by the observation that glycogen accumulation is inhibited by IPTG (data not shown). This strain was allowed to form biofilm for 24 hours in CFA medium in borosilicate tubes, in the absence of IPTG. Then, IPTG (1 mM final conc.) or sterile water was added directly to the culture medium along with ampicillin to maintain the plasmid, and biofilm was monitored in quadruplicate cultures. Induction of *csrA* resulted in the release of the biofilm over the following 4 to 6 hours (Fig. 3A). Because nutrient limitation, metabolite accumulation or other conditions in the spent medium might have affected the results, the dispersal experiment was repeated by

removing the spent medium from a 24 h biofilm, gently rinsing it with and incubating it further in fresh CFA medium containing ampicillin +/- IPTG. Again, *csrA* induction caused the biofilm to disperse (Fig. 3B). Lastly, a 24-hour biofilm was rinsed and the spent medium replaced with M63 salts solution, containing ampicillin, which lacked a carbon source, +/- IPTG. In this experiment, *csrA* induction also caused biofilm to disperse over the next few hours (Fig. 3C). Microscopic examination of cells released from the biofilms revealed that a small proportion of cells had become motile in the fresh CFA medium, but cells in spent medium or M63 salts were uniformly nonmotile (data not shown). Control experiments showed that IPTG does not affect either biofilm formation or dispersal in strains lacking an inducible *csrA* gene (data not shown). The growth curves of these strains were essentially similar showing that release was not triggered by cell death. These experiments, conducted under three different physiological conditions, indicate that *csrA* expression can serve as a general signal for biofilm dispersal in *E. coli*. Conversely, when 0.2% glucose was added to spent media along with ampicillin and +/- IPTG, the preformed biofilm did not release (Fig. 3D). The amount of biofilm formed in both the induced and uninduced biofilm increased 3-fold over 6 hours. This result suggests that nutritional cues may override some genetic signals.

Effects of *csrA* on biofilm formation are mediated via glycogen. Previously reported factors such as flagella, type I pili, curli fimbriae, and colanic acid have been shown to play a role in biofilm development in *E. coli* (30). The question of whether biofilm formation in a *csrA* mutant is mediated through these factors or any combination of these factors was addressed. Using the previously described biofilm assay, the effects of curli fimbriae, colanic acid, type I pili on biofilm formation. As previously reported, curli fimbriae and type I pili play a significant role in biofilm formation in the parent strain (Fig.4A). We confirmed that colanic acid plays no quantitative role in biofilm formation by crystal violet staining. In a *csrA* mutant, only type I pili significantly decreased biofilm formation. Interestingly, a *csrA* mutant lacking curli fimbriae, colanic acid, and type I pili forms a significant biofilm (Fig 4A). Growth curves conducted on these strains were similar indicating that the differences in biofilm formation were not due to growth defects (data not shown). Flagella have been reported to be important for initial biofilm attachment (30). The effect of motility on biofilm formation by a *csrA* mutant was tested. We obtained Δ*motB* mutant that does not inhibit flagellar biosynthesis but renders cells nonmotile. In the wild type strains, a disruption in motility virtually eliminated biofilm formation (Fig 4B). In a *csrA* motility mutant, the amount of biofilm formed significantly decreased but was not eliminated (Fig. 4B). Further studies revealed

the additive affect of type I pili deletion and motility defect in a wild type strain totally eliminated biofilm formation. However in the *csrA* mutant, there was apparently no additive affect of type I pili deletion and motility defect since the amount of biofilm formed was the same as the motility mutant alone. The data indicate that a *csrA* mutant will form a biofilm in the absence of previously reported extracellular factors. The trends seen were also observed when biofilms were grown in borosilicate glass tubes for 24 hours and also observed in 48 hour biofilm cultures grown on both glass and microtiter plates (data not shown).

RpoS (stationary phase sigma factor) and OmpR (outer membrane protein regulator) are regulators of biofilm formation (1,41). We assessed whether *rpoS* affects biofilm formation in a *csrA* mutant. We found that *rpoS* is important for biofilm formation in the parent *E. coli* strain but not in the *csrA* mutant strain (Fig. 4C). Studies on the effects of *csrA/rpoS* mutant strain on curli fimbriae and type I pili show that these factors are important in the parent strain but not significant in the *csrA* mutant strain (Fig. 4C). Even a combination *csrA* mutant with a *rpoS* mutation and curli fimbriae disruption, and a type I pili deletion will form a significant biofilm whereas in the parent strain biofilm has been eliminated. In a *csrA* mutant strain, OmpR has modest affects on biofilm formation. In a *csrA* mutant strain of *ompR*, *ompR*/curli fimbriae mutation, and

ompR/curli/type I pili show only a 20-30% decrease in biofilm formation (Fig. 4D). This suggests that none of these factors are important for biofilm formation in a *csrA* mutant. Interestingly, in the *csrA* mutant lacking *ompR* and type I pili there is little to no biofilm present; however, in this same strain when there is a curli mutation the amount of biofilm increases (Fig. 4D). This may be due to the fact that curli fimbriae are inhibitory to some factor necessary for biofilm formation in a *csrA* mutant lacking *ompR* and type I pili. The trends seen were also observed when biofilms were grown in borosilicate glass tubes for 24 hours and also observed in 48 hour biofilm cultures grown on both glass and microtiter plates (data not shown).

Originally, the *csrA* gene was recognized as a repressor of glycogen biosynthesis (35). Since none of the previously reported extracellular factors and regulators were essential for biofilm formation in a *csrA* mutant, thus the role of glycogen in biofilm formation was examined. While bacterial glycogen is generally presumed to serve as a carbon/energy reserve for survival in the stationary phase, no biological function has been rigorously established for this polymer (31). Furthermore, in a *csrA* mutant, both glycogen biosynthesis in the early stationary phase of growth and its subsequent catabolism are highly accelerated (43). This occurs because CsrA post-transcriptionally represses the *glgCAP* operon, which includes the biosynthetic genes *glgC* (ADP-glucose

synthetase) and *glgA* (glycogen synthase), as well as the gene encoding the degradative enzyme glycogen phosphorylase (*glgP*) (25). A *glgA* disruption caused both *csrA* wild type and mutant strains to become severely defective for biofilm formation (Fig. 5a). As expected, the *glgA*-encoding plasmid, pOP245, complemented the glycogen biosynthesis defect caused by the *glgA* mutation (data not shown). In contrast, this plasmid failed to restore biofilm formation. The possibility that glycogen catabolism may also be required for biofilm formation, and that the *glgA* transposon insertion had a polar effect on *glgP* expression, was examined by complementation with the *glgP*-encoding plasmid pJF02. While *glgP* alone did not restore biofilm formation, both *glgA* and *glgP* resulted in even more biofilm production than in the prototrophic parent (Fig. 5A). The observed requirement for both *glgA* and *glgP* for complementation indicate that glycogen synthesis and its subsequent turnover are both needed for biofilm formation, and defines the first distinct function for bacterial glycogen.

Because a *csrA* mutant overexpresses glycogen biosynthetic genes as well as the catabolic gene, *glgP* (35,37), we wondered which of these effects was significant for biofilm formation. Fig. 5B demonstrates that overexpression of either the biosynthetic genes or *glgP* from plasmids in a wild type strain enhances biofilm formation. Thus an increase in glycogen synthesis or in its subsequent turnover improves biofilm formation

indicating that both effects of CsrA are relevant. Based on these findings, we propose that intracellular glycogen, which is synthesized in the early stationary phase of growth and subsequently metabolized, is a carbon/energy source for the synthesis of extracellular factor(s) that are required for biofilm formation.

***csrA* expression during biofilm development**. To assess whether *csrA* expression is modulated during biofilm development in vivo, β-galactosidase activity encoded by a chromosomal *csrA*'-'lacZ translational fusion was monitored in planktonic and sessile cells. A result typifying several experiments is shown in Fig. 6. β-galactosidase activity in planktonic cells declined immediately prior to the appearance of the biofilm and for the following few hours, whereafter, it remained relatively constant. In the biofilm, a sharp decline in activity occurred during the first few hours of growth, such that activity was ultimately lower than in the unattached cells. As biofilm growth ceased, β-galactosidase activity increased moderately, and after 1.5 to 2 days of incubation, activity increased to pre-biofilm levels.

Discussion

It is clear that biofilm formation and eventual dispersal are complex processes and many of the factors that regulate these processes remain to be defined. Our studies demonstrate that CsrA can facilitate biofilm formation and dispersal in *E. coli*. A *csrA*

mutant very rapidly produces biofilm that differentiates into a complex multi-cellular structure. Surprisingly, biofilm formation by the *csrA* mutant is not completely dependent on any of the previously reported extracellular factors and regulators: type I pili, motility, colanic acid, curli fimbriae, OmpR, or RpoS. This would suggest an alternative pathway of biofilm formation through which *csrA* functions. Thus, studies on these functions might offer insight into other novel extracellular factors and regulators of biofilm development.

We found that the effects of CsrA are, in part, mediated through the cell's ability to utilize endogenous glycogen. It is not yet known how glycogen affects biofilm formation. Glycogen may be used solely as an energy source for the synthesis of essential proteins or extracellular adhesions necessary for biofilm formation. Our results indicate that global redirection of carbon metabolism, in response to the appropriate environmental conditions, facilitates the development of a biofilm.

Clearly, the participation of additional regulators of carbon metabolism in biofilm formation should be examined in other species, to determine whether this is a general principle. Perhaps the requirement for Crc in *P. aeruginosa* biofilm formation rests not only on its role in activating type IV pilus formation (29), but also on its influence on central carbon flux. Interestingly, the glycogen-like intracellular polymer of

Streptococcus mutans has long been recognized as a virulence factor for the formation of dental carries (19). This has been assumed to result from the prolonged secretion of organic acids generated from the metabolism of this polymer. However, the possibility that it provides carbon/energy for the synthesis of the extracellular glucans that are required for surface attachment and oral biofilm formation should now be considered.

Our results demonstrate that induction of *csrA* gene expression will cause a pre-formed biofilm to release under a variety of nutrient conditions and *csrA* is an important determinant of biofilm dispersal. It is not clear by what mechanism *csrA* will cause a biofilm to disperse. Interestingly, glucose, which we found to be inhibitory to initial biofilm formation (data not shown), promoting growth rather than release of a pre-formed biofilm by *csrA*. Nutritional cues have recently been shown to be important for biofilm formation. In *Pseudomonas aeruginosa*, it has been shown that iron will override the *lasI/lasR* quorum-sensing system control of oxidative stress response (8). In addition, a number of mutants defective for initial biofilm attachment in *Pseudomonas fluorescens* could be rescued by the addition of casamino acids, iron, glutamate and citrate (30). Thus, alternative pathways may be used under differing environments to initiate biofilm formation.

Overexpressing *csrA* from a plasmid completely blocked biofilm formation in *E. coli* K12 and inhibited biofilm formation in other enteric pathogens. These data suggest that other enteric pathogens behave similar to *E. coli* K-12 in that biofilm formation is decreased when *csrA* is overexpressed from a plasmid. However, data from overexpression studies should be taken with caution, since overexpressing any protein from a high copy plasmid may create artifacts that are not seen when using a corresponding mutant.

CsrA gene expression is not constant but is a dynamic within the biofilm, consistent with the hypothesis that CsrA is a switch for biofilm formation and dispersal. This fluctuation in CsrA expression levels may possibly lead to biofilm formation, in cases where CsrA expression levels are lower. However when CsrA levels are high, this could possibly be in preparation for cell dispersal. Perhaps IPTG-induction of *csrA* expression (Fig. 3) represents an extreme example of a typically more subtle release process occuring after ~1.5 days of growth. We suppose that, *in vivo*, an increase in *csrA* expression might facilitate either a shift from net accumulation to net dispersal of a biofilm, or a state in which cells are dividing and being continuously released from the biofilm.

Much of what we know about bacteria comes from studies on planktonic cells. Even studies on the efficacy of antibiotics are generally conducted on planktonic cells. However, bacteria mostly exist in biofilms. Research has now begun to focus on methods of preventing or eradicating biofilm infections. Because of antibiotic resistance, new studies should focus on genetic factors that can regulate biofilm formation and dispersal. A compound that mimics CsrA activity or increases *csrA* expression should inhibit biofilm formation and disperse antibiotic-sensitive planktonic cells from preexisting biofilm, without affecting host metabolism. We anticipate that ongoing studies to define the molecular mechanism of the CsrA protein may lead to new approaches for the treatment or prevention of biofilm-associated infections.

Finally, whatever structure(s) stabilize the biofilm, whether protein, polysaccharide or other, their synthesis and degradation or inactivation should be accomplished by distinct mechanisms that require different genes to be expressed. Thus, it is significant that CsrA impacts upon both of these processes. CsrA represents the first known repressor of biofilm formation and activator of dispersal, and genes that repress biofilm formation and activate dispersal will provide novel insight into the regulation of biofilm development.

