Multicopy Suppression of the *folA* null mutation in *Escherichia coli*

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ABSTRACT

In *Escherichia coli*, Dihydrofolate reductase coded by the *folA* gene plays a central role. It is required for the de novo synthesis of tetrahydrofolate and the recycling of dihydrofolate produced by cells synthesizing thymidylate. Deletion of the *folA* gene in E.coli K-12 strains is not lethal but generates auxotrophies when glycine or methionine is omitted from a combination of supplements containing pantothenate, thymidine, adenine and histidine.

We have isolated pSD6P, which contains part of the *csgD* sequence, as a multicopy suppressor of the glycine auxotrophy. Multicopy suppressors are genes that when overexpressed alleviates one or more growth requirements of a strain. *CsgD* codes for a transcriptional regulator required for the synthesis of curlin subunits that are used in the synthesis of extracellular matrix important for biofilm formation. Curli fibers are also thought to be important for infection of host cells and confer additional protection against damaging agents or predators in the environment.

We have shown that increasing the expression of the *glyA* gene can alleviate the glycine auxotrophy of a *folA* null strain. Serine hydroxymethyltransferase (SHMT), encoded by the *glyA* gene, is required for the conversion of serine to glycine. We have measured SHMT activity and shown that cells expressing *csgD* from multicopy plasmid have increased levels of activity. We have also monitored the effect of CsgD on transcription of genes involved in one-carbon metabolism using a lac-based reporter system. We have shown that the presence of CsgD increases the transcription of the *glyA* and *purU* genes. *PurU* codes for a formyltetrahydrofolate hydrolase and is important for balancing the cell's need for one-carbon units and glycine. The role of CsgD is not

limited to curli or one-carbon metabolism. Our results suggest that expression of *hmp*, a gene adjacent to *glyA*, is slightly induced. *Hmp* codes for a flavohaemoglobin with denitrification properties. Interestingly, the *csgD*- mediated induction of the *glyA* gene but not of hmp requires the known regulators (MetR and PurR) of *glyA* transcription.

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1. INTRODUCTION

Folic acid and folate are often used interchangeably referring to a water-soluble B-complex vitamin. Folic acid is made of a 4-[(pteridin-6-yl) methyl amino] benzoic acid skeleton conjugated with one or more L glutamate units (Figure1). The number of glutamates dictates binding affinity of folate cofactors to folate dependent enzymes and the ability for the cell or organelle to retain the vitamin. The coenzyme forms are the reduced products of folic acid, which act as acceptors and donors of one-carbon units in a variety of biochemical reactions essential for the cell reproduction such as amino acid, vitamin, and nucleotide biosynthesis.

Understanding folate metabolism is of tremendous importance. Imbalances or dysfunctions in folate metabolism have been linked to severe illness such as cardiovascular diseases, several cancers, Alzheimer's disease and Down's syndrome. The precise metabolic events leading to these diseases are not well understood. However, folic acid, in its native form, or as a dietary or pharmacological supplement, has been credited with beneficial role in preventing or alleviating a number of disorders (Lucock., 2000). For instance, it has been demonstrated that periconceptional use of folic acid supplementation and/or food fortification prevents neural tube defects such as spina bifida in newborn babies and possibly other congenital malformations (Fleming, Mutchninik and Romero., 2001).

The use of antifolate drugs in treatment of diseases is of equal importance. The antifolate drug, methotrexate, is used to treat cancer, rheumatoid arthritis and psoriasis. Other medications known to be folate antagonists are the antibiotic trimethoprim, the antimalarial pyrimethanine, triamterene for blood pressure and sulfasalazine as an anti –

