CsgD, a regulator of curli and cellulose synthesis, also regulates serine hydroxymethyltransferase synthesis in Escherichia coli K-12

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The homologous CsgD and AgfD proteins are members of the FixJ/UhpA/LuxR family and are proposed to regulate curli (thin aggregative fibres) and cellulose production by Escherichia coli and Salmonella enterica serovar Typhimurium, respectively. A plasmid containing part of the csgD gene was isolated during a screen for multicopy suppressors of glycine auxotrophy caused by deleting the folA gene in E. coli. The sequence of the plasmid suggests it encodes a chimaeric protein. Plasmids containing the intact csgD or agfD gene also caused suppression. Cells transformed with the recombinant plasmids contained higher serine hydroxymethyltransferase (SHMT) activity than controls. The increase could also be monitored by assaying β-galactosidase activity from a reporter strain with part of the SHMT gene, glyA, fused to lacZ. The increase in SHMT activity was sufficient to correct the glycine auxotrophy of strains lacking folA. The recombinant plasmids also enabled K-12 strains that are not curli-proficient to make curli. Curlin, the major component of curli, contains more glycine than normal E. coli proteins. It is proposed that CsgD upregulates glyA to facilitate synthesis of curli. It is suggested that recombinant plasmids produce enough CsgD or chimaeric protein to titrate out a ligand that switches CsgD into its inactive form. As a result, sufficient active CsgD is present to activate genes in its regulon. It is concluded that CsgD increases expression of the glyA gene either directly or indirectly.

INTRODUCTION

Commensal strains of Escherichia coli are found in the lower intestine of many different animal species (Souza et al., 1999). Pathogenic strains of E. coli can cause diarrhoea of varying severity, urinary tract infections and sepsis in humans, and a variety of diseases in domestic animals. Because of their wide geographical distribution and primary habitat, they are also common in soil and water contaminated with faeces. Their numbers can increase in these habitats given a favourable environment and relatively low predation (Solo-Gabriele et al., 2000).

Thus, E. coli switches between colonizing an animal host and water or soil. Colonization of a new habitat by bacterial cells is enhanced by their ability to attach to surfaces; such attachment is facilitated by fimbriae. E. coli can produce fimbriae called curli (Olsén et al., 1989). Salmonella enterica produces homologous fimbriae called thin aggregative fibres or SEF17 (Collinson et al., 1991; Römling et al., 1998a). We will use curli to refer to these fimbriae in both E. coli and S. enterica. Curli promote binding to a variety of host proteins, increase internalization of bacterial cells by eukaryotic cells in culture and trigger events leading to septic shock (Olsén et al., 1989, 1998; Sjöbring et al., 1994; Ben Nasr et al., 1996; Bian et al., 2000; Gophna et al., 2001; Uhlich et al., 2002). Curli also promote clumping of bacterial cells in culture and binding to abiotic surfaces such as glass and polystyrene, making them important for biofilm formation (Vidal et al., 1998; Römling et al., 1998b; Austin et al., 1998; Prigent-Combaret et al., 2000).

Although E. coli, S. enterica serovars Typhimurium (Salty) and Enteritidis, and Shigella species have the genes required for curli synthesis, the expression of curli is not common to all strains. Many strains contain insertion sequence disruptions or deletions in the genes encoding curli components and assembly factors, or mutations elsewhere that block curli formation (Olsén et al., 1993b; Arnqvist et al., 1994; La Ragione et al., 1999; Uhlich et al., 2001; Sakellaris et al., 2000). Natural isolates of enterohaemorrhagic, enterotoxigenic and sepsis E. coli strains from humans, and isolates from avian and bovine infections, are frequently curliated, whereas those from enteroinvasive and enteropathogenic strains are not (Olsén et al., 1989, 1993a; Maurer, 1998; Bian et al., 2000; Uhlich et al., 2001). E. coli K-12 strains generally are not curli-proficient but can produce curli as a result of mutations (Arnqvist et al.,...
1992, 1994; Vidal et al., 1998). Expression of curli in E. coli and Salty normally occurs during stationary phase and requires growth at low temperature and low osmolarity, but some strains and S. enterica serovar Enteritidis can express curli at 37 °C (Olsén et al., 1989; Collinson et al., 1991; Römling et al., 1998b; Uhlich et al., 2001).

The csg (agf) genes in S. enterica are required for curli synthesis. They are organized in two adjacent divergently transcribed operons. The csgBA (agfBA) operon contains the gene (agfB) for a nucleator protein and the gene (agfA) for curli, the major protein component of the curli fibre. The csgDEFG (agfDEFG) operon contains a putative regulatory gene (csgD), a gene (csgG) encoding a lipoprotein involved in secretion of curlin and CsgB, a gene (csgE) encoding a chaperone-like protein and a gene (csgF) encoding a second nucleator protein (Collinson et al., 1996, 1997; Hammar et al., 1995, 1996; Loferer et al., 1997; Römling et al., 1998a; Chapman et al., 2002).