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Table 1. Bacterial strains, phage, or plasmids used in this study

Strain, phage, or plasmid	Relevant Genotype	Reference or Source
E. coli		
MG1655	F⁻λ⁻	Michael Cashel
CF7789	MG1655 Δ *lacI-Z*(*MluI*)	Michael Cashel
KSA712	CF7789 Δ (λ *att-lom*)::bla Φ(*csrA'-'lacZ*) 1 (hyb) $Amp^R Kan^S$	This Study
AAECO&@	MG1655 Δ *fimB-H*	Ian Blomfield
DHB6521	(F⁻λInch1(kan^R)Δ*lac*(MS265) *mel* Nal^R *supF*58 ($suIII^+$)	9
JM101	(*supE* thiΔ (*lac-proAB*)[F' *traD*36 *proAB lac* I^q Z ΔM115]	Lab Strain
MC4100	F⁻Δ(*argF-lac*)U169*rps*L *relA flh*D *deoC ptsF rbsR*	30
MM5057	Δ *motB*, *uvrC*-279::Tn*10*	Mike Manson
MHR 204	MC4100 *csgA*2::Tn*105*	20
RH106	*rpoS*::Tn*10*	R. Hengge-Aronis
SG20043	MC4100 *cpsE*::Tn*10*	Valerie Stout
TK821	MC4100 *ompR*331::Tn*10*	42
P18	*E. coli* clinical isolate	21

TR1-5, TR[a]	*csrA::kanR*	21
glgA[b]	*glgA::kanR*	This Study
DJ1	MG1655 *csgA*2::Tn*105*	This Study
DJ2	TRMG1655 *csgA*2::Tn*105*	This Study
DJ3	MG1655 *cpsE*::Tn*10*	This Study
DJ4	TRMG *cpsE*::Tn*10*	This Study
DJ6	TRAAEC072	This Study
DJ7	AAECO72 *csgA*2::Tn*105*	This Study
DJ8	TRAAECO72 *csgA*2::Tn*105*	This Study
DJ9	AAECO72 *cpsE*::Tn*10*	This Study
DJ10	TRAAECO72 *cpsE*::Tn*10*	This Study
DJ11	MG1655 *csgA*2::Tn*105 cpsE*::Tn*10*	This Study
DJ12	TR MG1655 *csgA*2::Tn*105* *cpsE*::Tn*10*	This Study
DJ13	AAECO72 *csgA*2::Tn*105* *cpsE*::Tn*10*	This Study
DJ14	TRAAECO72 *csgA*2::Tn*105* *cpsE*::Tn*10*	This Study
DJ21	MG1655 Δ*motB uvrC*-279::Tn*10*	This Study
DJ22	AAECO72 Δ*motB uvrC*-279::Tn*10*	This Study

DJ24	TR Δ*motB uvrC*-279::Tn*10*	This Study
DJ25	TRAAECO72 Δ*motB* *uvrC*-279::Tn*10*	This Study
DJ30	MG1655 *rpoS*::Tn*10*	This Study
DJ31	TR MG1655 *rpoS*::Tn*10*	This Study
DJ32	MG1655 *rpoS*::Tn*10* *csgA*2::Tn*105*	This Study
DJ33	TR MG1655 *rpoS*::Tn*10* *csgA*2::Tn*105*	This Study
DJ34	AAECO72 *rpoS*::Tn*10*	This Study
DJ35	TR AAECO72 *rpoS*::Tn*10*	This Study
DJ36	AAECO72 *csgA*2::Tn*105* *rpoS*::Tn*10*	This Study
DJ37	TRAAECO72 *csgA*2::Tn*105* *rpoS*::Tn*10*	This Study
DJ40	MG1655 *ompR*333::Tn*10*	This Study
DJ41	TR MG1655 *ompR*333::Tn	This Study
DJ42	MG1655 *ompR*333::Tn*10* *csgA*2::Tn*105*	This Study
DJ43	TR MG1655 *ompR*333::Tn*10* *csgA*2::Tn*105*	This Study
DJ44	AAECO72 *ompR*333::Tn*10*	This Study

DJ45	TR AAECO72 *ompR*333::Tn*10*	This Study
DJ46	AAECO72 *ompR*333::Tn*10* *csgA*2::Tn*105*	This Study
DJ47	TR AAECO72 *ompR*333::Tn*10* *csgA*2::Tn*105*	This Study
Other		
P5	*Citrobacter freundii* clinical isolate	21
EF302	*E. coli* O157:H7 clinical isolate	John Foster
ATCC14028	*Salmonella typhimurium*	Craig Altier
Bacteriophages		
P1*vir*	Strictly lytic P1; forms clear plaques	Carol Gross
Plasmids		
pUC19	cloning vector, Amp^R	35
pBR322	cloning vector, Tet^R	35
pCSR10	*csrA* gene, 0.5kb *Dde*I from λ 446 in pUC19 Amp^R	35
pCSRH^-19	produces a recombinant csrA protein under IPTG induction Amp^R	24
pOP12	containing *asd* and *glgCAP* in pBR322 Tet^R	36
pOP245	*glgA* in pBR322 Tet^R	36

pJF02	*glgP* in pUC19, Amp^R	45
pMLB1034	for construction of '*lacZ* translational fusion Amp^R	35
pTR151P1	PstI subclone from pTR151 (*csrA::kan*R allele in pUC19, Amp^R)	35

[a] Indicates the wild type (*csrA*+) allele has been replaced by *csrA::kanR* allele

[b] Strains names with the suffix *glgA* contained a *glgA::kanR* insertion

Fig. 1. Biofilm formation by wild type and *csrA* mutant strains of *E. coli*i;**A)** Growth in polystyrene microtiter wells of planktonic cells of the wild type strain MG1655 or its *csrA* mutant, filled squares or triangles, respectively. Biofilm formation in the same wells is shown as open squares or triangles, respectively. **B)** Nomarski interference of biofilm formed by MG1655 in the left panels (10, 16 and 24 h, top to bottom) or its *csrA* mutant (3, 10, and 24 h) in the right panels. **C)** A topographical image of the *csrA* mutant biofilm is shown in the left panel (white scale bar 14 μm; virtual color code depicts biofilm height above the microscope slide), along with a 2 μm thick cross-section at a depth of 6 μm in the right panel (scale bar 10 μm), and a cross-section of a sagital view tilted ($\Theta = 45°$, $\Phi = 30°$) in the bottom panel (scale bar 20 μm), as visualized by confocal microscopy.

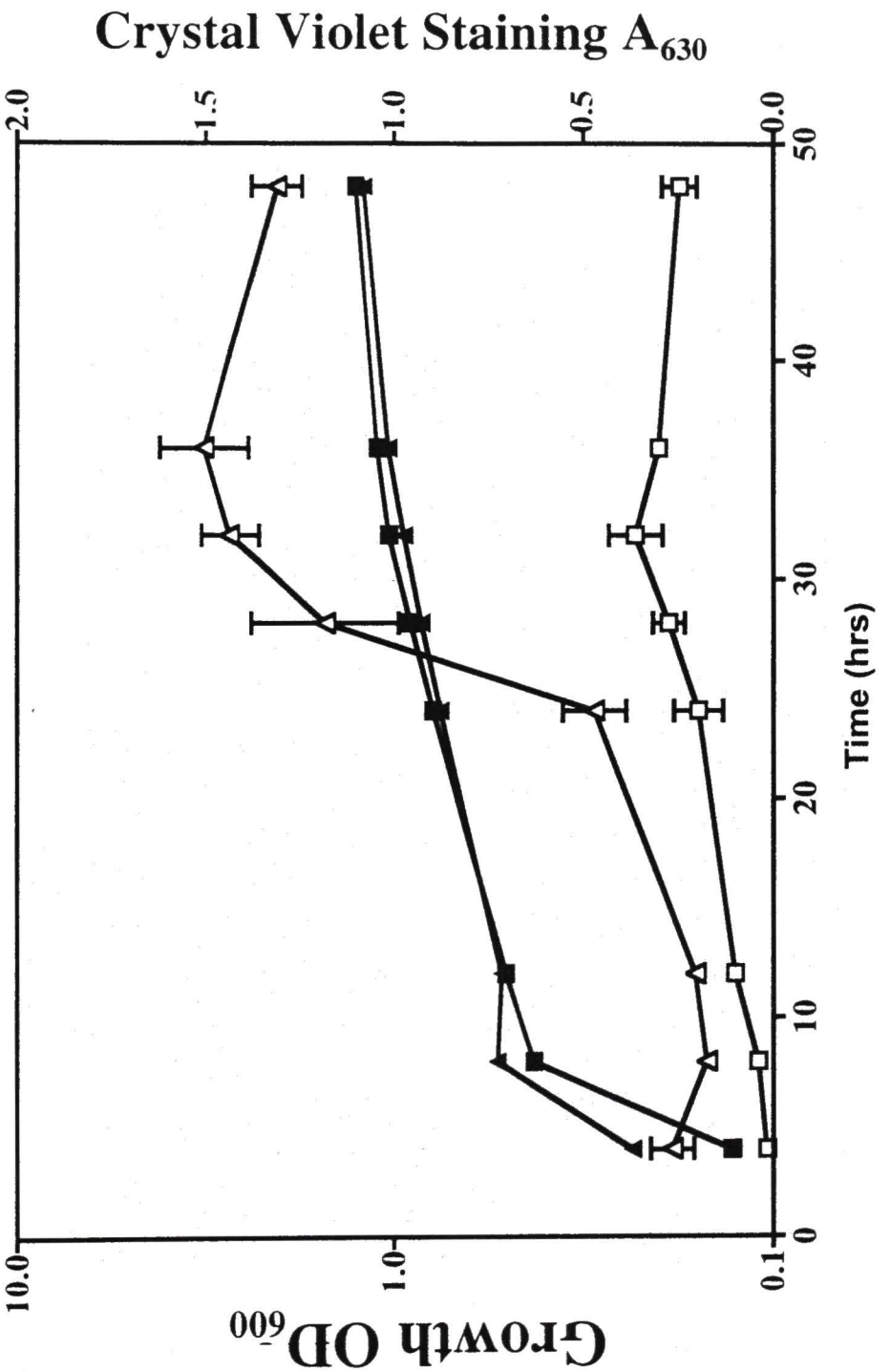

Crystal Violet Staining A_{630}
2.0
1.5
1.0
0.5
0.0
50
40
30
20
10
0
Time (hrs)
10.0
1.0
0.1
Growth OD_{600}