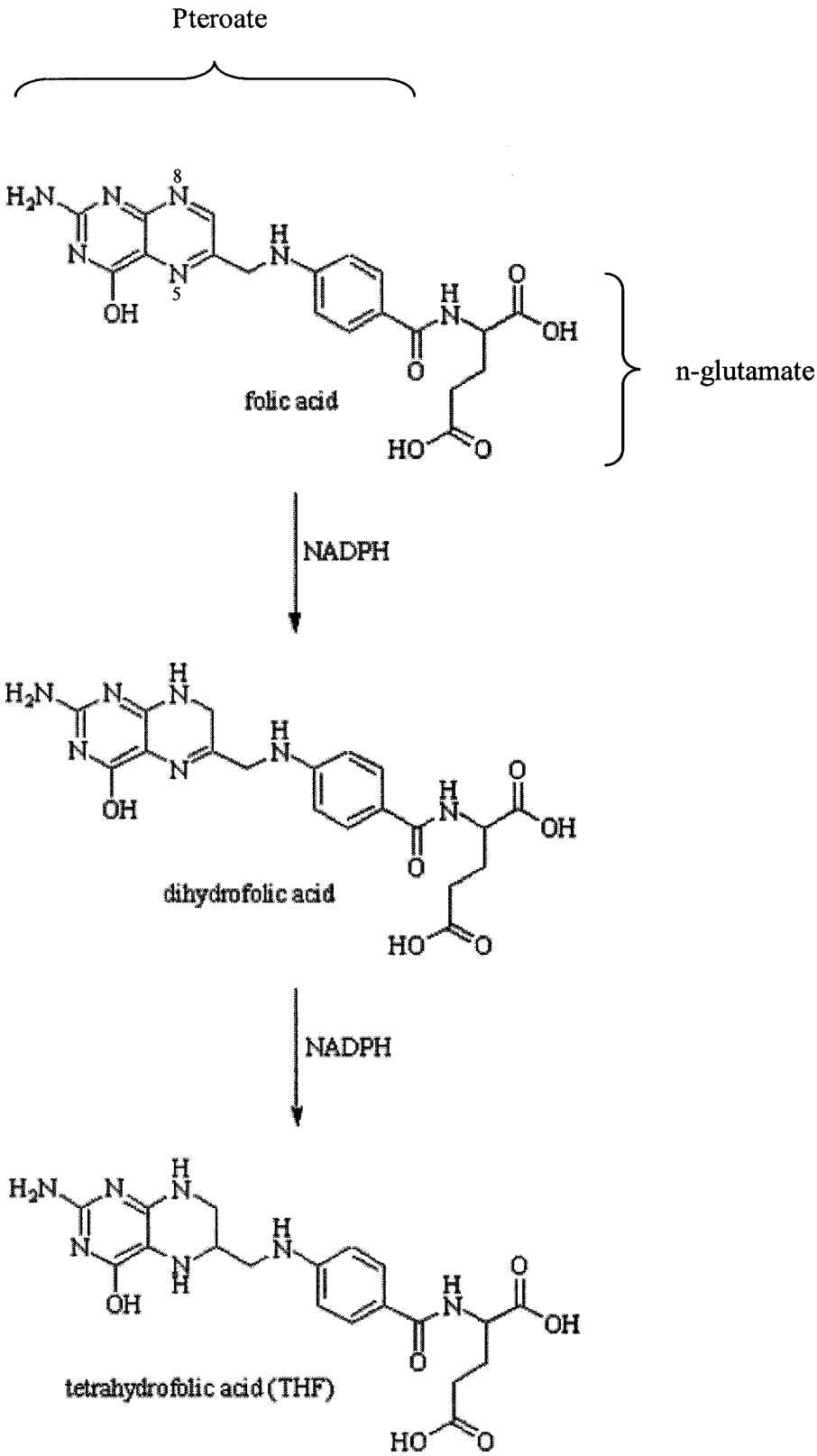


Figure 1: Folic Acid.
(http://chemed.chem.purdue.edu/organic/orgapp/vitamins/folic.html)

Helicobacter pylori in treatments of certain types of ulcers (Zhang and Rathod 2002; Ulrich , Robien and Sparks 2002).

Despite the importance of folate, major gaps exist in our understanding of folate metabolism in humans. This is because folate metabolism is very complex and much of the human evidence comes from observational studies. Conversion of folates to cofactors is similar in all organisms, but folates are obtained in different ways. Mammals must acquire their folate from diet; Plants and some bacteria, like *Escherichia coli,* can synthesize it de novo but can (with the exception of *E.coli*) use dietary folate to produce cofactors. Thus *Escherichia coli,* with its highly developed genetics, its ability to synthesize folates de novo and the availability of its entire genome sequence, should make a good simple model of folate metabolism.

1.1 <u>De novo synthesis of tetrahydrofolate</u>

In prokaryotes the precursors of folates, pteridine and p-amino benzoic acid (pABA), are respectively synthesized from the nucleotide GTP and chorismate. Dihydropteroate synthase catalyzes the condensation of pteridine with pABA to form dihydropteroate. Dihydropteroate is then converted to dihydrofolate (DHF) by the addition of a glutamate by dihyrofolate synthetase, which is part of the bifunctional enzyme dihydrofolate synthetase/folylpolyglutamate synthetase coded by the *folC* gene. Dihydrofolate reductase (DHFR) coded by the *folA* gene then reduces DHF to tetrahydrofolate (THF) in an NADPH dependent reaction (Figure 2). To date, this is the only enzyme known to reduce DHF to THF in vivo.

Figure 2: Biosynthesis of C_1-THF and Folate dependent pathways.

FolA: DHFR

GlyA: SHMT

MetF: Methylene-THF reductase

ThyA: Thymidilate synthetase

PanB: Ketopantoate hydroxymethyltransferase

GCV: Glycine cleavage enzymes

FolD: Methylene-tetrahydofolate dehydrogenase/ methenyl-tetrahydrofolate cyclohydrase