Changes in the csgD (agfD) promoter region can influence curli formation. Some isolates of the enteroinvasive E. coli O157:H7 show a phase shift between curliated and non-curliated cells that results from a single base pair change in the csgD promoter (Uhlich et al., 2002). A change in the agfD promoter in Salty enables the cells to make curli at 37 °C (Römling et al., 2000).

The csgD-encoded protein is homologous to proteins of the FixJ/UhpA/LuxR family, suggesting that it is regulatory protein (Hammar et al., 1995). This role is supported by the observations that inactivation of csgD prevents the transcription of the csgBA operon (Hammar et al., 1995) and of adrA, a putative regulatory gene required for cellulose synthesis (Römling et al., 2000; Zogaj et al., 2001). Cellulose is found in the matrix of E. coli and Salty biofilms. The FixI/UhpA/LuxR family is characterized by a DNA-binding C-terminal domain and an N-terminal receiver domain. Depending on the regulator, the receiver domain is either phosphorylated or binds homoserine lactone or other small molecules (Kahn & Ditta, 1991; Sitiukov et al., 1995; Baikalov et al., 1996; Ducros et al., 2001). As yet there is no evidence for DNA binding by CsgD, so it is not known whether it controls the csgBA operon and the adrA gene directly or indirectly. Similarly, it is not known what ligand modulates its function. Expression of csgD or agfD increases in late exponential phase and is influenced by temperature, osmolarity and the availability of nutrients, oxygen and iron (Römling et al., 1998b; Prigent-Combaret et al., 2001; Gerstel & Römling, 2001).

In this study, we show that csgD is a multicyclic suppressor of the glycine auxotrophy of a strain lacking dihydrofolate reductase (DHFR) activity. DHFR is required for the de novo synthesis of tetrahydrofolate (THF) and for the recycling of dihydrofolate produced during thymidylate synthesis. THF is converted to methylene THF during glycine synthesis or degradation. Methylenetetrahydrofolate is required for thymidylate and pantothenate biosynthesis and is the precursor for methyl-THF and formyl-THF. Methyl-THF is required for methionine biosynthesis. Formyl-THF is used for purine (and, indirectly, histidine) synthesis and for the formulation of Met-trNAfmet (reviewed by Matthews, 1996).

Strains with the DHFR gene (folA) deleted grow slowly on rich or supplemented minimal medium. The supplements required depend on the strain background, but most strains will grow with thymidine or a combination of folate end-products (FEP: thymine, glycine, histidine, methionine, adenosine and pantothenic acid) (Ahrweiler & Frieden, 1988; Howell et al., 1988; Hamm-Alvarez et al., 1990; Krishnan & Berg, 1993; Herrington & Chirwa, 1999). When glycine or glycine and histidine are omitted from the mixture the cells grow extremely slowly, and when methionine or methionine and histidine are omitted the cells do not grow. Under these conditions the purine pathway is repressed and this could reduce turnover of formyl-THF (Herrington & Chirwa, 1999). We show here that plasmids containing csgD sequences enabled faster growth in the absence of glycine. This results from an increased expression of glyA, the gene encoding serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), suggesting that csgD also regulates glyA.

METHODS

Bacterial strains and plasmids. E. coli K-12 strains and plasmids are listed in Table 1.

Media and growth conditions. Minimal medium A with glucose (GM), and Luria–Bertani (LB) medium containing 50 g thymidine ml⁻¹ (LB-Thy) (Miller, 1992) were used routinely. Media were solidified with 15 g agar l⁻¹. GM was supplemented with amino acids and thymidine at 50 μg ml⁻¹, adenine at 30 μg ml⁻¹ and pantothenate at 1 μg ml⁻¹. Media containing subsets of FEP were identified by indicating the missing FEP. For example, FEP-His-Met is GM with glycine, adenine, pantothenate and thymidine, but not histidine or methionine. Any compounds required by strains were also supplied. Ampicillin (100 μg ml⁻¹) was always added when growing ampicillin-resistant transformants. Chloramphenicol (25 μg ml⁻¹), spectinomycin (100 μg ml⁻¹), streptomycin (20 μg ml⁻¹) and tetracycline (25 μg ml⁻¹) were added when required. Congo red plates (Hammar et al., 1995) with added thymidine were used to test curli proficiency. Liquid cultures were grown at the indicated temperatures with shaking. Growth on solid media was tested by spotting 10 μl of dilutions of overnight cultures grown in LB-Thy and by monitoring colony formation (Herrington & Chirwa, 1999).

Genetic and molecular techniques. P1 transductions (Miller, 1992) were performed with either P1CM or P1vir. P1CM lysates prepared from JC1089 were used to lysogenize other strains as needed. P1vir was obtained from C. G. Cupples, Concordia University, Montreal, Canada.