Figure 1A

Figure 1B

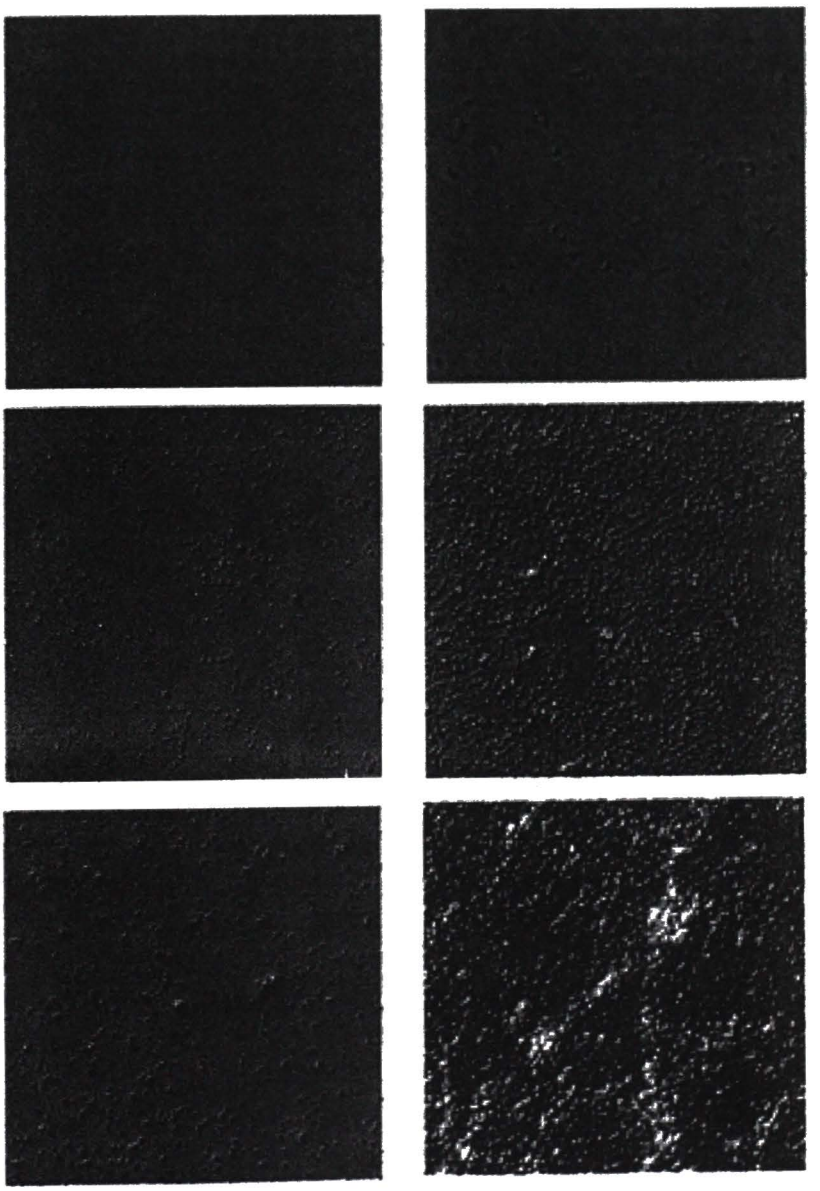


Figure 1C

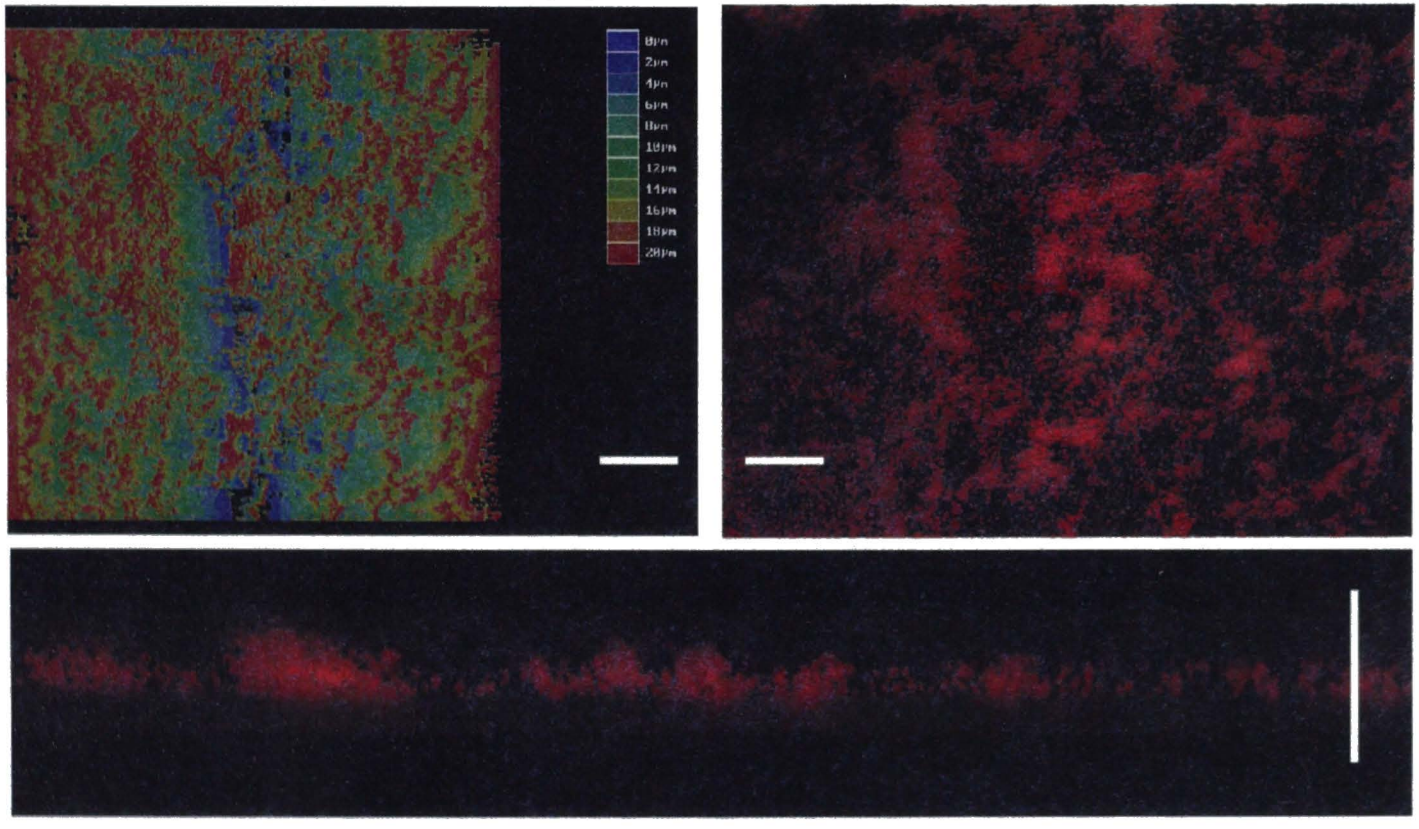


Fig. 2. Effects of *csrA* on biofilm formation during 24 h growth in microtiter wells. A) MG1655 (bars 1-3) or its *csrA* mutant (4-6) containing plasmids pCSR10 (*csrA*$^+$) (2, 5) or pUC19 (3,6). B) Urinary tract pathogens *E. coli* P18 (1-3) and *Citrobacter freundii* P5 (4-6), containing plasmids pCSR10 (*csrA*$^+$) (2,5) or pUC19 (3,6). C) Food-borne pathogens *E. coli* O157:H7 (1-3) and *Salmonella typhimurium* ATCC14028 (4-6) containing plasmids pCSR10 (*csrA*$^+$) (2), pSTCSR5 (*csrA*$^+$) (5), or pUC19 (3,6). Each bar shows the average and standard error of three separate experiments, and * denotes a significant difference between strains ($P<0.0001$).

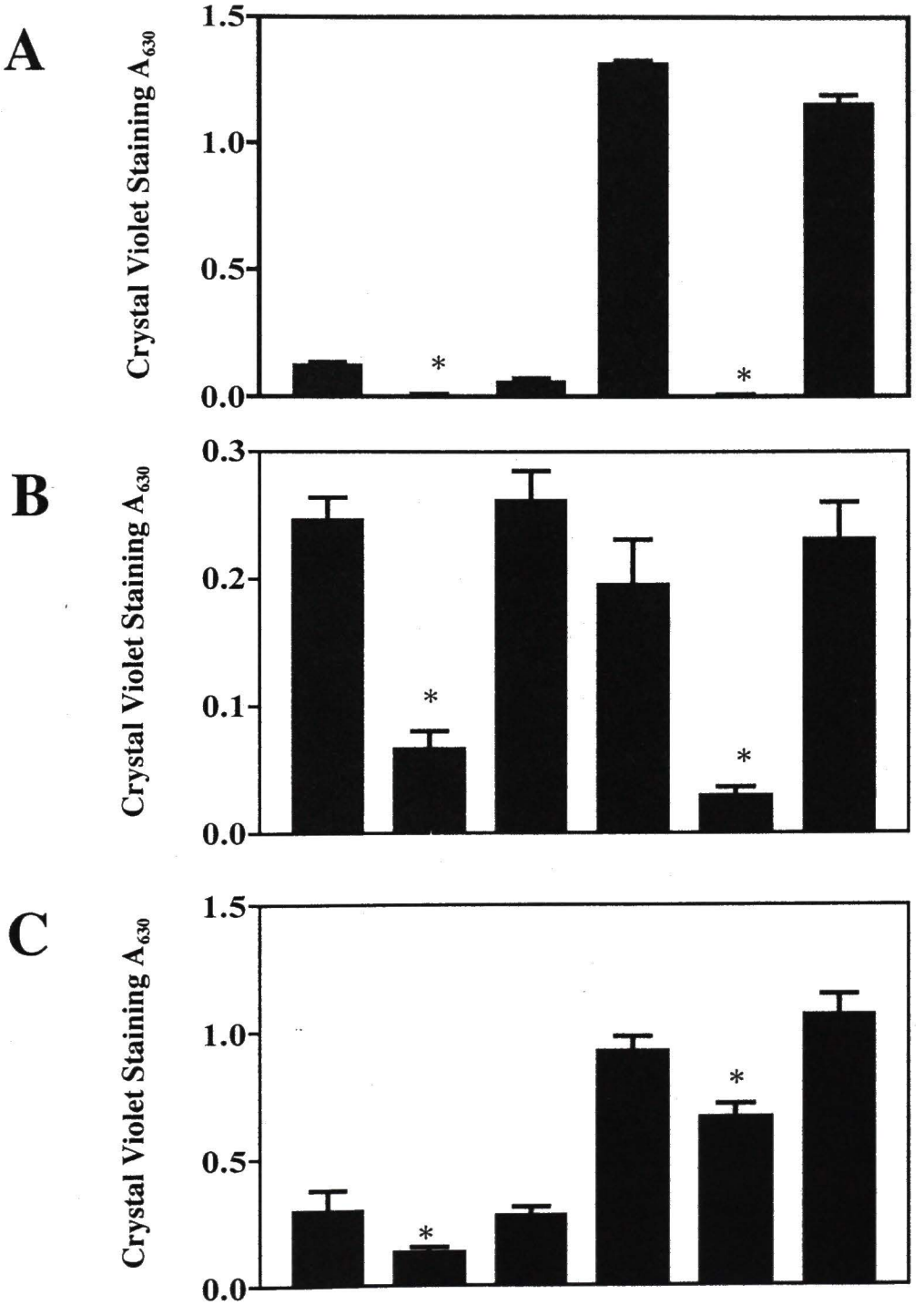

A
Crystal Violet Staining A_{630}
1.5
1.0
0.5
0.0
*
*
B
Crystal Violet Staining A_{630}
0.3
0.2
0.1
0.0
*
*
C
Crystal Violet Staining A_{630}
1.5
1.0
0.5
0.0
*
*

Fig. 3. Dispersal of bacteria from pre-formed biofilm by *csrA* induction. A strain containing an IPTG-inducible *csrA* gene, TRJM101[pCSRH6-19], was allowed to form a 24 h biofilm, whereafter **A)** ampicillin was added in addition to IPTG (1 mM final) or sterile deionized water was added directly to the medium. **B)** the medium was discarded and fresh CFA with ampicillin was added +/- IPTG. **C)** the medium was replaced with a M63 salts solution with ampicillin +/- IPTG. **D)** 0.2% glucose with ampicillin and IPTG (1mM final) or sterile deionized water was added directly to the medium. The biofilm remaining at the indicated times following *csrA* induction is shown. Each value represents an average of at least two independent experiments, with quadruplicate samples, and * denotes a significance difference between strains ($P<0.0001$).