PurU: Formyl-THF hydrolase

Fmt: Met-tRNA fmet formyltransferase

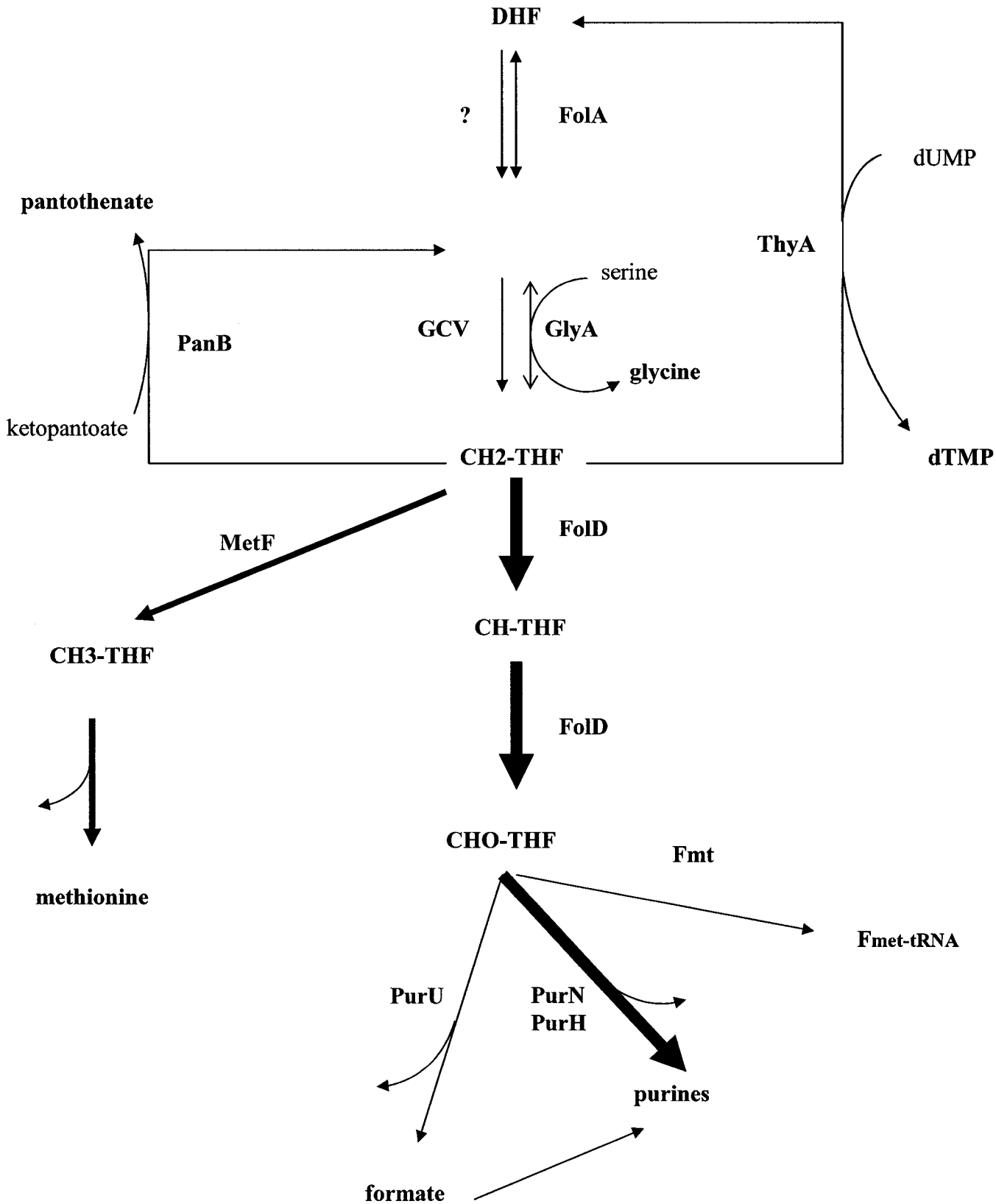
THF: Tetrahydrofolate

CH2-THF: Methylene-THF

CH-THF: Methenyl-THF

CHO-THF: Formyl-THF

CH3-THF: Methyl-THF


DHF
?
FolA
dUMP
pantothenate
ThyA
serine
GCV
GlyA
PanB
glycine
ketopantoate
CH2-THF
dTMP
MetF
FolD
CH-THF
CH3-THF
FolD
CHO-THF
Fmt
methionine
Fmet-tRNA
PurU
PurN
PurH
purines
formate

1.2 Formation and Interconversion of folate cofactors

Methylene-THF is generated from the conversion of serine to glycine by serinehydroxymethyltransferase (SHMT) encoded by the *glyA* gene or from glycine cleavage to form CO_2 and NH_3 (Figure 3). Glycine synthesis and its regulation will be reviewed in chapter 1.6. The methylene group is then oxidized or reduced to form the other one- carbon substituted THF (C_1-THF) (Figures 2,3). Folylpolyglutamate synthetase (FPGS) can then add one or more glutamate residues to C_1-THF. The enzyme has a higher affinity to formyl-THF-mono-glutamate and methylene-THF-di-glutamate. The addition of a polyglutamate tail to folate and derivatives plays an important role in the retention of intracellular folates and enzyme regulation. Most folate dependent enzymes have a higher affinity for polyglutamated- C_1-THF (Green, Nichols and Matthews,1986).

Methylene-THF is required for the synthesis of pantothenate in the intermediary reaction catalyzed by ketopantoate hydroxymethyltransferase which transfers the methylene group to ketoisovalerate to produce ketopantonoate and THF. Methylene-THF is also required for the methylation of deoxyuridine monophosphate (dUMP) to form deoxythymidylate or thymidylate (dTMP). dTTP is used in DNA synthesis. This is the only C_1-THF dependent reaction to produce DHF, rather than THF. As a result DHF has to be reduced again by DHFR before it can undergo another cycle. Thymidylate synthase activity is not strongly regulated. Significant amounts of DHF are generated during the de novo synthesis of dTMP even when cells are supplemented with exogenous thymine.

Methylene-THF is reduced to methyl-THF by methylene-THF reductase coded by the *metF* gene. Methyl-THF is used by homocysteine transmethylase to convert

Figure 3: Synthesis of C_1-THF and Glycine. (Underlined indicates the gene involved)