Normally, cells were rendered competent for transformation using CaCl₂ (Sambrook, 1989). To avoid heat shock, the one-step PEG method (Chung et al., 1989) was used to make strain GS162 glyA–lacZ and its derivatives competent.

Strain construction. P1vir-mediated transduction was used to transfer the purR6::::Tn10 mutation from strain SO5052 to strain MH829. We constructed strains MH910 and MH911 by, respectively, transducing csgA2::::Tn105 and csgG1::::Tn105 mutations into
Strain or plasmid | Description | Source* or reference
--- | --- | ---
**Strains**
FB10186 | csgD::Tn5<KAN-I-SceI> | F. Blattner
GS162, glyA–lacZ | ΔlacI69 pheA905 araD129 rpsL thi | G. Staufer; Lorenz & Stauffer (1995)
JC10289 | recA::Tn10 P1CM | M. Belfort; Csonka & Clark (1979)
MG1655 | Wild-type | M. Singer
MH829 | ΔfolA::kan3 thyA(ts) argE3 rna λ | Herrington & Chirwa (1999)
MH894 | ΔfolA::kan3 thyA(ts) argE3 rna purR λ | This study
MH901 | ΔlacI69 pheA905 araD129 rpsL thi csgA::Tn10 glyA–lacZ | This study
MH902 | ΔlacI69 pheA905 araD129 rpsL thi csgG::Tn10 glyA–lacZ | This study
MH910 | ΔfolA::kan3 thyA(ts) argE3 rna csgA::Tn10 | This study
MH911 | ΔfolA::kan3 thyA(ts) argE3 rna csgA::aadA | This study
MH917 | thyA(ts) argE3 rna ΔfolA::aadA csgD::Tn5<KAN-I-SceI> | This study
MH937 | thyA(ts) argE3 rna ΔfolA::aadA csgD::Tn5<KAN-I-SceI> | This study
MH938 | ΔlacI69 pheA905 araD129 rpsL thi csgD::Tn5<KAN-I-SceI> (glyA–lacZ) | This study
MHR204 | araD139 Δ(argF–lac)U169 rpsL150 relAI flbB deoC ptsF25 rbsR csgA::Tn105 | S. Normark; Hammar et al. (1995)
MHR210 | araD139 Δ(argF–lac)U169 rpsL150 relAI flbB deoC ptsF25 rbsR csgG::Tn105 | S. Normark; Hammar et al. (1995)
SO502 | lacZ608(4Am) purR6::Tn10 rpsL thi | Coli Genetic Stock Center; Kilstrup et al. (1989)
**Plasmids**
pBR322 | Vector | B. R. Glick; Sutcliffe (1978)
pCP994 | pKK233-2 with a 697 fragment containing the csgD ORF | C. Prigent-Combaret; Vidal et al. (1998)
PCSGD | Salty csgD gene inserted into pWSK29 | U. Römling; Römling et al. (1998a)
pGS29 | 3-34 kb SalI–EcoRI fragment containing the glyA gene inserted into pBR322 | E. B. Newman; Stauffer et al. (1981)
pKK233-2 | Vector | C. G. Cupples; Amann & Brosius (1985)
pS66P | 210 bp HindIII csgD fragment in pUC18 | This study
pUC18 | Vector | C. G. Cupples; Messing, 1983; Yanisch-Perron (1985)

*F. Blattner, University of Wisconsin, Madison, WI, USA; M. Belfort, Wadsworth Center, Albany, NY, USA; C. G. Cupples, Concordia University, Montreal, Canada; B. Glick, University of Waterloo, Waterloo, Canada; E. B. Newman, Concordia University, Montreal, Canada; S. Normark, Karolinska Institute, Stockholm, Sweden; C. Prigent-Combaret, INSA, Lyon, France; U. Römling, Karolinska Institute, Stockholm, Sweden; M. Singer, University of California at Davis, USA; G. Staufer, University of Iowa, Iowa City, USA; Coli Genetic Stock Center, Yale University, Princeton, NJ, USA; Mary Berlyn, Curator (strain was obtained from B. Bachmann).

strain MH829 with P1vir lysates made from strains MHR204 and MHR210. Strains MH901 and MH902 were obtained by transferring these mutations to strain GS162, glyA–lacZ. P1CM lysates prepared on strain FB10186 were used to transduce the csgD::Tn5<KAN-I-SceI> mutation to strains MH917 and GS162, glyA–lacZ. Construction of the spectinomycin-resistant folA null mutant MH917 was similar to that of strain MH829 (Herrington & Chirwa, 1999).

Isolation and identification of multicopy suppressors. Strain χPh43 is a double lysogen for Mu cts and the mini-Mu replicon MudHI4042 which confers chloramphenicol resistance on the host strain (Groisman & Casadaban, 1987). Lysates from strain χPh43 were used to prepare mini-Mu lysogens of MG1655. Mini-Mu lysates prepared on strain MG1655 were then used to transduce strain MH829/Mu cts at 31°C. Chloramphenicol-resistant colonies were selected and then were replica-plated to screen for growth on various media.