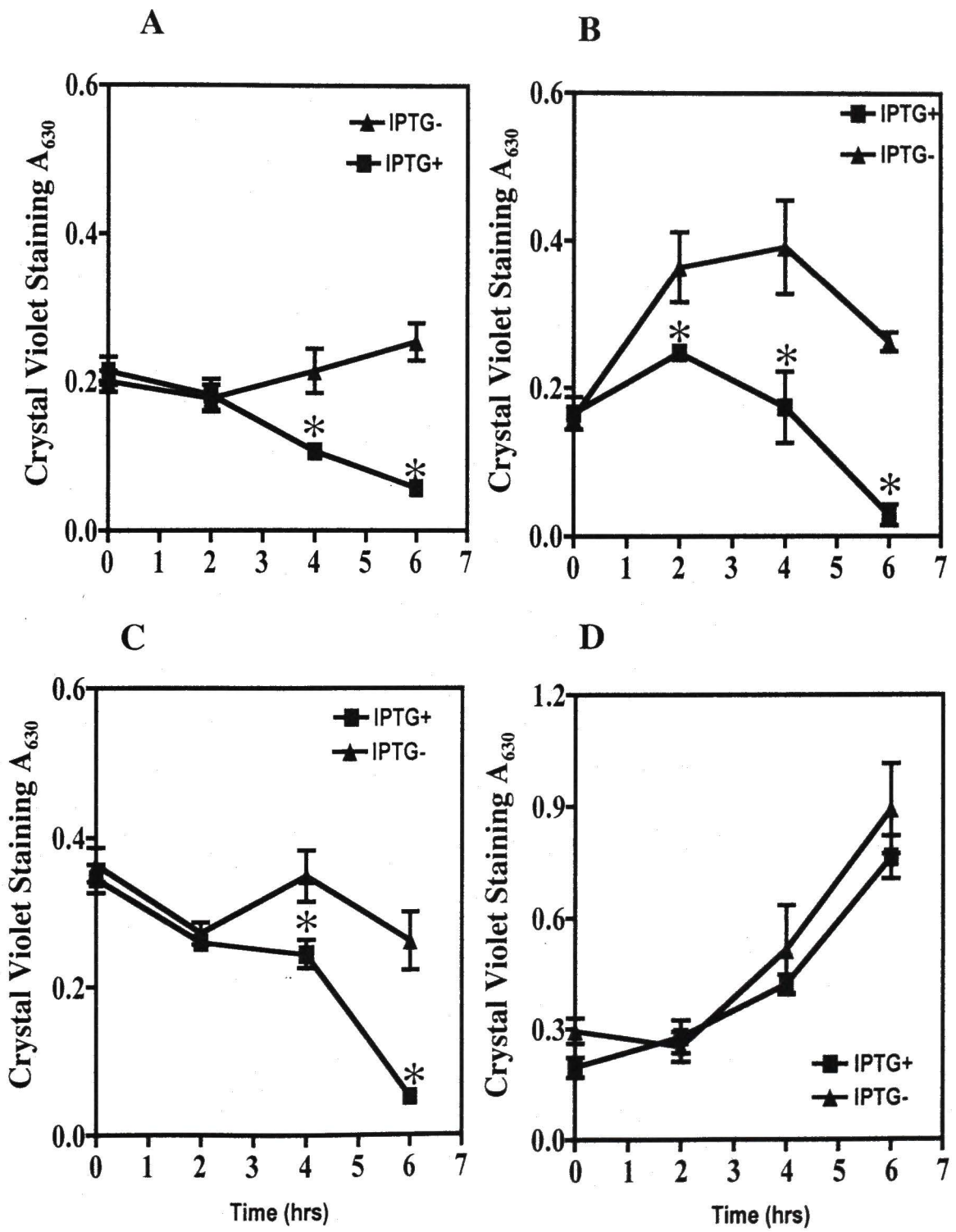
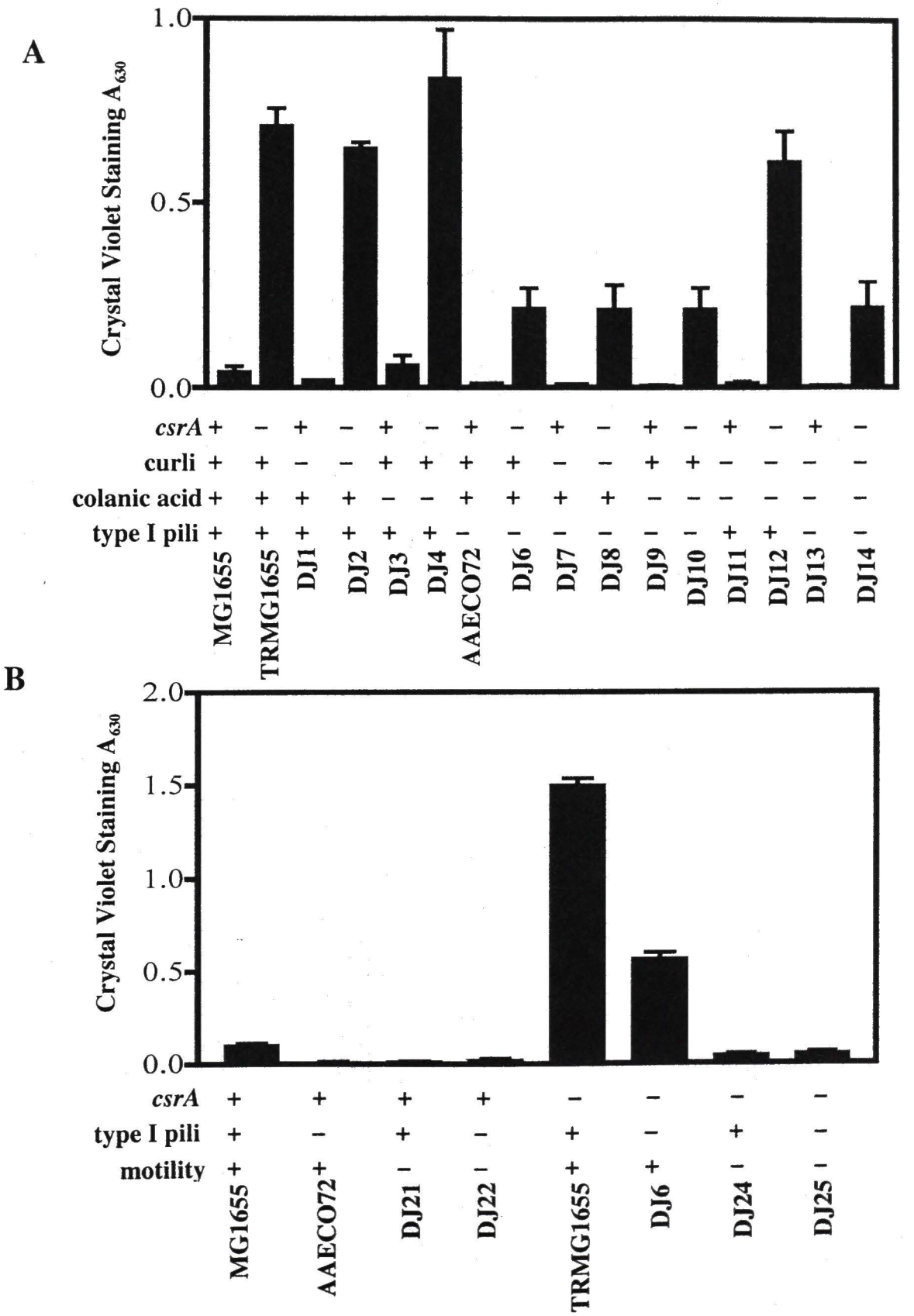
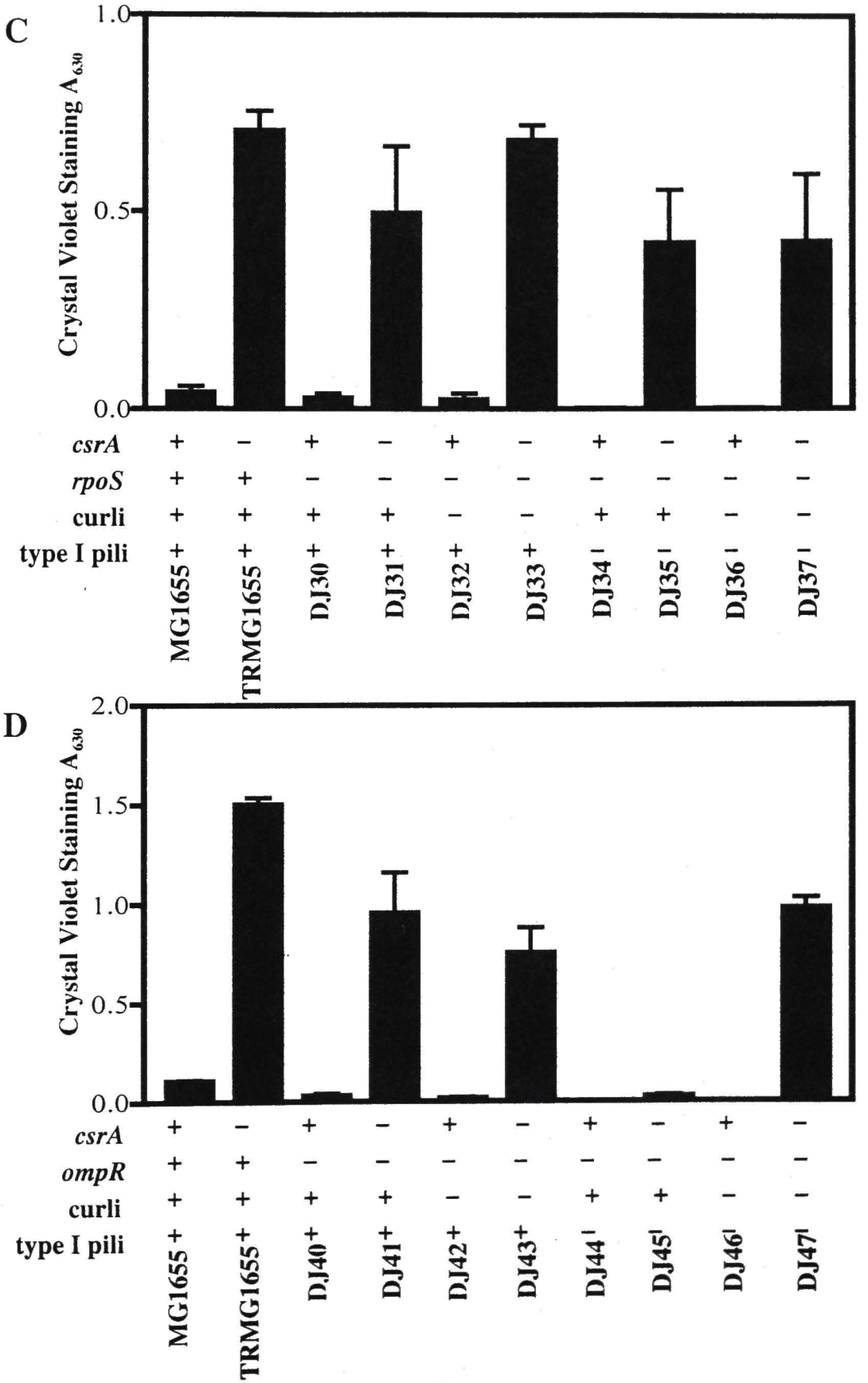

A
B
C
D
Crystal Violet Staining A_{630}
IPTG-
IPTG+
Time (hrs)
0.0
0.2
0.4
0.6
0.9
1.2
0 1 2 3 4 5 6 7
*

Fig. 4. Effects of extracellular factors and global regulators on biofilm formation in *E. coli* strain MG1655 and its isogenic *csrA* mutant. Crystal violet (CV) staining of biofilms formed with mutations in **A)** curli fimbriae, colanic acid, type I pili, or in combination. **B)** type I pili, motility, or both. **C)** RpoS, curli fimbriae, type I pili or combinations of these mutations. **D)** OmpR, curli fimbriae, type I pili or combinations of these mutations. Each bar shows an average and standard error of three separate experiments ($P<0.0001$).


A
Crystal Violet Staining A_{630}
1.0
0.5
0.0
csrA + − + − + − + − + − + − + − + −
curli + + − − + + + + − − + + − − − −
colanic acid + + + + − − + + + + − − − − − −
type I pili + + + + + + − − − − − − + + − −
MG1655
TRMG1655
DJ1
DJ2
DJ3
DJ4
AAEC072
DJ6
DJ7
DJ8
DJ9
DJ10
DJ11
DJ12
DJ13
DJ14
B
Crystal Violet Staining A_{630}
2.0
1.5
1.0
0.5
0.0
csrA + + + + − − − −
type I pili + − + − + − + −
motility + + − − + + − −
MG1655
AAEC072
DJ21
DJ22
TRMG1655
DJ6
DJ24
DJ25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curli + + + + – – + + – –
type I pili + + + + + + I I I I
MG1655
TRMG1655
DJ30
DJ31
DJ32
DJ33
DJ34
DJ35
DJ36
DJ37
D
Crystal Violet Staining A_{630}
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1.5
1.0
0.5
0.0
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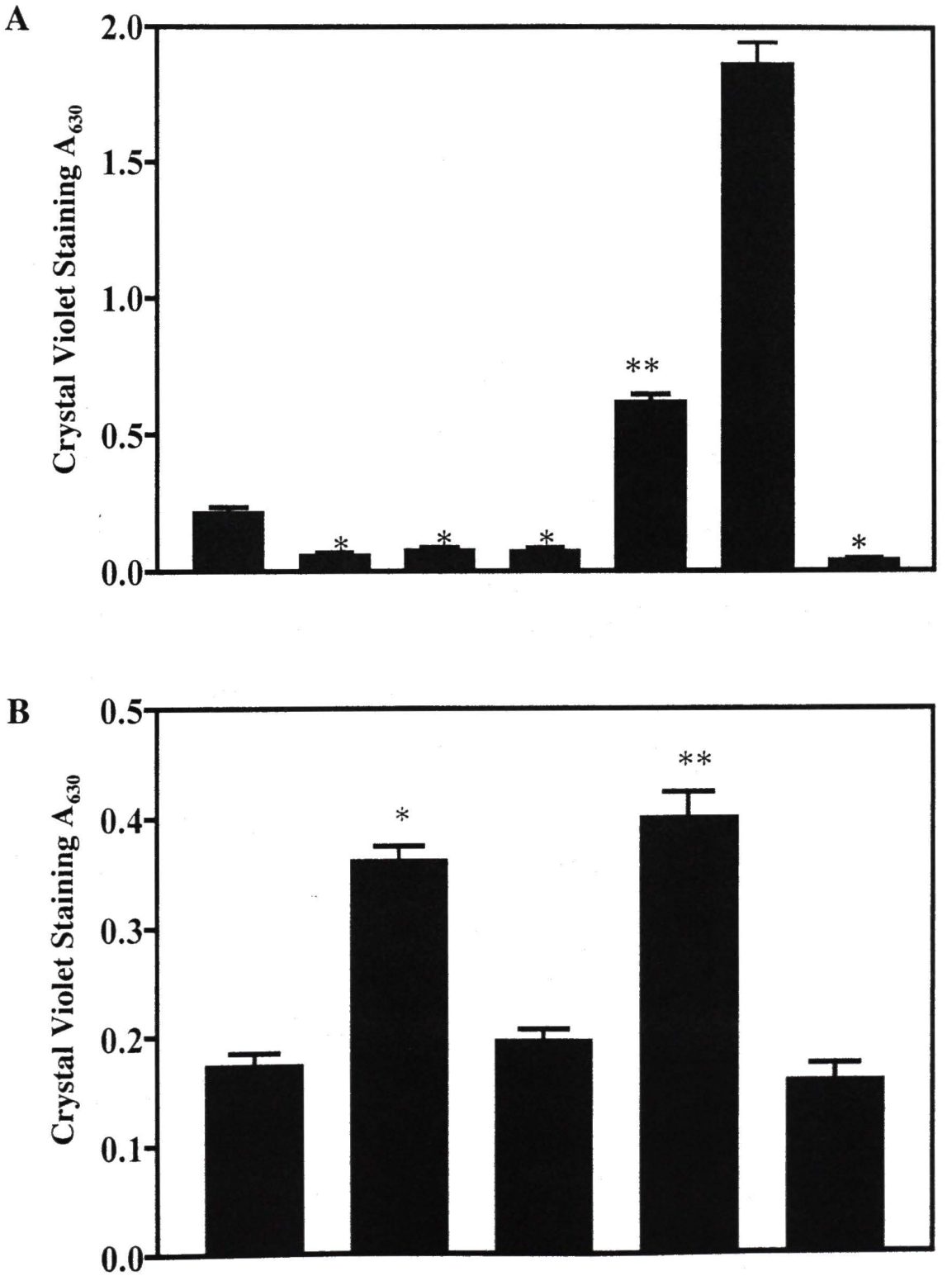
ompR + + – – – – – – – –
curli + + + + – – + + – –
type I pili + + + + + + I I I I
MG1655
TRMG1655
DJ40
DJ41
DJ42
DJ43
DJ44
DJ45
DJ46
DJ47