SerA : phosphoglycerate dehydrogenase

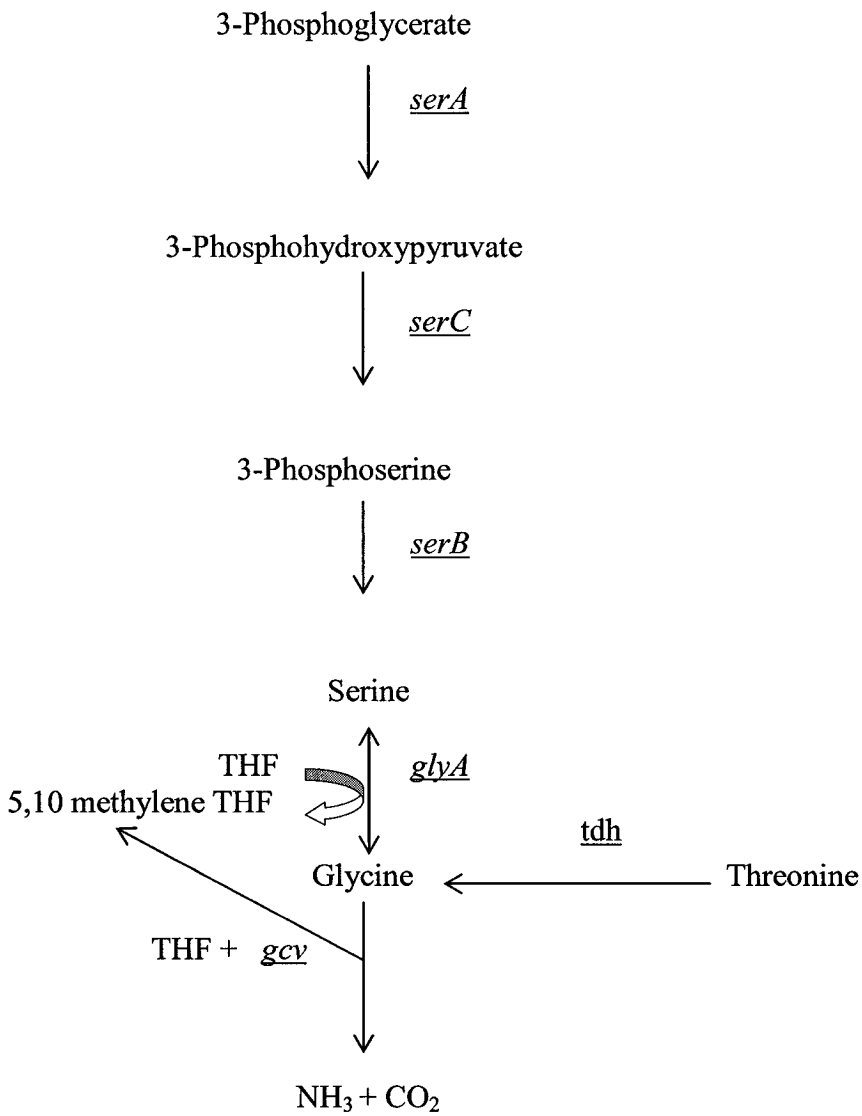
SerC : 3-phosphoserine aminotransferase

SerB: phosphoserine phosphatase

GlyA: Serine Hydroxymethyltransferase

Tdh: Threonine dehydrogenase

Gcv: Glycine cleavage enzymes


3-Phosphoglycerate
serA
3-Phosphohydroxypyruvate
serC
3-Phosphoserine
serB
Serine
THF
5,10 methylene THF
glyA
tdh
Glycine
Threonine
THF + gcv
NH3 + CO2

homocysteine into methionine, which is required for protein synthesis and as a precursor for S-adenosyl-methionine (SAM), a methyl donor in many reactions. Homocysteine transmethylases are coded by the *metE* and *metH* genes and differ in their requirements for enzyme activity. The *metE* gene product uses polyglutamated methyl-THF whereas the *metH* gene product can use both monoglutamated and polyglutamated methyl-THF and is vitamin B12 dependent.

The genes required for methionine biosynthesis in *E.coli* are scattered throughout the chromosome but form a regulon (Figure 4) (Urbanowski and Stauffer, 1987 a). The *metJ* gene product represses expression of the met regulon with the exception of *metH(*Urbanowski and Stauffer, 1987 b; Wu, Urbanowski and Stauffer,1992). SAM acts as co-repressor. Transcription of *metE* and *metH* genes is activated by the DNA-binding protein MetR (Urbanowski and Stauffer, 1987(a); Urbanowski *et al.*, 1987(b)). The activation requires homocysteine as co-activator. High levels of homocysteine inhibit *metR*-mediated induction of *metH* expression (Urbanowski and Stauffer 1989). The m*etE* and *metF* genes are repressed when cells are grown in the presence of vitamin B_{12}. This is because the MetH/B_{12} complex depletes the intracellular levels of homocysteine, thus competing with MetE (Wu, Urbanowski and Stauffer, 1992., Shoeman et al., 1985). The repression is relieved by the addition of homocysteine to the growth media containing B_{12}. Homocysteine is also involved as a co-repressor in the autoregulation of MetR synthesis (Maxon *et al*, 1989). Expression of the *metR* gene is also repressed by the MetJ protein (Urbanowski et al 1987 (a)).

Methylene-THF is oxidized to methenyl-THF, which is then converted to formyl-THF by the bifunctional enzyme methylene-THF dehydrogenase/methenyl-THF