Suppressing plasmids were restricted and the resulting fragments were ligated into pUC18. DH5α was transformed with the ligation mixture. Recombinant plasmids were tested for their effect on growth of strain MH829. Inserts in suppressor plasmids were sequenced by the York University Core Molecular Facility, Toronto, Canada.

Enzyme assays. Cells were grown at 37°C to an OD600 between 0.4 and 0.8 for all enzyme assays. Threonine dehydrogenase assays were measured in toluene-permeabilized cells (Ravnikar & Somerville, 1987). The SHMT- (Taylor & Weissbach, 1965) and glycine-cleavage activities were assayed in crude extracts prepared by sonication cells. Glycine cleavage was assayed by a modification of the SHMT assay. The reaction mixture contained 50 mM potassium phosphate, pH 7.4, 5 mM DTT, 1-14 mM THF, 1 mM pyridoxal phosphate and 2-14Cglycine (0-16 mGm M⁻¹). Reactions were incubated at 32°C. Reactions were stopped and analysed as for SHMT. Protein was measured by the modified bicinchoninic acid.

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procedure (Brown et al., 1989). β-Galactosidase activity in reporter strains for glyA (Lorenz & Stauffer, 1995) and goT (Jourdan & Stauffer, 1998) expression was assayed as described by Miller (1992) using the SDS/chloroform lysis method.

Curli formation. Curli proficiency was assayed on Congo red plates (Hammar et al., 1995), by the ability of cells to bind to polystyrene (Vidal et al., 1998) and by the binding of Congo red in solution (Gophna et al., 2001). Congo red binding units were 1000 (ΔA500/OΔD600) where ΔA500 was the difference between the A500 value of the Congo red solution without cells and the A500 value measured after cells had been incubated in the solution and then removed. OD600 measurements for these assays were made on Milton Roy Spectronic 1001 Plus apparatus, using a 1 cm path length. Cells were diluted in 0-9 % saline.

RESULTS

A plasmid containing part of the csgD gene is a multicyclic suppressor

A mini-Mu lysate prepared on the wild E. coli strain MG1655 was used to transduce strain MH829/Mu cts to chloramphenicol resistance. A total of 59 195 chloramphenicol-resistant colonies were screened on FEP-Gly, FEP-Met, FEP-His-Met and FEP-His-Gly. The majority of colonies did not grow on FEP-Gly or FEP-Met but grew slowly on FEP-His-Gly and FEP-His-Met. Fifty of these grew on FEP-Gly and on FEP-Met and 16 grew on FEP-Gly but not on FEP-Met. This suggested that different suppressors were obtained in this screen. Plasmids were isolated and transformed into strain MH829/Mu cts to confirm the phenotype.

Mini-Mu plasmids can carry insertions of up to 22·3 kb (Groisman & Casadaban, 1987). To reduce the size of the inserts, HindIII fragments of suppressing plasmids were subcloned into the high-copy-number vector pUC18. The resultant recombinant plasmids were tested for their ability to correct the growth defect of strain MH829.

One recombinant plasmid, pSD6P, allowed growth on FEP-Gly but not on FEP-Met. It contained a 210 bp insert. The sequence of the insert was determined and a BLAST (Altschul et al., 1997) search of the E. coli genome sequence indicated that it matched part of the csgD gene (GenBank accession no. AE000205). When pSD6P was analysed for ORFs, two were observed. One corresponded to the bla gene of pUC18 (GenBank accession no. L08752). The other, which is in the opposite orientation to the sequence encoding the lacZ α fragment, initiated at the complement of nt 499 of pUC18, spanned the insert and terminated at complement of nt 143 of pUC18. The predicted chimaeric protein contains 70 aa corresponding to the central region of CsgD (Fig. 1). The presence of a weak promoter located at the complement of nucleotide 746 of pUC18 suggests the chimaeric ORF could be transcribed.

If suppression results from expression of a chimaeric protein, changing the orientation of the insert in pSD6P should eliminate suppression. When pSD6P was cut with HindIII, ligated, and transformed into strain MH829, approximately 50 % of the transformants did not grow on FEP-Gly. One non-suppressing plasmid was sequenced and shown to have the insert in the opposite orientation to pSD6P. These results suggest that a chimaeric protein made by pSD6P was responsible for suppression.

Plasmids containing intact csgD genes suppress Colony formation on LB-Thy and FEP-Gly was monitored in strain MH829 transformed with different plasmids containing csgD or agfD sequences and with control plasmids. All transformants produced small colonies within 24 h on LB-Thy (Fig. 2a and Table 2). Transformants with pSD6P (partial csgD), pCP994 (intact csgD coding region) or pCSGD (agfD) from S. enterica serovar Typhimurium formed colonies on FEP-Gly within 4 days. In contrast, the pUC18 control made no colonies (pUC18) and the pKK233-2 control made extremely small colonies after 5 days (Fig. 2b and Table 2). The efficiency of plating on LB-Thy and on FEP-Gly was similar. These results indicate that multiple copies of the intact csgD gene also suppress the glycine auxotrophy.