Fig. 5. Effects of glycogen synthesis and catabolism on biofilm formation. Isogenic derivatives of strain MG1655, defective for *glgA*, *csrA*, or both genes were compared. Plasmids pOP245, pJF02, or pOP12 encode *glgA* , *glgP*, or *asdglgBXCAP'* (deleted for most of *glgP*), respectively. **A)** Effects of a polar *glgA* mutation. The strain identities for bars 1-7 were MG1655, *glgA* mutant, *glgA* mutant containing pOP245, pJF02, or both plasmids, the *csrA* mutant, and the *csrA glgA* double mutant, respectively. **B)** Overexpression of glycogen biosynthetic or catabolic genes. Lanes 1-5 show MG1655 containing no plasmid, pOP12, pBR322 (vector control for pOP12), pJF02, and pUC19 (vector control for pJF02), respectively. Each bar shows the average and standard error of three separate experiments. The * and **: denote significant differences with respect to the parent strain ($P<0.0001$).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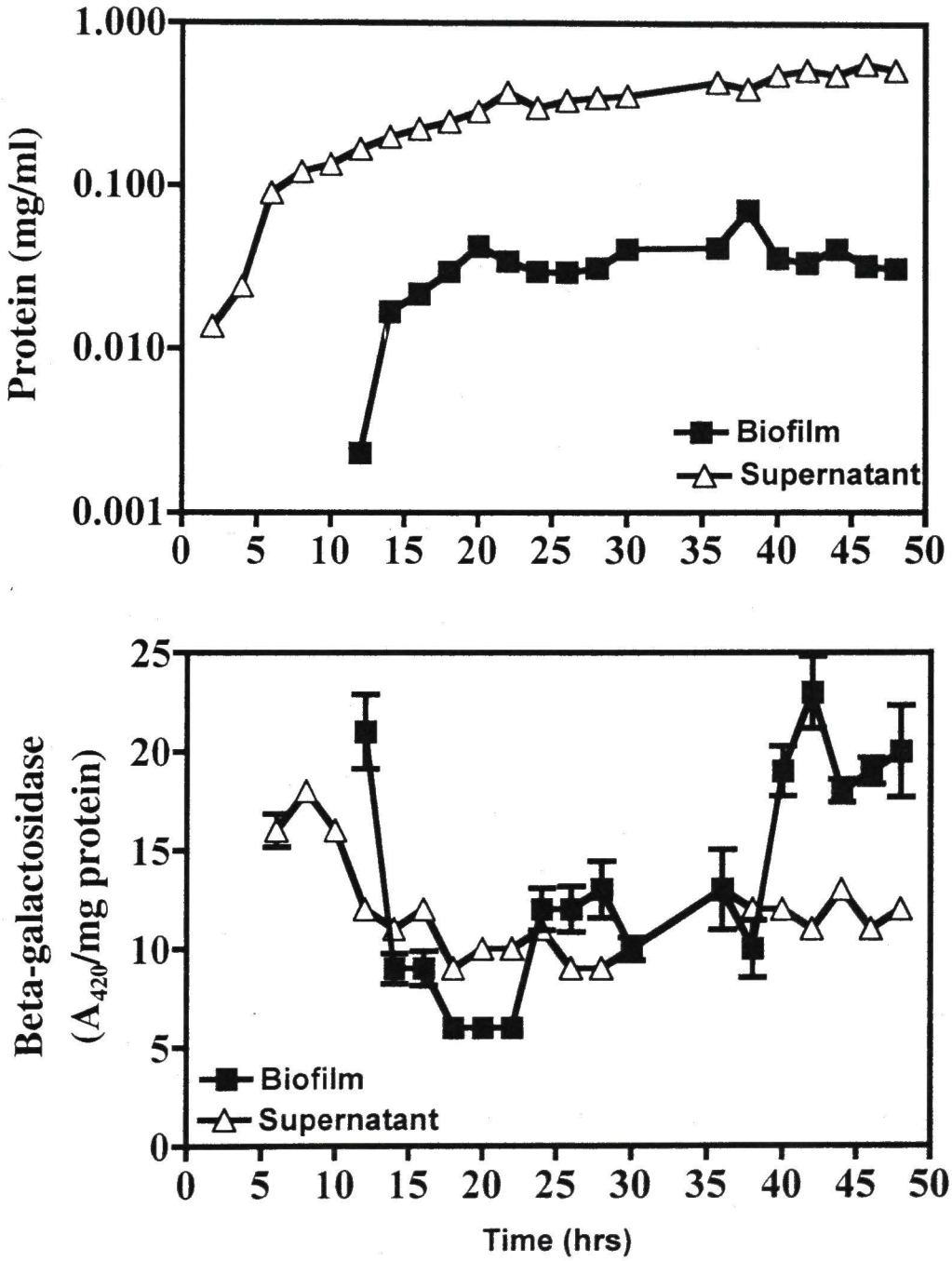
*
*
*
**
*
B
Crystal Violet Staining A_{630}
0.5
0.4
0.3
0.2
0.1
0.0
*
**

Fig. 6. Expression of a *csrA'-'lacZ* translational fusion in biofilm and planktonic cells of strain KSA712. Closed squares and open triangles represent determinations conducted on biofilm and planktonic cells, respectively. The average values (+/- standard error) were determined in duplicate samples. Error bars are not visible where the standard error was less than the area occupied by a given symbol.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0.010
0.001
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5
10
15
20
25
30
35
40
45
50
Biofilm
Supernatant
25
20
15
10
5
0
Beta-galactosidase
(A_{420}/mg protein)
Biofilm
Supernatant
Time (hrs)

Chapter IV
Supporting Documents

Introduction

In the natural environment bacteria attach to a variety of abiotic surfaces such as glass, plastic and metals (Costerton *et al.*, 1995). The precise mechanism by which bacteria adhere to inert surfaces is unknown (Vidal *et al.* , 1998). In *E. coli*, the extracellular factors type I pili, motility, curli fimbriae, and colanic acid have been proposed to play roles in biofilm formation (Pratt, 1998; Vidal *et al.*, 1998; Danese, 2000). In addition, the global regulatory factors OmpR and RpoS have been postulated to activate biofilm formation in *E. coli* (Vidal *et al.*, 1998 and Adams, 1999). The roles of these extracellular factors and regulators on biofilm formation in a *csrA* mutant was addressed in Chapter III of this dissertation. We used microtiter plates as a surface for attachment by biofilm bacteria because it is a convenient and sterile way to study biofilm formation (Loo *et al.*, 2000). To confirm that the differences observed in the crystal violet adherence assays on the extracellular factors and regulators in Chapter III were due to differences in potential of the various strains for greater attachment and growth of biofilms and were not unique to the polystrene surface, all strains were tested using borosilicate glass tubes as well as in microtiter plates. In addition, we wanted to know if

the same trends would be observed at later time points so the crystal violet adherence assay was allowed to proceed for 48 hours. These assays were performed in microtiter plates and borosilicate glass tubes to again confirm that any noted effects were not dependent on the availability of a specific type of surface.

Methods

Quantitative Biofilm Assay. Overnight cultures were inoculated 1:100 in fresh medium. In the microtiter plate assay, inoculated cultures were grown in a 96-well polystyrene microtiter plate. Growth of planktonic cells was determined by absorbance at 600 nm or total protein assay. Biofilm was measured by discarding the medium, rinsing the wells with water (3X) and staining bound cells with crystal violet (BBL, Cockeyville, MD). The dye was solubilized with 33% acetic acid (EM Science, Gibbstown, NJ) and absorbance at 630 nm was determined using a microtiter plate reader (DynaTech, Chantilly, VA). All comparative analyses were conducted between strains that were incubated within the same microtiter plate to minimize variability. In order to confirm that effects on biofilm formation were not surface-specific, cultures were grown and tested simultaneously in new borosilicate glass test tubes (18 mm). Each experiment

was performed at least in triplicate, and the data were analyzed by Tukey Multigroup Analysis (StatView-SAS Institute Inc., Cary, NC).

Results

Biofilm formation was similar on borosilicate glass tubes or microtiter (polystrene) plates over 24 hours. In comparing biofilm formation between microtiter plates and borosilicate glass tubes, it was found similar trends in biofilm formation occurred. In a wild type strain, curli fimbriae and type I pili are important for biofilm formation (Fig. 1A). Similarly, it was found that only type I pili decrease biofilm formation in a *csrA* mutant (Fig. 1A). Interestingly, motility was important for biofilm formation in a wild type as well as *csrA* mutant on borosilicate glass as was previously seen on microtiter plates (Fig. 1B). The differences observed in a wild type and *csrA* mutant with a RpoS mutation were similar on glass or plastic (Fig. 1C). Even in a *csrA/rpoS* mutant curli fimbriae and type I pili had similar affects on biofilm formation on borosilicate glass tubes (Fig. 1C). In a *csrA* mutant strain of *ompR*, *ompR*/curli fimbriae mutation, and *ompR*/curli/type I pili there is no decrease in biofilm formation. This decrease may not be significant since these *csrA* mutants still form a prolific biofilm.

Similar trends were observed at 48 hours in borosilicate glass tubes or microtiter plates. Using the same adherence assay, similar trends were observed over a 48 time

period for effect of curli fimbriae, type I pili and colanic acid on wild type *E. coli* and its isogenic *csrA* mutant (Fig. 2A and Fig. 3A). Likewise motility was important for biofilm formation in the wild type and *csrA* mutant on both glass and plastic surfaces (Fig. 2B and Fig. 3B). We observed that *rpoS* was important for wild type biofilm formation but not the *csrA* mutant on both glass and plastic at 48 hours (Fig. 2C and Fig. 3C). Even combinations of curli/type I pili had no affect on biofilm formation in a *csrA* mutant on glass or plastic (Fig. 2C and Fig. 3C). On glass or plastic, OmpR was important for biofilm formation in the wild type strain but not the *csrA* mutant (Fig. 2D and Fig. 3D). A *csrA* mutant strain with mutations in *ompR*/curli, *ompR*/type I pili and *ompR*/curli/type I pili decreased biofilm formation relative to the *csrA* mutant on the glass surface at 48 hours, it did not eliminate it (Fig. 2D). In contrast there was no difference in biofilm formation at 48 hours in a *csrA* mutant strain with mutations in *ompR*/curli, *ompR*/type I pili and *ompR*/curli/type I pili relative to the *csrA* mutant (Fig. 3D). The difference observed on glass may be because the *csrA* mutant has more biofilm formed on glass relative to the microtiter plate (plastic).

Discussion

Our data confirm that the differences seen in amount of biofilm formed were not surface dependent, since the same trends could be observed for glass and plastic at 24 hours. Although these experiments were conducted simultaneously, the only valid comparison can be made between trends since borosilicate glass tubes have more surface area than microtiter plates. The same trends were observed at 48 hours suggesting that the defects in biofilm formation could be observed at later time points. This suggests that these mutations not only affect initial biofilm attachment but also attachment of the biofilm as it grows. Taken together these data suggest that the observed differences in biofilm formation are not dependent upon surface as the same results were observed in borosilicate glass tubes as well as microtiter plates. The same trends were observed at later time points suggesting that these defects affect overall biofilm attachment and growth.

References

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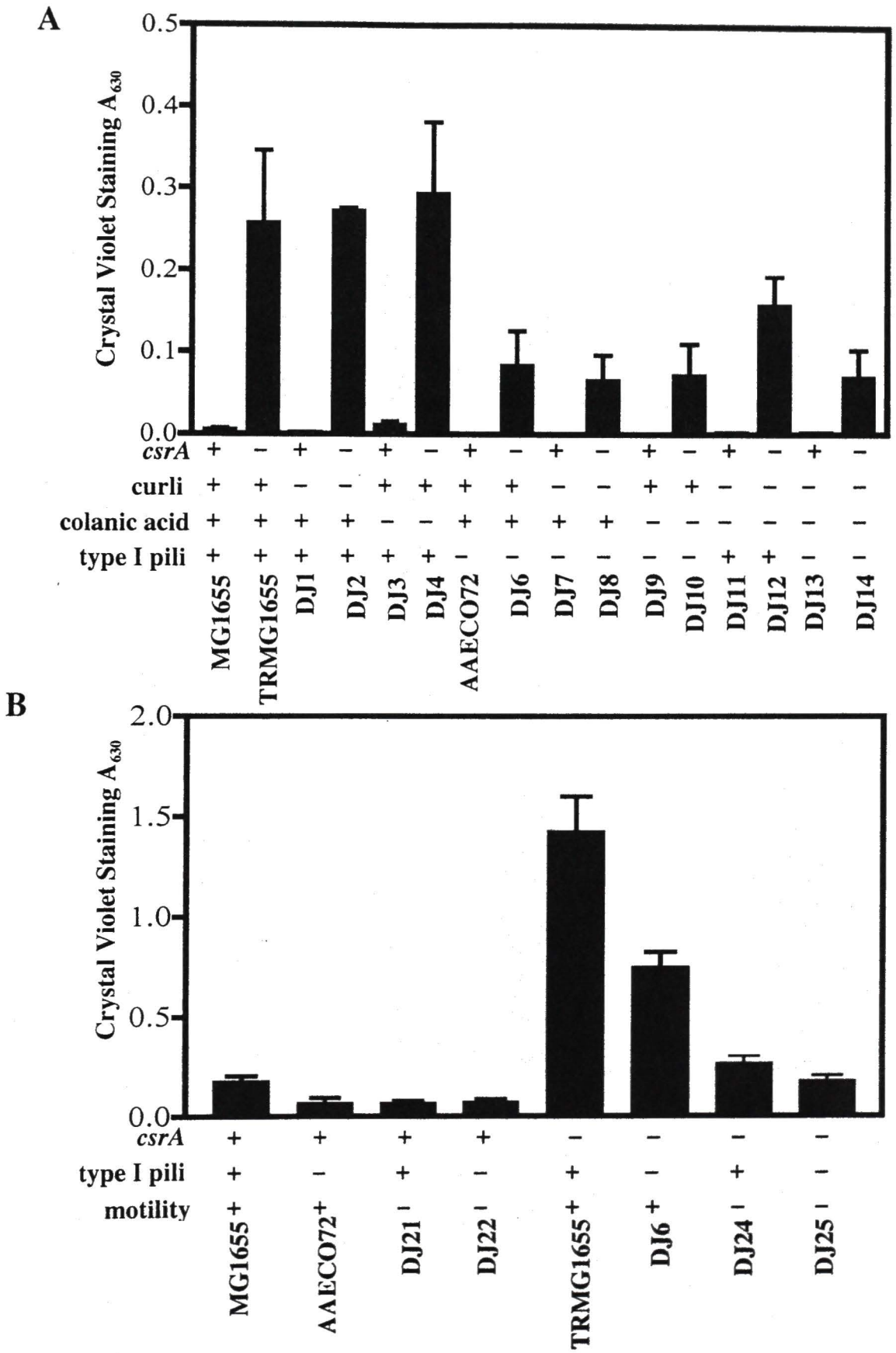
3) **Danese, P., L.A. Pratt, and R. Kolter**. 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. **182**: 3593-3596.