Figure 4. Methionine biosynthetic pathway in *E. coli.*

MetA: Homoserine transsuccinylase

MetB: Cystathione-γ- synthase

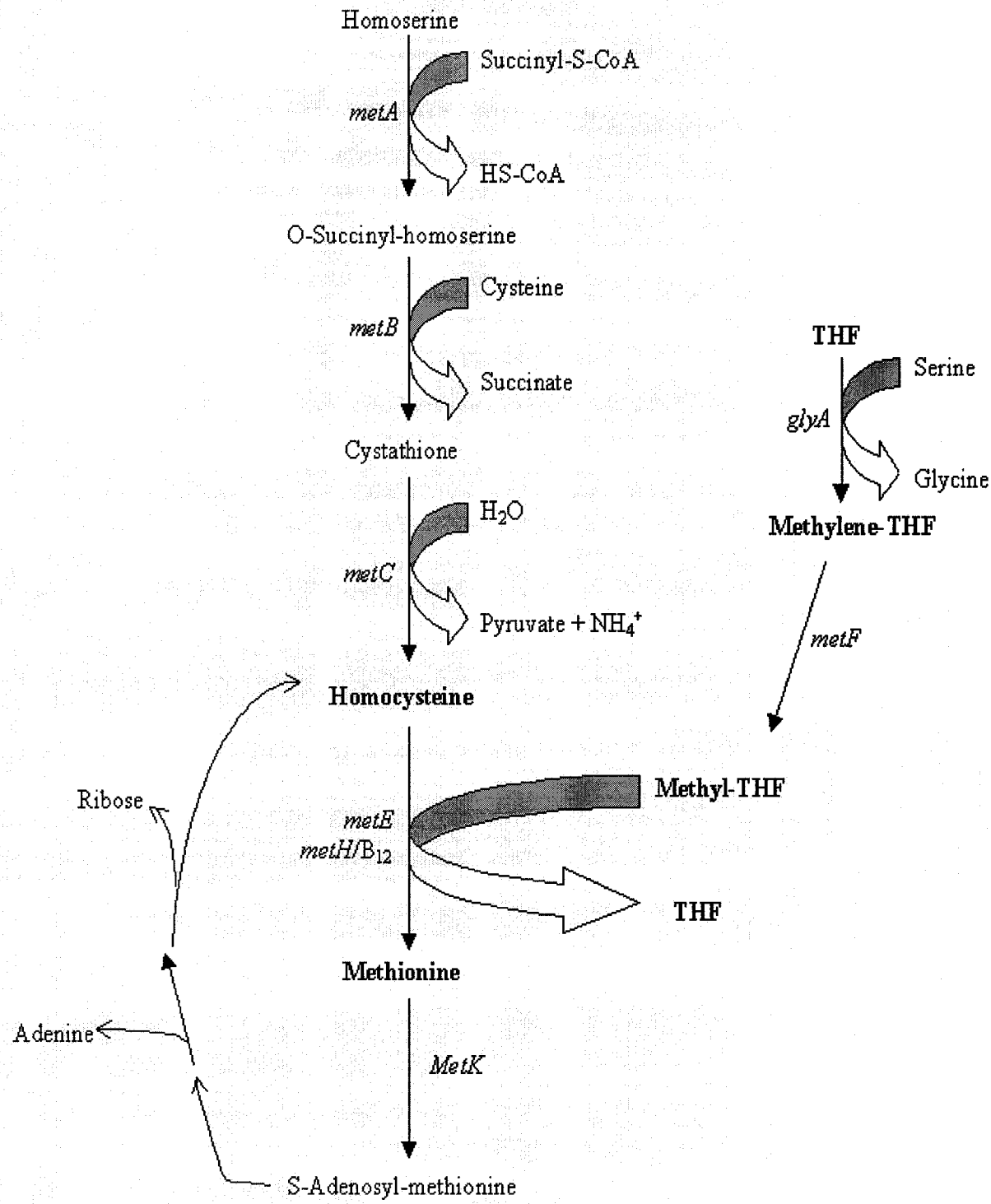
MetC: β-Cystathionase

MetE, MetH: Homocysteine transmethylase

MetF: Methylene-THF reductase

MetK: Methionine adenosyltransferase

GlyA: Serine Hydroxyl-methyl transferase


Homoserine
Succinyl-S-CoA
metA
HS-CoA
O-Succinyl-homoserine
Cysteine
metB
Succinate
Cystathione
H2O
metC
Pyruvate + NH4+
Homocysteine
THF
Serine
glyA
Glycine
Methylene-THF
metF
Methyl-THF
metE
metH/B12
THF
Methionine
MetK
S-Adenosyl-methionine
Ribose
Adenine

cyclohydrase encoded by the *folD* gene. Formyl-THF is the cofactor for two enzymes (5-phosphoribosylglycinamide (GAR) transformylase and 1- (5-phosphoribosyl)-5-amino-4-imadazolecarboxamide (AICAR) transformylase) involved in purine biosynthesis (Figure 5). The synthesis of a purine ring starts with the amination of phosphribosyl pyrophosphate (PRPP), a reaction catalyzed by glutamine –PRPP amidotransferase encoded by the *purF* gene. Glycine is then added to the 5-phosphoribosylamine to form GAR. This reactions is catalyzed by the enzyme GAR synthetase coded by the *purD* gene. GAR must be formylated prior to being used in a subsequent reaction leading to the synthesis of AICAR. GAR transformylase coded by the *purN* gene transfers the formyl group from formyl-THF to GAR. There exists an alternative GAR transformylase coded by the *purT* gene that uses formate instead formyl-THF (Marolewski, Smith and Benkovic, 1994). The formate is generated by formyltetrahydrofolate hydrolase encoded by *purU* (Nagy, McCorkle and Zalkin, 1993) which has also been proposed to balance THF/C1-THF pools in response of glycine and methionine needs in the cell.

AICAR transformylase (*purH)* then catalyzes the transfer of a formyl group from formyl-THF to AICAR in a reaction leading to the synthesis of inosine monophosphate (IMP). IMP is the branch-point for purine biosynthesis since it serves as a precursor for either adenosine monophoshate (AMP) or guanosine monophoshate (GMP) leading to ATP and GTP respectively (Rohlman and Matthews, 1990).

Histidine biosynthesis shares a metabolic link with purine biosynthesis. Histidine is made up of 5 carbons that are derived from PRPP, an intermediate in purine and pyrimidine biosynthetic pathways, and a carbon atom that stems from ATP. The ATP atoms not incorporated into histidine are eliminated as AICAR, which can be recycled

Figure 5: De novo purine and histidine biosynthesis

The *pur* regulon:

PurF: Glutamine-PRPP amidotransferase

PurD: phosphoribosylglycineamide synthetase

PurN: GAR Transformylase

PurU: formyltetrahydrofolate hydrolase

PurT:* GAR Transformylase (uses formate instead of C_1-THF)

PurL: 5'-phosphoribosylformylglycinamide amidotransferase

PurM: aminoimidazole ribonucleotide synthetase

PurE: phosphoribosylaminoimidazole carboxylase

PurC: phosphoribosylaminoimidazole-succinocarboxamide synthetase

PurB: Adenylosuccinate lyase *PurH:* AICAR transformylase

Abbreviations:

PRPP: 5-phosphoribosyl-α-pyrosphosphate

PRA: 5-phosphoribosylamine GAR: 5-phosphoribosylglycinamide

FGAR: 5-phosphoribosyl-N-formylglycinamide

FGAM: 5-phosphoribosyl-N-formylglycinamidine

AIR: 5-phosphoribosylaminomidazole

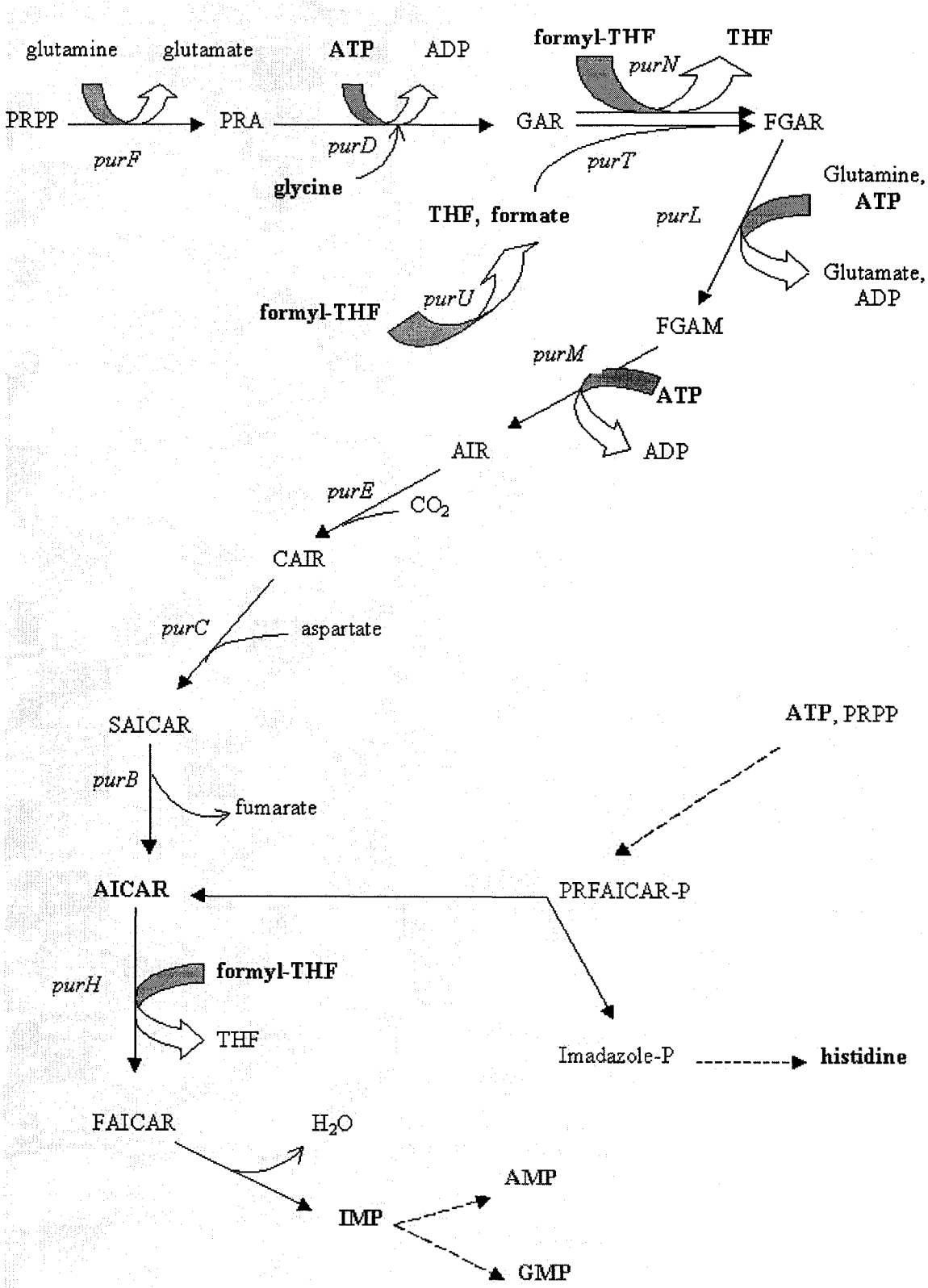
CAIR: 1- (5-phosphoribosyl)-5- amino-4-carboxymidazole

SAICAR: 1- (5-phosphoribosyl)-4-(N succinocarboxamide)-5-aminomidazole

AICAR: 1- (5-phosphoribosyl)-5-amino-4-imadazolecarboxamide

FAICAR: 1- (5-phosphoribosyl)-5-formamido-4-imadazolecarboxamide

PRFAICAR-P: phosphoribulosylformimino-AICAR-phosphate


glutamine
glutamate
ATP
ADP
formyl-THF
THF
PRPP
PRA
GAR
FGAR
purF
purD
purN
purT
glycine
THF, formate
purL
Glutamine,
ATP
Glutamate,
ADP
formyl-THF
purU
FGAM
purM
ATP
AIR
ADP
purE
CO2
CAIR
purC
aspartate
SAICAR
ATP, PRPP
purB
fumarate
AICAR
PRFAICAR-P
purH
formyl-THF
THF
Imadazole-P
histidine
FAICAR
H2O
AMP
IMP
GMP

into purines (Figure 5).

The genes involved in the de novo purine biosynthesis are also scattered but form a regulon in *E.coli* (He *et al.*, 1990). The synthesis of purines is regulated both at the gene level and at the enzyme level. The main control points of enzyme activities regulation are the rate limiting steps, which are the first two steps in the purine-committed pathway. The synthesis of PRPP is feedback inhibited by purines mainly AMP and GMP. On one hand, glutamine –PRPP amidotransferase is subject to feed back inhibition by derivatives of AMP and GMP (ADP,ATP, GDPand GTP) (Zhou *et al.*, 1993, Messenger and Zalkin 1979). On the other hand the amidotransferase enzyme activity is enhanced by the accumulation of intracellular PRPP(Kim *et al.*, 1996) . At the gene level, the expression of the *pur* genes and related pathways is down-regulated by purines (IMP and GMP).This repression is mediated by the autoregulated protein PurR with hypoxanthine and guanine acting as corepressors (Meng *et al.*, 1990).

Histidine biosynthesis involves eight genes that form a regulon (Winkler, M.E., 1996). There is feedback inhibition by high levels of histidine on the first enzyme on the pathway, ATP- phosporibosyltransferase, coded by the *hisG* gene. Expression of the *his* operon is regulated by the attenuation mechanism through the concentration of aminoacylated histidine transfer ribonucleic acid (Bruni *et al*, 1980; Meyers *et al* ., 1975)

Formyl-THF is also crucial for initiation of protein synthesis through the formylation of methionine bound to the initiator tRNA by the *fmt* gene product Met-tRNA formyltransferase. Deficiencies or perturbation in folate metabolism have been linked to reduced rates of protein synthesis initiation and reduced translational accuracy (Basso and Herrington 1994).

1.3 Consequences of Inactivating the *folA* gene

Albeit the pivotal role played by DHFR in folate metabolism, deletion of the *folA* gene is not lethal but alters the growth requirements of the cell (Hamm-Alvarez *et al.*, 1990; Krishnan and Berg 1993; Herrington and Chirwa, 1999). Reduced folates have been detected in *folA* null mutant (Hamm-Alvarez *et al.*, 1990), although in limited quantities compared to $folA^+$ (Herrington and Chirwa, 1999). This suggests that another enzyme or pathway exists in *E. coli* for the reduction of DHF. A possible candidate could be dihydropteridine reductase, which has been shown to have in vitro DHFR activity (Hamm-Alvarez et al., 1990; Vasudevan, Paal and Armarego, 1992). A second protein, the bacterial flavohaemoglobin encoded by the *hmp* gene, possesses dihydropteridine reductase activity (Vasudevan *et* al., 1991) but has not been tested for DHFR activity. Recently, a novel protein (FolM) coded by the *ydgB* gene (renamed *folM*) was shown in E.*coli* to possess DHFR activity and to completely complement a Δ*folA* mutation . The FolM protein is unrelated to the previously isolated DHPR (Giladi *et al.*, 2003).