Transformed cells were grown in liquid FEP-Gly containing ampicillin with varying amounts of glycine. In the absence of glycine or at low concentration, the yield of cells transformed with pCP994 was higher than those transformed with PPK233-2 (Fig. 3). Similar results were obtained with pSD6P transformants (data not shown).

SHMT activity is increased by expression of CsgD from a plasmid

The csgD gene could reduce the requirement for exogenous glycine by increasing synthesis or decreasing degradation. Most of the glycine made by E. coli is produced from serine by SHMT (Stauffer, 1996) with the concomitant production of 5,10-methylene THF from THF. To test whether csgD sequences modified the expression of SHMT, we assayed SHMT activity in transformants of
strains MH828 (folA+ thyATS), MH829 (ΔfolA::kan3 thyA105) and GS162glyA–lacZ (folA+ thyA+). SHMT activity was significantly and reproducibly 1.5- to 3-fold higher in pSD6P and pCP194 transformants compared to control transformants when MH829 and GS162glyA–lacZ transformants were grown in GM+Thy and when MH828 and GS162glyA–lacZ transformants were grown in FEP-Gly (Table 3). Although pSD6P transformants of strain MH828 grown in GM+Thy had higher activity than control transformants in the experiment shown (Table 3), this difference was not observed in other experiments.

SHMT activity was higher in the ΔfolA strain MH829 than in the folA+ strains. Similar results were observed in a wild-type strain when DHFR was inhibited by trimethoprim (Stauffer, 1996). This could be a response to limited glycine, methionine and purine synthesis when DHFR is not available.

Growth in the presence of purines and methionine normally represses SHMT (Mansouri et al., 1972; Miller & Newman, 1974; Greene & Radovich, 1975). We observed repression on FEP-Gly with strain GS162glyA–lacZ transformants, but not with the strain MH828 transformants. This suggests that the thyA mutation in strain MH828 affected the regulation of SHMT. Under the conditions used, strains with the thyA(ts) allele normally express 5% of wild-type thymidylate synthase activity (Herrington & Chirwa, 1999). The reduced demand for methylene THF for thymidylate synthesis could be perceived by the cell as a signal to provide more formyl-THF for protein synthesis; hence the upregulation of SHMT.

### Table 2. Growth of strain MH829 and derivatives on FEP-Gly

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Test plasmid</th>
<th>Control plasmid</th>
<th>Growth*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No plasmid</td>
<td>Test plasmid</td>
<td>Control plasmid</td>
</tr>
<tr>
<td>MH828</td>
<td>folA+</td>
<td>None</td>
<td>None</td>
<td>1†</td>
</tr>
<tr>
<td>MH829</td>
<td>ΔfolA::kan3</td>
<td>None</td>
<td>None</td>
<td>5†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pSD6P</td>
<td>pUC18</td>
<td>NA  NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCP994</td>
<td>pKK233-2</td>
<td>4  NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCSGD</td>
<td>pUC18</td>
<td>4  5‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pGS29</td>
<td>pBR322</td>
<td>4  4</td>
</tr>
<tr>
<td>MH894</td>
<td>ΔfolA::kan3 purR6::Tn10</td>
<td>None</td>
<td>None</td>
<td>4  NA</td>
</tr>
<tr>
<td>MH910</td>
<td>ΔfolA::kan3 csgA2::Tn105</td>
<td>pCP994</td>
<td>pKK233-2</td>
<td>4  5‡</td>
</tr>
<tr>
<td>MH937</td>
<td>ΔfolA::aadA csgD::Tn5&lt;KAN-I-SceI&gt;</td>
<td>pCP994</td>
<td>pKK233-2</td>
<td>4  5‡</td>
</tr>
<tr>
<td>MH937</td>
<td>ΔfolA::aadA csgD::Tn5&lt;KAN-I-SceI&gt;</td>
<td>pSD6P</td>
<td>pUC18</td>
<td>5  5‡</td>
</tr>
<tr>
<td>MH911</td>
<td>ΔfolA::kan3 csgG1::Tn105</td>
<td>pCP994</td>
<td>pKK233-2</td>
<td>4  5‡</td>
</tr>
</tbody>
</table>

*Growth is expressed as the days required for colony formation on FEP-Gly with the highest dilution of cells spotted; –, no colonies observed at 5 days; NA, not applicable. The highest dilution of all strains formed colonies on LB-Thy at 1 day.
†Herrington & Chirwa (1999).
‡Colonies were barely visible at 5 days.
Table 3. SHMT activity in transformants