4) **Loo, C.Y., Corliss, D.A., and Ganeshkumar, N.** 2000. *Streptococcus gordo*nii biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. **182**: 1374-1382.

5) **Pratt, L.A., and Kolter R**. 1998. Genetic analysis of *Escherichia c*oli biofilm formation: role of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30**: 285-293.

6) **Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejueune**. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: involvement of a new *ompR* allele that increases curli expression. J. Bacteriol. **180**: 2442-2449.

Fig. 1. Effects of extracellular factors and global regulators on biofilm formation in *E. coli* strain MG1655 and its isogenic csrA mutant in borosilicate glass tubes. Crystal violet (CV) staining of biofilms formed with mutations in: **A)** curli fimbriae, colanic acid, type I pili, or in combination; **B)** type I pili, motility, or both; **C)** RpoS, curli fimbriae, type I pili or combinations of these mutations; **D)** OmpR, curli fimbriae, type I pili or combinations of these mutations. Each bar shows an average and standard error of three separate experiments ($P<0.0001$).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curli + + − − + + + + − − + + − − − −
colanic acid + + + + − − + + + + − − − − − −
type I pili + + + + + + − − − − − − + + − −
MG1655
TRMG1655
DJ1
DJ2
DJ3
DJ4
AAEC072
DJ6
DJ7
DJ8
DJ9
DJ10
DJ11
DJ12
DJ13
DJ14
B
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1.0
0.5
0.0
csrA + + + + − − − −
type I pili + − + − + − + −
motility + + − + − + − + − + − + − + − +
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DJ21
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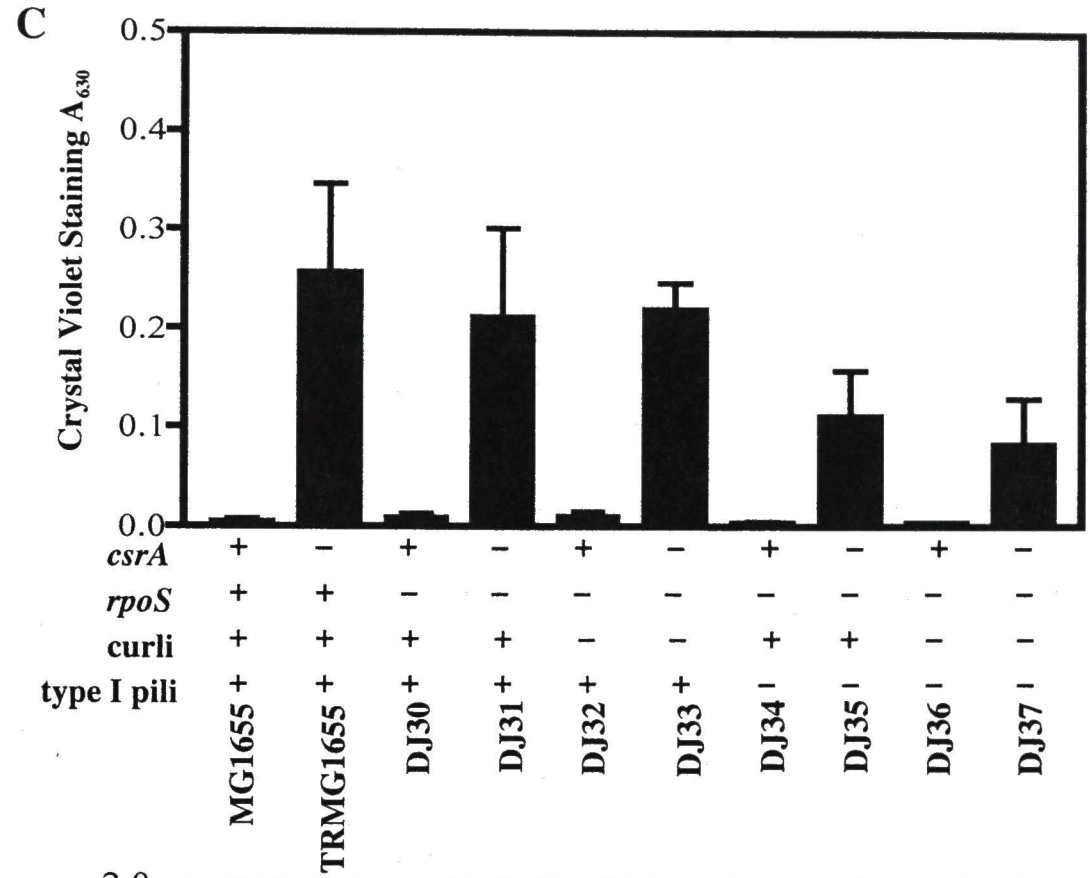
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rpoS + + − − − − − − − −
curli + + + + − − + + − −
type I pili + + + + + + − − − −
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DJ31
DJ32
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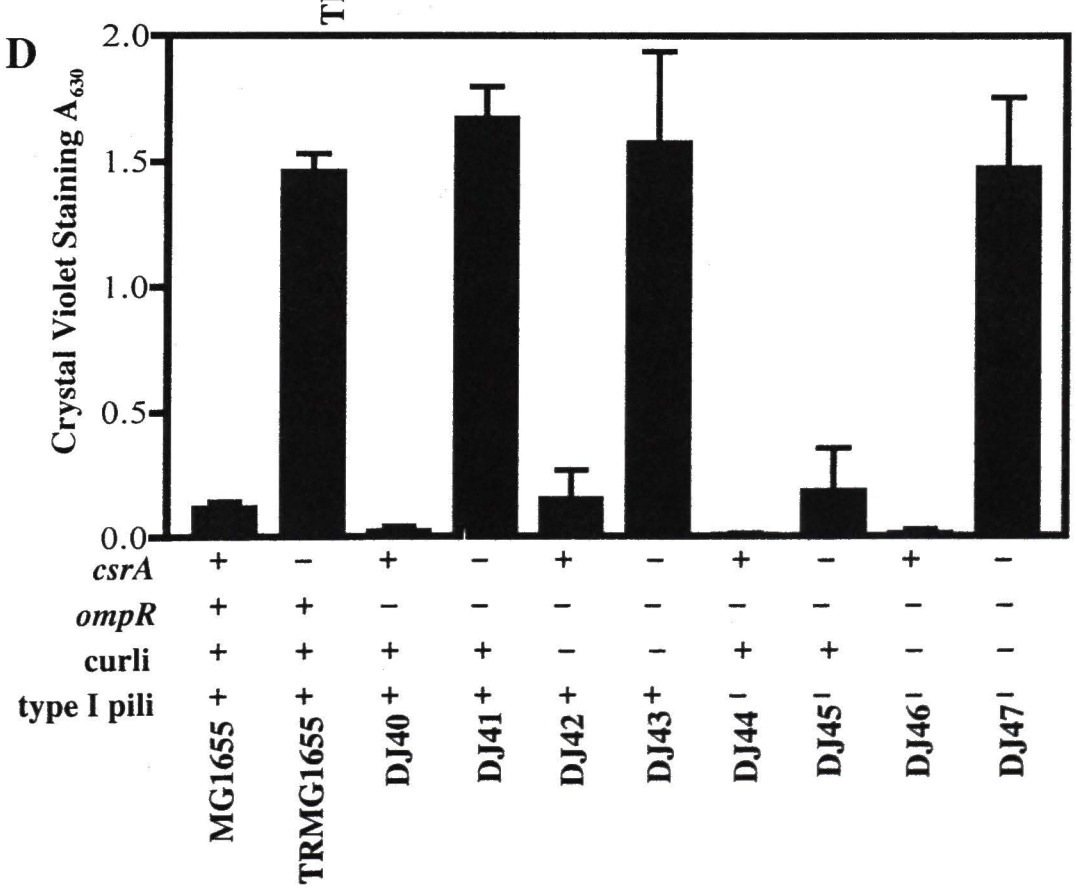
DJ34
DJ35
DJ36
DJ37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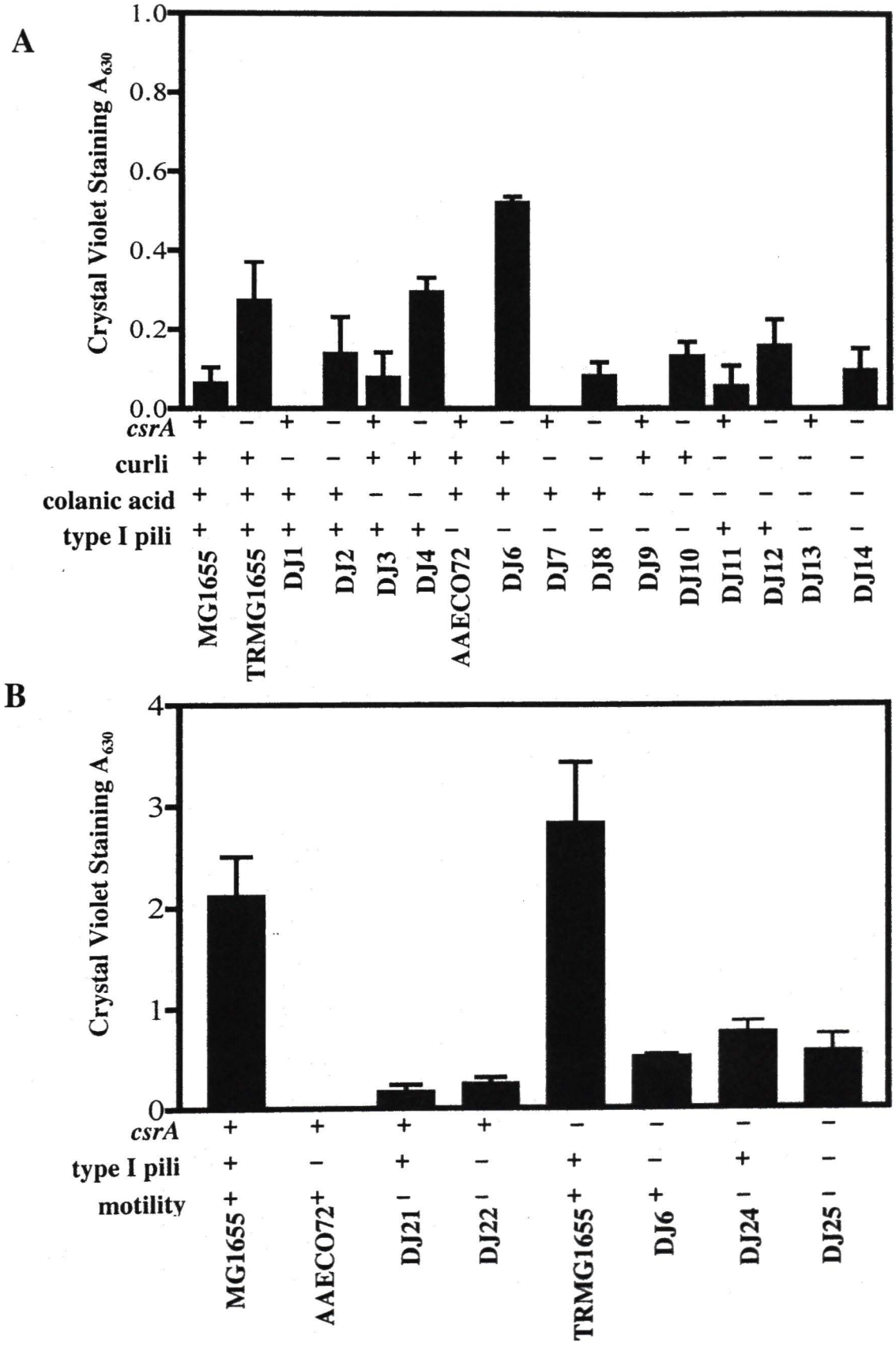
ompR + + − − − − − − − −
curli + + + + − − + + − −
type I pili + + + + + + − − − −
MG1655
TRMG1655
DJ40
DJ41
DJ42
DJ43
DJ44
DJ45
DJ46
DJ47