When otherwise wild type, *folA* null mutant will grow on rich media or minimal media with yeast extract, but not with folate end products (FEP: thymine, glycine, histidine, methionine, adenine and pantothenic acid) (Herrington and Chirwa, 1999). In contrast, *folA* null mutants that were also $thyA^-$ were able to grow on minimal glucose with thymidine or with some combination of FEPs.

Thymidylate synthetase activity is not strongly regulated; therefore in a $thyA^+$ cell thymidylate must be synthesised even in the presence of FEP. Generation of DHF during thymidylate synthesis depletes the total folate pool in Δ*folA* $thyA^+$ strains in such a way growth is no longer supported on minimal media.

The double mutants (*ΔfolA thyA*$^-$) can not grow when glycine or methionine is omitted from the FEP (Herrington and Chirwa, 1999). The auxotrophy of cells lacking DHFR activity on minimal media with these combinations of FEP suggests that THF is limiting and that sufficient one-carbon derivatives are appropriately distributed to all folate dependent biosynthetic reactions only in some conditions.

These auxotrophies could partially or totally be alleviated if there is an increase in the synthesis of THF provided by an alternative pathway, by shifts in the distribution of folates, by reducing the demand for folates, or by eliminating the requirement for fMet-tRNA in the protein synthesis. To that effect, this thesis describes the isolation and characterisation of one multicopy suppressor of the glycine auxotrophy in a Δ*folA* strain. Multicopy suppressors are genes that alter the requirement for some or all FEP when expressed on a multicopy plasmid. The suppressor gene is *csgD* and I show here that it upregulates two genes involved in one–carbon metabolism and that it also induces the *hmp* gene.

The following sections describe what is currently known of the biochemistry, regulation and genetics of one carbon metabolism and the regulation of *hmp* gene. The regulation and role of curli will also be described because *csgD* codes for a putative regulator of curli synthesis.

1.4 Serine biosynthesis and its regulation

For cells grown on glucose as the carbon source, the major source of one carbon units is the β carbon of serine. Serine is synthesised from 3 phosphoglyceraldehyde, an intermediate in the glycolytic pathway (Figure 3). Serine is required in the biosynthesis

of cysteine, tryptophan and glycine. There is a secondary pathway for serine synthesis through threonine utilisation using the glycine cleavage system (GCV). Threonine is converted to glycine by threonine dehydrogenase encoded by the *tdh* gene. Glycine cleavage produces methylene THF. Serine hydroxymethltransferase (SHMT) catalyses the condensation of the C_1 unit from methylene-THF with a second unit of glycine to produce serine (Figure 2) (Stauffer G 1996 (a); Voet and Voet 1990).

Regulation of the genes involved in serine metabolism is complex and poorly understood. Three enzymes, coded by *serA, serC* and *serB* genes, are involved in the biosynthesis of serine. High levels of serine inhibit phosphoglycerate dehydrogenase (*serA)*, the first enzyme in the serine synthesis pathway. The role of cyclic AMP (cAMP) receptor protein (CRP) and its cofactor cAMP in the serine pathway is to date ambiguous. There have been reports indicating repression of *serA* expression by cAMP (Stauffer, 1996 (a)). Other studies propose that CRP activates *serA* expression, but its role is modulated by the leucine responsive regulatory protein (Lrp) involved in the transcriptional control of many amino acid biosynthetic genes, since the effect of CRP is less in the absence of Lrp (Yang *et al.*, 2002). Two independent promoters P1 and P2, located respectively at 45 bp and 137 bp upstream of the translation start site of *serA,* have been identified. Binding of Lrp to the stronger promoter (P1) activates serine biosynthesis, while binding to the weaker promoter (P2) represses by interfering with RNA polymerase binding. *SerC* expression is also activated by Lrp, but there is uncertainty as to whether CRP has an activating or inhibitory effect (Yang *et al.*, 2002). *Nac*, the nitrogen-regulated gene encoding a lysR-type transcription factor, represses *serA* expression under nitrogen limiting conditions (low intracellular glutamine concentration).

The exact mode of action of Nac protein has not been deciphered, though two overlapping consensus Nac binding sites have been identified in the *serA* promoter region (Blauwkamp and Ninfa, 2002).

1.5 Regulation of GCV

As mentioned in the previous section, the glycine cleavage system (GCV) constitutes part of an alternative pathway for biosynthesis of serine. It is also necessary to maintain appropriate glycine and C_1 units levels by degrading the excess glycine. *E. coli* mutants in GCV excrete the excess glycine, whereas E.coli strains with mutations in both GCV and serine biosynthetic pathway can not grow on media supplemented with glycine whereas those blocked in serine biosynthesis can (Wilson, Steiert and Stauffer, 1993). The GCV consists of four proteins. GcvP catalyzes the decarboxylation of glycine to CO_2 and an aminomethyl group. GcvH contains a covalently bound lipoic acid prosthetic group that serves as an electron sink and carrier of the aminomethyl moiety. GcvT transfers the one carbon unit from GcvP to THF and releases ammonia. GcvL encoded by *lpd* is an NAD^+-dependent FAD lipoamide dehydrogenase used to reoxidize the reduced lipoic acid prosthetic group. GcvP, H and T form an operon (Stauffer, 1996 (a)). Regulation of the Gcv operon is mainly controlled by five different factors: GcvA, Lrp, PurR, GcvR and CRP.

GcvA, an autoregulated protein, positively regulates the expression of the gcv operon in glycine supplemented media (Ghrist and stauffer, 1998). Mutations in *gcvA* prevent glycine induction of GCV system. Lrp has also been implicated in both the

activation and repression of *gcv* expression. Although multiple binding sites upstream of the *gcv* promoter region have been identified, the precise mode of action is unknown. Mutations in the *lrp* gene lead to very low levels of Gcv enzymes (Wilson, Steiert and Stauffer, 1993).