<table>
<thead>
<tr>
<th>Medium</th>
<th>Strain</th>
<th>SHMT activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pUC18</td>
</tr>
<tr>
<td>GM + Thy</td>
<td>MH828</td>
<td>7 ± 0.3</td>
</tr>
<tr>
<td>GM + Thy</td>
<td>MH829</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>FEP-Gly</td>
<td>MH828</td>
<td>20 ± 0.8</td>
</tr>
<tr>
<td>GM</td>
<td>GS162ΔglyA–lacZ</td>
<td>ND</td>
</tr>
<tr>
<td>FEP-Gly</td>
<td>GS162ΔglyA–lacZ</td>
<td>ND</td>
</tr>
</tbody>
</table>

*SHMT activities (nmol min⁻¹ mg⁻¹) are means ± SD obtained by averaging the activities from at least two cultures done in duplicate. Values for MH828 and MH829 transformants are from one experiment and those for GS162ΔglyA–lacZ transformants were from a separate experiment. Repeated experiments gave similar results except for strain MH828 grown in GM + Thy, where the difference was not reproducible. Values for the control and csgD-containing plasmids were compared using Student’s t-test; *indicates conditions in which the csgD plasmid significantly increased the activity in this experiment and in other trials; ND, not determined.

Expression of β-galactosidase from a glyA–lacZ fusion is also increased

Strain GS162ΔglyA–lacZ is lysogenized with the glyA reporter, ΔglyA–lacZ. This phage contains the entire hmp–glyA intergenic region and the sequence encoding the first 50 aa of SHMT fused in-frame with the lacZ gene (Lorenz & Stauffer, 1995). β-Galactosidase activity (Table 4) in strain GS162ΔglyA–lacZ transformed with pKK233-2 and pCP994 paralleled SHMT activity (Table 3). In all media tested, the presence of pCP994 elicited 1.5- to 3-fold more β-galactosidase activity than the control plasmid pKK233-2. A similar but smaller increase (1.3–1.4-fold) in β-galactosidase activity was observed with pSD6P transformants compared to pUC18 transformants (data not shown).

Both the SHMT activity assay and the assay of β-galactosidase from the glyA–lacZ fusion indicated that SHMT activity was increased by the presence of csgD or the chimaeric protein. This was not simply caused by overproduction of any protein. We observe suppression only with some recombinant plasmids although many would be expected to overexpress protein. Moreover, deliberate high-level expression of luciferase in E. coli actually reduces glyA expression (Oh & Liao, 2000).

Increased SHMT activity was sufficient for growth of strain MH829 on FEP-Gly

Several lines of evidence indicate that increasing the SHMT activity above a threshold level is sufficient to enable strain MH829 to make enough glycine and methylene THF to grow on FEP-Gly. SHMT activity could be increased by

Table 4. Expression of β-galactosidase from the ΔglyA–lacZ fusion

Transformants were grown in the media indicated. β-Galactosidase activities were normalized against the activity of control plasmid pKK233-2 transformants that were grown without FEP. One hundred per cent activity for strain GS162ΔglyA–lacZ was 7100 Miller units (Miller, 1992); for strain MH901, 8750 Miller units; and for strain MH902, 12 000 Miller units. The SD was less than 15% in all samples. Data are from two representative experiments in which two cultures of each transformant were assayed in duplicate. Strain GS162ΔglyA–lacZ was assayed under all growth conditions in one experiment, and strains MH901 and MH902 were assayed in the other. Experiments were reproduced at least twice.
transforming cells with the glyA-containing plasmid, pGS29 (Stauffer et al., 1981), by inactivating the PurR repressor by mutation of purR (Steiert et al., 1990), or by growth in FEP-His-Gly (Table 4). Strain MH829 grows on FEP-His-Gly (Herrington & Chirwa, 1999) and grew on FEP-Gly when transformed with pGS29 or made purR− (Table 2), indicating that the increase in SHMT activity was sufficient to compensate for the lack of glycine in the medium.

Are there alternative sources of glycine in cells transformed with a csgD-containing plasmid?

Glycine can also be produced from threonine via threonine dehydrogenase (Fraser & Newman, 1975; Ravnikar & Somerville, 1987) or can be spared by reducing glycine cleavage (Stauffer, 1996). We assayed threonine dehydrogenase activity and glycine-cleavage activity in transformants of MH829 and β-galactosidase activity in transformants of GS162::glyT–lacZ. These activities were very low, and there were no reproducible differences between control and csgD transformants when transformants were grown in FEP-Gly (data not shown). Therefore, glycine was not provided by either of these routes.

Are curli produced in transformants?