Fig. 2. Effects of extracellular factors and global regulators on biofilm formation in *E. coli* strain MG1655 and its isogenic csrA mutant in borosilicate glass tubes for 48 hours. Crystal violet (CV) staining of biofilms formed with mutations in: **A)** curli fimbriae, colanic acid, type I pili, or in combination; **B)** type I pili, motility, or both; **C)** RpoS, curli fimbriae, type I pili or combinations of these mutations; **D)** OmpR, curli fimbriae, type I pili or combinations of these mutations. Each bar shows an average and standard error of three separate experiments ($P<0.0001$).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type I pili
MG1655
TRMG1655
DJ1
DJ2
DJ3
DJ4
AAECO72
DJ6
DJ7
DJ8
DJ9
DJ10
DJ11
DJ12
DJ13
DJ14
B
Crystal Violet Staining A_{630}
4
3
2
1
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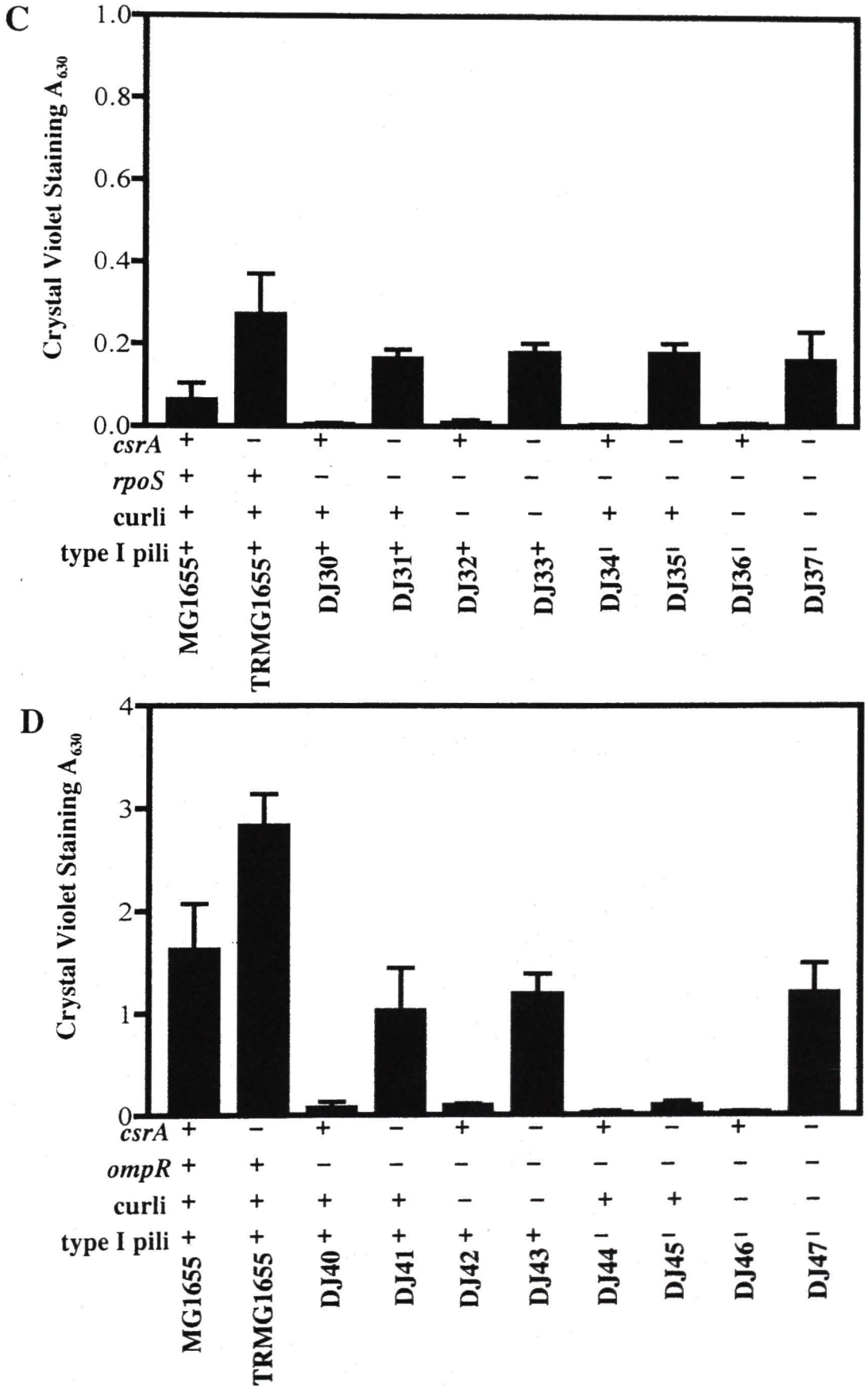
DJ6
DJ24
DJ25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curli + + + + − − + + − −
type I pili + + + + + + − − − −
MG1655+
TRMG1655+
DJ30+
DJ31+
DJ32+
DJ33+
DJ34I
DJ35I
DJ36I
DJ37I
D
Crystal Violet Staining A_{630}
4
3
2
1
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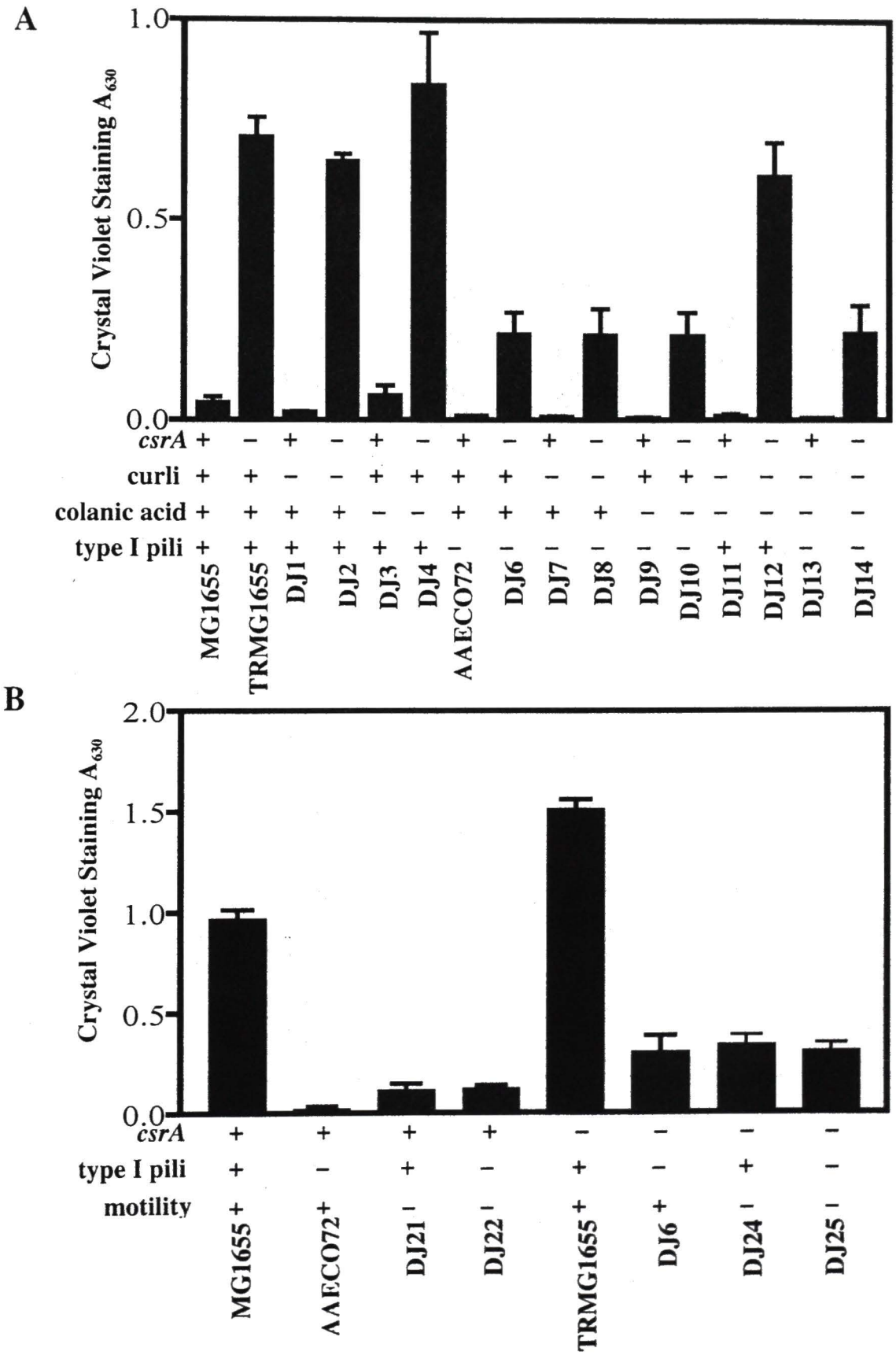
ompR + + − − − − − − − −
curli + + + + − − + + − −
type I pili + + + + + + − − − −
MG1655 +
TRMG1655 +
DJ40 +
DJ41 +
DJ42 +
DJ43 +
DJ44 I
DJ45 I
DJ46 I
DJ47 I

Fig. 3. Effects of extracellular factors and global regulators on biofilm formation in *E. coli* strain MG1655 and its isogenic csrA mutant in microtiter plates for 48 hours. Crystal violet (CV) staining of biofilms formed with mutations in: **A)** curli fimbriae, colanic acid, type I pili, or in combination; **B)** type I pili, motility, or both; **C)** RpoS, curli fimbriae, type I pili or combinations of these mutations; **D)** OmpR, curli fimbriae, type I pili or combinations of these mutations. Each bar shows an average and standard error of three separate experiments ($P<0.0001$).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curli + + – – + + + + – – + + – – – –
colanic acid + + + + – – + + + + – – – – – –
type I pili + + + + + + – – – – – – + + – –
MG1655
TRMG1655
DJ1
DJ2
DJ3
DJ4
AAECO72
DJ6
DJ7
DJ8
DJ9
DJ10
DJ11
DJ12
DJ13
DJ14
B
Crystal Violet Staining A_{630}
2.0
1.5
1.0
0.5
0.0
csrA + + + + – – – –
type I pili + – + – + – + –
motility + + – – + – + –
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DJ21
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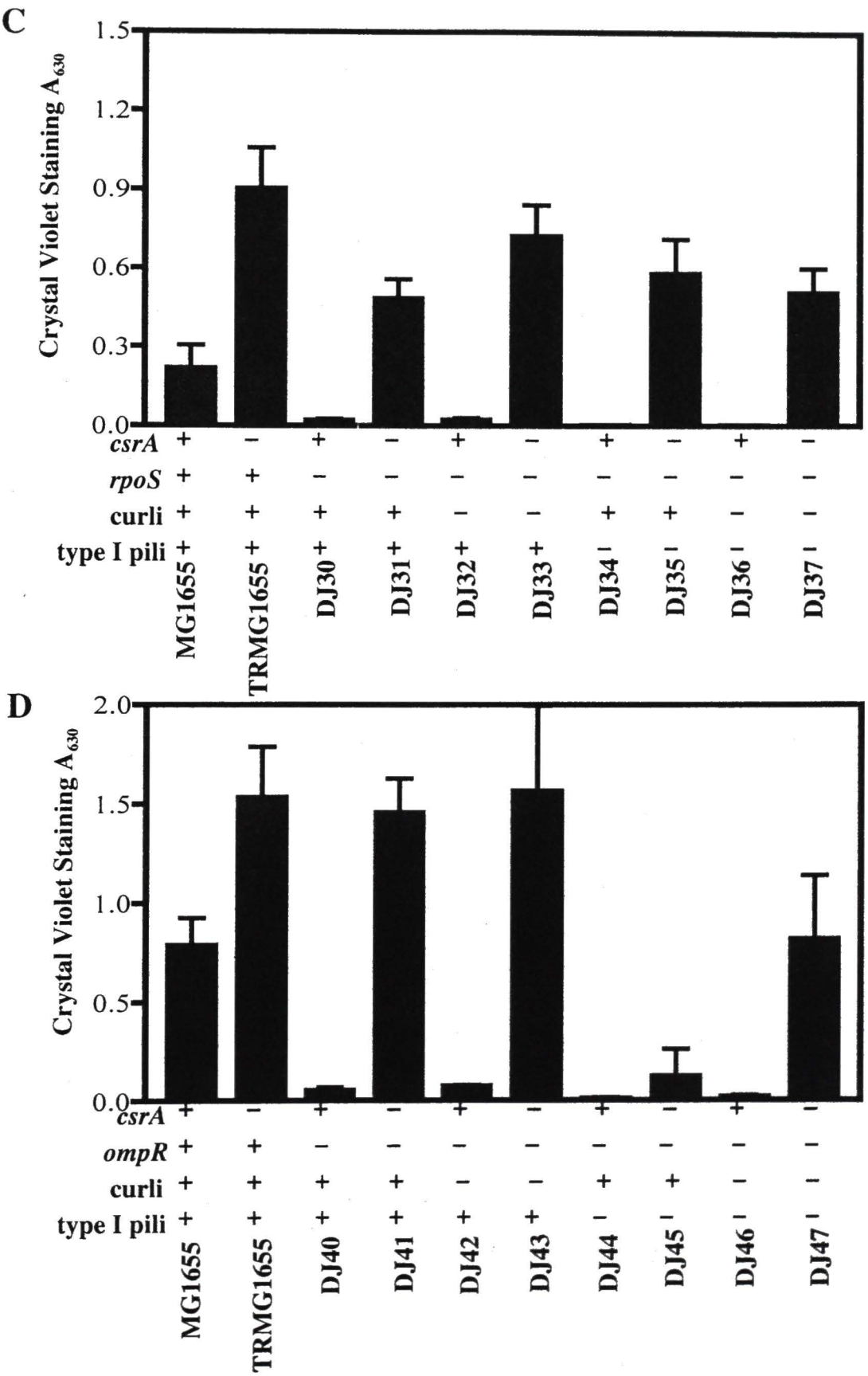
DJ6
DJ24
DJ25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0.9
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rpoS + + − − − − − − − −
curli + + + + − − + + − −
type I pili + + + + + + I I I I
MG1655
TRMG1655
DJ30
DJ31
DJ32
DJ33
DJ34
DJ35
DJ36
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D
Crystal Violet Staining A_{630}
2.0
1.5
1.0
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csrA + − + − + − + − + −
ompR + + − − − − − − − −
curli + + + + − − + + − −
type I pili + + + + + + I I I I
MG1655
TRMG1655
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Chapter V