Curli are not expressed by most laboratory strains of E. coli, but expression of csgD from a plasmid induces their formation (Prigent-Combaret et al., 1999). We tested for curli formation by spotting cells on Congo red plates (Hammar et al., 1995), by measuring Congo red binding (Gophna et al., 2001) and by monitoring binding to polystyrene (Prigent-Combaret et al., 1999). On Congo red plates, spots of strains MH829 (folA::kan3) and MH828 (folA+) were white, indicating that they do not produce curli (data not shown). Both strains transformed with the control plasmids pUC18 and pCP994 pKK233-2 were white, whereas pSD6P and pCP994 transformants were red (Fig. 2c and data not shown). Strain MH828 transformed with control plasmids pUC18 and pKK233-2 bound 49 ± 6-5 and 47 ± 0-1 Congo red units, respectively, whereas pSD6P and pCP994 transformants bound 67 ± 7-0 and 96 ± 32 units, respectively. The csgD plasmids also promoted binding to plastic whereas the control plasmids pUC18 and pCP994 pKK233-2 were white, whereas pSD6P and pCP994 transformants were red (Fig. 2c and data not shown). Strain MH828 transformed with control plasmids pUC18 and pKK233-2 bound 67 ± 7-0 and 96 ± 32 units, respectively. The csgD plasmids also promoted binding to plastic whereas the control plasmids did not (data not shown). These results indicate that our strains made curli when the csgD plasmids were present.

Does strain MH829 have to produce curli to grow on FEP-Gly?

To determine whether the ability to make curli is necessary for growth on FEP-Gly or increasing glyA expression we constructed csgA− and csgG− derivatives of strains MH829 and GS162::glyA–lacZ. Both genes are essential for curli biogenesis. Transformants of strains MH910 (folA (folA csgA)) and MH911 (folA csgG) were tested for growth on FEP-Gly (Table 2). The csg mutations did not affect growth. β-Galactosidase activity was higher in pCP994 transformants of the glyA–lacZ fusion strains MH901 and MH902 than in control transformants (Table 4). We conclude that formation of curli fibres was not necessary for the CsgD-mediated suppression of glycine auxotrophy or elevation of glyA expression.

Is chromosomally encoded CsgD involved?

To determine if the chromosomal csgD gene was required for suppression by the plasmids pSD6P and pCP994, we constructed csgD− derivatives of strains MH917 and GS162::glyA–lacZ, and transformed the resultant strains with pSD6P, pCP994 and control plasmids. The pCP994 transformants of strain MH937 (folA::aadA csgD+) grew as well as transformants of MH829 (Table 2) and were red on Congo red plates (data not shown). In contrast, pSD6P transformants grew poorly and did not stain as strongly on the Congo red plates. The activity of β-galactosidase in pCP994 transformants of the csgD− reporter strain MH938 was higher than in the controls (Table 5) and the magnitude of the increase was similar to that observed in the csgD+ strain GS162::glyA–lacZ (Table 4). In contrast, pSD6P enhanced the activity slightly in two of the three growth conditions tested (Table 5). Taken together, these results suggest that the chimaeric protein coded by pSD6P is not sufficient for optimum suppression, curli formation and glyA expression, and that it influences the expression or activity of the chromosomally encoded CsgD protein.

DISCUSSION

Multicopy plasmids expressing either the intact csgD gene, a chimaeric form of csgD or the glyA gene suppressed the glycine auxotrophy of strain MH829. These plasmids all increase the amount of SHMT in the cell. Strain MH829 lacks DHFR activity and is auxotrophic for glycine when histidine, adenine, thymidine, methionine and pantetheine are present (Herrington & Chirwa, 1999). In spite of

Table 5. β-Galactosidase activities in strain MH938

<table>
<thead>
<tr>
<th>Suplement</th>
<th>Normalized β-galactosidase activity in strain MH938 transformed with:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>pUC18</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>FEP-Gly</td>
<td>44</td>
</tr>
<tr>
<td>FEP</td>
<td>74</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org 531
the lack of DHFR, folA mutants can still produce some THF (Hamm-Alvarez et al., 1990; Herrington & Chirwa, 1999). The distribution of reduced folates in the cell presumably depends on the growth conditions. In FEP-Gly, methyl-THF and formyl-THF pools could be elevated relative to the THF pool because these cofactors would not be turned over via methionine and purine synthesis. This could limit synthesis of glycine, and restrict protein synthesis, further limiting turnover of formyl-THF. An increase in the amount of SHMT could increase glycine synthesis, thereby enhancing protein synthesis and turnover of formyl-THF.

The glycine auxotrophy of strain MH829 can also be alleviated by mutations resulting in curli proficiency. Curli-proficient mutants of strain MH829 grew on FEP-Gly, and some mutants selected for growth on FEP-Gly were curli-proficient (T. MacRae, C. Zambrana, N. T. Chirwa and M. B. Herrington, unpublished results). We have not yet tested whether glyA is upregulated in these mutants, but it is reasonable to expect that some could express higher levels of SHMT.

We propose that upregulation of glyA is an integral response to signals eliciting curli formation. Cells make curli in stationary phase, suggesting that the demand for amino acids is high at a time when their availability could be limited. Glycine makes up 11.5% of the amino acid residues in total E. coli protein (Neidhardt & Umbarger, 1996). Currin, the major protein of curli, contains 1.7 times as much glycine, and cells make a lot of curli, suggesting the demand for glycine is particularly high. Thus, increasing the cell’s ability to make glycine could facilitate its production of curli.

Expression of csgD is highly regulated. A variety of environmental factors including nutrient limitation, low osmotic strength, low temperature, microaerophilic conditions and iron limitation increase expression of csgD or its Salt homologue, agfD (Römling et al., 1998b; Prigent-Combaret et al., 2001; Gerstel & Römling, 2001). At least four regulatory proteins (RpoS, OmpR, Cpx and Mlr) control CsgD expression (Arnqvist et al., 1994; Brown et al., 2001; Prigent-Combaret et al., 2001) (Fig. 4). AgfD is detectable by Western blotting in late exponential and stationary phases, but transcript measurements and reporter constructs suggest low levels of AgfD and CsgD are made even during exponential phase (Prigent-Combaret et al., 2001; Gerstel & Römling, 2001).

CsgD is a homologue of the two-component FixJ/UhpA/LuxR family of proteins characterized by a DNA binding (helix–turn–helix) domain in the C-terminal region and a receiver domain in the N-terminal region of the proteins. Depending on the protein, the receiver domain contains a phosphorylation site or binds a small molecule. The helix–turn–helix domain of CsgD is a good fit to the consensus (obtained from the Conserved Domain Database; Marchler-Bauer et al., 2002) for the family. The chimaeric protein made by pSD6P does not contain the domain (Fig. 1), indicating that glyA activation and curli formation do not involve the binding of the chimaeric protein to DNA. This suggests CsgD activation could involve both protein–DNA and protein–protein interactions. Comparison of AgfD with homologues suggests that it does not contain a phosphorylation site similar to that of the homologues (Römling et al., 2000).

In the cell, CsgD presumably can have ligand bound to it (CsgD[L]) or not (CsgD[ ]). We propose that CsgD[ ] activates transcription of target genes whereas CsgD[L] is inactive (Fig. 4). Normally, growing cells contain very little CsgD (Prigent-Combaret, 2001) so CsgD[L] predominates. In curli-proficient cells, activation of csgDEFG transcription by OmpR, MlrA or other activators results in increased amounts of CsgD so that the active form predominates, CsgD[ ] then activates transcription of csgBA, adrA and possibly other genes. In curli non-proficient cells, CsgD[L] predominates because either ligand levels are high or csgD expression remains very low.

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**Fig. 4.** Regulatory roles of CsgD. CsgD[L] represents the protein with bound ligand and CsgD[ ] the protein with no ligand. Gene names in the dashed boxes indicate genes partially or completely dependent on RpoS for transcription (Olse ´ n et al., 1993b; De Wulf et al., 1999; Römling et al., 2000; Brown et al., 2001). Solid arrows are used to indicate gene–protein relationships and the two states of CsgD. Patterned arrows indicate activation and patterned lines with a bar represent repression. Patterns are used to differentiate between known regulatory circuits that have been identified by mutants (Hammar et al., 1995; Römling et al., 2000; Brown et al., 2001; Zogaj et al., 2001) or by mutants and DNA binding studies (Stauffer, 1996; Dorel et al., 1999; Prigent-Combaret et al., 2001) and possible circuits proposed in this study. Dotted lines, mutants; dash–dot lines, hypothetical; dashed lines, mutants and DNA binding.
When curli non-proficient cells are transformed with pCP994, the amount of CsgD made is higher than the amount of ligand so CsgD[ ] will activate curli synthesis and glyA expression. In cells transformed with pSD6P, the chimaeric protein will bind the ligand and there will be a mixture of chimaeric protein and chromosomally encoded CsgD in the activator form. The net activation will be weaker than in cells transformed with pCP994 because the chimaeric protein is a poor activator. This is demonstrated by eliminating CsgD. In the csgD null mutant, the response to pSD6P was weaker than in the csgD+ strain.

How does CsgD regulate glyA expression? CsgD[ ] could activate glyA directly. Alternatively, it could alter known regulation either by binding one of the ligands or by altering expression of one of the regulatory proteins. MetR and its co-activator, homocysteine, activates transcription of glyA and PurR and its co-repressor, guanine or inosine, represses transcription. Other, as yet unidentified, factors also influence glyA transcription ((Stauffer, 1996). A search of the Conserved Domain Database (Marchler-Bauer, 2002) using the N-terminal region of CsgD returned no hits, suggesting that the receiver domain does not resemble any functional important region of the protein.

Our results are the first to demonstrate that CsgD alters the expression of a gene not directly involved in multicellular behaviour. Our observation that a chimaeric protein containing only 70 aa of CsgD is sufficient to stimulate curli formation is of interest because it defines a functionally important region of the protein.

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