CONCLUSIONS

Previously, CsrA was shown to repress biofilm formation in a qualitative study. This is the first study to quantitatively reveal that CsrA can function to repress biofilm formation. Evidence in this dissertation indicates that: 1) *csrA* is widely distributed among eubacteria; 2) biofilm formation is accelerated in a *csrA* mutant; 3) a *csrA* mutant will form a biofilm in the absence of each previously reported extracellular factor or regulator; 4) *csrA* expression levels are dynamically regulated during biofilm development; 5) redirection of carbon flux through glycogen metabolism is essential for optimal biofilm formation; 6) overexpression of CsrA represses biofilm formation in *E. coli* K-12 and related pathogenic bacteria; and 7) overexpression of *csrA* can cause the dispersal of a preformed biofilm under a variety of nutritional conditions.

CsrA appears to be confined to the eubacteria since *csrA* homologs have not been found in the archaebacteria or in the eukaryotes. This study revealed a widespread distribution of *csrA* among eubacteria. Homologs of *csrA* were found in four of the seven major divisions of eubacteria: Spirochaetales, Thermotogales, Firmicutes, particularly the

bacilli, and several subdivisions of Proteobacteria. The *csrA* gene is found in the Firmicutes (gram positive bacilli), which has been proposed to be one of the closest subdivision to the eubacterial progenitor, would suggest *csrA* evolved early in the eubacteria. CsrA homologs were not found in certain eubacteria: some Firmicutes, particularly the cocci (*Staphylococcus aureus*), α and β-Proteobacteria (*Agrobacterium tumefaciens* and *Neisseria meningitidis* respectively), and Cyanobacteria (*Synechococcus spp.*). It is interesting that homologs of *csrA* have not been found in the intracellular parasites: *Chlamydia trachomatis*, *Rickettsia proazekii,* and *Mycobacterium leprae.* This would suggest that these bacteria have found specialized niches that do not require *csrA.* Additionally, *csrA* homologs have not been found in *Mycoplasma genitalium*, the smallest free-living organism, but this is not surprising since it lacks virtually all other regulatory genes. Bacteria tend to maintain genes that confer a selective advantage in the natural environment. CsrA may confer a selective advantage by allowing bacteria with a *csrA* homolog to live in numerous environments. Bacteria with *csrA* homologs may be able to lower *csrA* expression to form a biofilm in harsh environments. With such a wide distribution of *csrA* in the eubacteria, it may be possible to develop antimicrobial drugs that would selectively eradicate microbial infections in medicine.

Biofilm formation in a *csrA* mutant was substantially accelerated. Our data indicate that the parent and *csrA* mutant had similar planktonic growth as indicated by their growth curves. The *csrA* mutant was more accelerated in biofilm formation than the parental strain even though they both produced biofilm primarily in stationary phase of growth. In the wild type and *csrA* mutant, it was found that biofilm formation did not accelerate until late stationary phase (28 hours). This lag in biofilm formation was again notice in experiments using the *csrA'-`lacZ* translational fusion, where in *csrA* expression levels decreased prior to biofilm growth in stationary phase. Previous studies have shown that glycogen synthesis and catabolism is repressed by *csrA*. The lag in biofilm formation may be attributed to the need for intracellular glycogen metabolism. We have shown that glycogen synthesis and glycogen turnover is essential for biofilm formation in the wild type strain MG1655 and its isogenic *csrA* mutant. Glycogen is made in the transition from exponential to stationary phase and subsequently degraded. The *csrA* mutant accumulates high levels of glycogen (up to 50% dry weight) and this may account for the increase seen in biofilm formation over the wild type strain in the growth curve. We have shown in a wild type strain that if either glycogen synthesis (*glgA*) or glycogen catabolism (*glgP*) is increased, biofilm formation will increase. It is not known whether

glycogen is used in biofilm formation as an energy store or whether glycogen functions as a precursor of one or more adhesins or factors needed for biofilm formation.

Using an inducible *csrA* gene, we examined the effect of overexpressing the *csrA* gene in a preformed biofilm. It was shown that overexpressing *csrA* caused dispersal of a preformed biofilm under a variety of nutrient conditions. To our knowledge, this is the first experiment to directly demonstrate biofilm dispersal under the influence of a regulatory gene. Using a *csrA'-`lacZ* translational fusion, *csrA* expression levels were found to increase in cells beyond 1.5 to 2 days in culture, in possible preparation for biofilm dispersal. One of the limitations in using borosilicate glass tubes or microtiter plates is that along with biofilm cells that are adherent there are also planktonic cells. This makes it difficult to get accurate data of cells that have dispersed from the biofilm since planktonic cells are still dividing. Even when replacing spent media with new media or a minimal salt, it is still difficult to get accurate data on dispersed cells, since adding the media also disrupts the biofilm and you have cells that are growing in the media. By using a flow cell to grow a biofilm, it may be possible to accurately assess biofilm dispersal by CsrA.

When glucose was added to a preformed biofilm and dispersal was induced by the overexpression of *csrA*, there was net biofilm growth. Apparently, an environmental

signal, in this case carbon (glucose), overcomes the dispersal signal. Thus, glucose seems to override the effects of CsrA on biofilm dispersal, suggesting bacterial response to nutritional cues has a strong effects on biofilm dispersal. To propagate to other sites, it is essential for cells to be dispersed from a biofilm. Perhaps a substantial supply of available carbon provides an environment that may facilitate the bacteria remaining in the biofilm and not dispersing. The environmental signals that affect CsrA, and therefore biofilm formation and dispersal, are unknown. Perhaps such processes also involve *csrB* regulation, but this remains to be addressed.

A *csrA* mutant will form a biofilm in the absence of each previously reported extracellular factors such as motility, type I pili, colanic acid, or curli fimbriae. Type I pili and motility are important for biofilm formation in a *csrA* mutant since each decreases biofilm formation in a *csrA* mutant; however, a *csrA* mutant still forms a significant biofilm in their absence. The wild type strains as well as the *csrA* mutants apparently form biofilms independently of surface properties since essentially the same results were found in borosilicate glass or polystyrene (microtiter plates). Similar trends were also observed when biofilm formation was quantified at 24 or 48 hours. The finding that a *csrA* mutant forms biofilm in the absence of motility, type I pili, curli fimbriae, or colanic acid strongly suggests that other factors not previously known to

participate in biofilm formation are involved. Future studies should reveal novel genes involved in biofilm formation in the *csrA* mutant.

The overexpression of the *csrA* gene repressed biofilm formation in the wild type and *csrA* mutant strain of *E. coli* K-12. Similarly, the overexpression of the *E. coli* K-12 *csrA* gene inhibited biofilm formation, although more modestly in urinary catheter strains and food-borne pathogens. It is not clear why CsrA exhibited weaker inhibition of biofilm formation in the enteric pathogens. It could be that these strains: 1) do not have the same number of copies of the plasmid encoding *csrA*; 2) the *csrA* gene is not properly expressed in these strains, or 3) these species have acquired new genes and the regulation of their biofilm formation has been changed. It would be interesting to generate *csrA* mutants of pathogenic strains and more thoroughly study biofilm development.

The role of CsrA in biofilm development is complex. CsrA activates flagella biogenesis yet represses biofilm formation. This is paradoxical since flagella are important for initial biofilm formation and a *csrA* mutant is nonmotile in most growth media. However, a *csrA* mutant is motile in CFA (Colony Forming Antigen) medium, which was the primary growth medium used in these studies. Glycogen, which is made in transition to stationary phase and later degraded, is needed for biofilm formation, but is repressed by CsrA. A possible scenario by which CsrA influences biofilm formation is

that CsrA represses biofilm formation through its repression of glycogen synthesis and glycogen catabolism. Both of these metabolic pathways are necessary for biofilm formation since our data indicate that even when glycogen synthesis is up regulated in a strain that has a polar mutation on the catabolic gene, *glgP*, there is no biofilm formation. Likewise when catabolism (*glgP*) is up regulated in a glycogen deficient strain (*glgA*), there is no biofilm formed until glycogen is synthesized (*glgA*) and catabolized (*glgP*). Why are glycogen synthesis and catabolism necessary? Glycogen is an intracellular molecule, so it is not likely to be an adhesion factor. Preliminary mutagenesis studies in our laboratory by Xin Wang indicate that there are novel extracellular factors that decrease biofilm formation in a *csrA* mutant. It may be that intracellular glycogen synthesis and catabolism drives the formation of these factors by providing the precursors needed for the expression of these extracellular factors. CsrA indirectly represses these factors through its repression of glycogen. Therefore, flagella are not essential for biofilm formation in a *csrA* mutant, and that other novel factors, synthesized through glycogen metabolism, play a major role in biofilm formation by the *csrA* mutant.

In considering a potential model for biofilm formation based upon these studies, planktonic cells are rapidly growing when there is an abundance of nutrients. However, when one or more nutrients start to become limiting, bacteria produce flagella. In

laboratory culture, this occurs in late exponential phase of growth, and may permit the cells to look for alternative nutrient supplies or to form a biofilm. These free-swimming or planktonic cells utilize their newly synthesized flagella to approach the surface (abiotic or tissue). During this time CsrA levels are relatively high since CsrA activates flagella biogenesis. CsrA levels decrease to signal for surface attachment and initiate biofilm formation. For stable biofilm attachment to the surface, an extracellular adhesion factor such as type I pili is needed. In some cases, bacteria tend to express extracellular adhesion factors before they have made contact with the surface, thereby aggregating together or clumping in a liquid medium. When the bacteria clump, they may or may not be able to make contact with a surface. Furthermore, clumping may not provide the necessary growth conditions to form a stable, mature biofilm. Even though glycogen synthesis occurs when *csrA* expression levels are high, there is positive regulation of glycogen synthesis at this time, such as cAMP -dependent stimulation of *glgCAP* transcription, which allows for glycogen synthesis and glycogen catabolism. Biofilm formation then proceeds as glycogen is catabolized. When the biofilm becomes limited for surface area, CsrA levels could increase to allow for dispersal of cells from the biofilm. Perhaps the dramatic effects of increasing CsrA levels by IPTG induction is

represented, in nature, by a more subtle process whereby a limited number of bacteria are being released from the biofilm.

The regulation of biofilm development by CsrA presents intriguing possibilities for the manipulation of eubacteria that form biofilms. I can envision the design of a possible mimic of CsrA, which can be used to cause release of cells from a biofilm. The resulting planktonic cells are expected to be much more sensitive to antibiotics than cells in a biofilm. Previous studies have indicated that biofilms can not be engulfed by macrophages, thus the macrophages release their oxidative burst on surrounding tissue. Dispersed cells would be less likely to result in tissue damage, since the macrophages will be able to engulf and eradicate the planktonic cells. Finally, it may be of use to increase biofilm formation in certain settings by using a CsrA antagonist. A shift to increase biofilm formation, by the decrease of CsrA or increase in intracellular glycogen metabolism, could be used to protect agricultural crops. In agriculture, bacterial biofilms protect certain plants from fungal infections.

The role of CsrA in biofilm development is a novel and provocative finding but many interesting questions still remain. What factors are essential for biofilm formation in a *csrA* mutant? Future studies will look for factors that affect biofilm formation in a *csrA* mutant. Also of interest are the environmental signals that affect biofilm formation

in a *csrA* mutant since in a *csrA* mutant biofilm formation is still temporally regulated. Mutagenesis studies should find novel factors affecting biofilm formation and environmental signaling in a *csrA* mutant. Although CsrA inhibits biofilm formation in some related enteric species, the effect of a *csrA* mutation in these strains is unknown. Future studies could focus on the role of CsrA in biofilm development of related enteric pathogens particularly those that utilize biofilm formation as a virulence factor. CsrA homologs in certain plant and animal pathogens repress the production of virulence factors. However, no study has addressed the role of *csrA* in biofilm formation in these species.

My studies have shown that CsrA serves as a critical global regulator of biofilm development. The studies, in this dissertation, should provide insight not only into novel mechanisms of biofilm formation but also into the regulation of biofilm dispersal. Maybe, through these studies, novel treatments of biofilm infection may be found. In addition, it is anticipated novel ways to genetically alter biofilm so that they are beneficial to industry and agriculture may be discovered.