PurR decreases by two-fold the expression of a gcvT-lacZ fusion protein when cells are grown in the presence of purines (Wilson, Steiert and Stauffer, 1993). The PurR interacts with the gcv promoter region at approximately -2 to +15 region, which is a region that matches the PurR consensus-binding sequences. Interestingly, GcvA was also involved in purine mediated repression. Mutations in the *gcvA* gene prevent repression of *gcv* by purines. This pathway is not well understood (Wilson, Steiert and Stauffer, 1993), but GcvR, a negative regulator of the expression of the *gcv* operon, was shown to interact with GcvA to prevent activation of the operon. However, it is not clear how glycine or purine modulate respectively GcvA activation or GcvA-GcvR interaction (Wonderling and Stauffer, 1999; Ghrist, Heil and Stauffer, 2001).

CRP and cAMP have been recently shown also to regulate expression of the *gcv* operon. Two binding sites are available for the CRP, one centered near -313 (site1) and the other at -140 (site2) relative to the transcriptional start of the gcv operon (Wonderling and Stauffer, 1999). Mutations of the binding sequences have revealed that only site1 is required for regulation. It has been postulated that CRP's role is to antagonize GcvA repression of the *gcv* operon or to prevent the formation or function of GcvA, GcvR and Lrp complex (Wonderling and Stauffer, 1999).

1.6 Regulation of SHMT synthesis

Serine hydroxymethyltransferase (SHMT) encoded by the *glyA* gene converts serine and THF to methylene-THF (Figure 3). This reaction is the major source of glycine, although threonine aldolase could provide an alternative route. The gene for thermostable low specificity L-threonine aldolase (*ItaE*) has been cloned and characterised. This enzyme cleaves threonine to glycine and acetaldehyde. *E. coli ItaE glyA* double mutants have a slower growth rate than an *ItaE* strain that grows at the wild-type rate. Regulation of this enzyme is still under investigation (Liu *et al.*, 1998).

Regulation of SHMT is complex and involves control on gene expression. The transcriptional regulation of SHMT expression has been extensively studied. Addition to the growth media of compounds directly involved in C_1 metabolism such as serine, glycine, methionine, purines and pyrimidines, results in a decrease of *glyA* expression even under glycine limitation (Matthews, 1996). In *S. typhimurium* enhanced repression of *glyA* by a combination of compounds suggests a cumulative repression. *S. typhimurium* singly auxotrophic for these compounds did not exhibit derepression of *glyA* on media with limiting concentration of the required compound (Stauffer and Brenchley, 1977).. This is indicative of an indirect interaction of these compounds in the regulatory mechanism. However, derepression is observed when wild type strains are grown in media containing trimethoprim, an inhibitor of DHFR, or when a purine auxotroph is starved for purines. These results point towards a role of purine and/or FEP in the regulation of SHMT activity (Stauffer, Baker and Brenchley, 1974).

There are no known conditions where SHMT levels goes to zero, but two major systems play a critical role in regulating SHMT expression. These are the methionine and purine mediated regulation.

Glycine and methionine syntheses are interconnected with the formation of methyleneTHF derived from serine through the SHMT reaction. The methylene-THF is subsequently reduced to methyl THF by methylene-THF reductase and is used as a methyl donor in the biosynthesis of methionine. The importance of methionine in regulating *glyA* was suggested by the reduced levels of SHMT in *metF* and *metE* (methionine synthetase) mutants on media with limiting concentrations of methionine (Stauffer and Brenchley, 1977). *MetK* (SAM synthase) and *metJ* (repressor of methionine biosynthesis) mutants also have altered SHMT regulation. In a wild type background, the addition of excess serine, glycine, methionine, adenine, guanine and thymine resulted in a 2.5-8 fold decrease in SHMT levels (Stauffer, Baker and Brenchley, 1974), whereas it had no effect on *metK* and *metJ* mutants (Stauffer and Brenchley, 1977). Both mutants express 80% of wild type levels of SHMT when grown in unsupplemented media or minimal media supplemented with the six compounds. Thus SHMT could be partially regulated by the methionine pathway through SAM and *metJ* (Stauffer and Brenchley, 1977).

The DNA binding protein, MetR, a member of the LysR family regulatory proteins, positively regulates *glyA* expression. The interaction requires homocysteine, a methionine pathway intermediate, as a co-regulator (Plamann and Stauffer, 1989). SHMT activities under methionine limiting conditions are 1.6 fold higher in $metR^+$ background than in $metR^-$. Addition of methionine to the media does not alter SHMT expression in

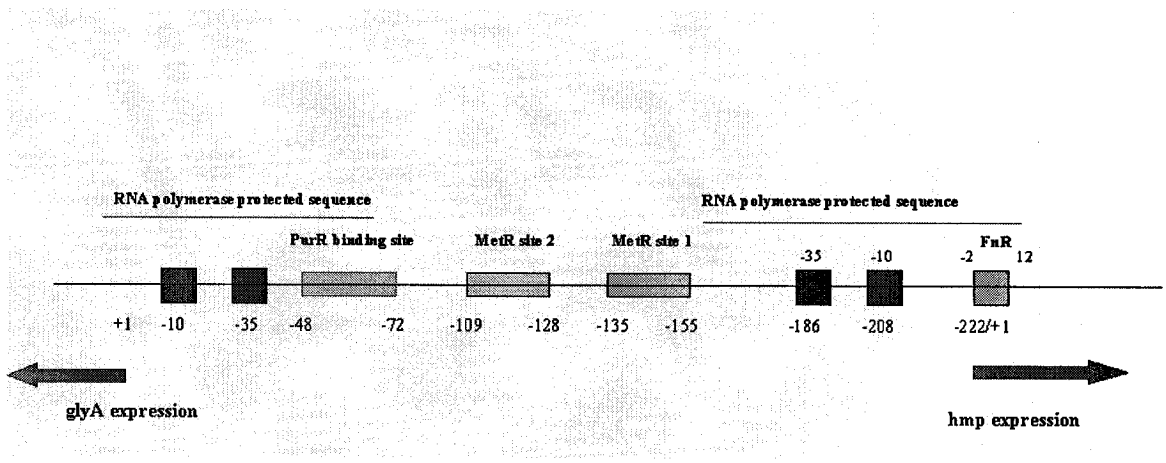


Figure 6: The *glyA* control region.

metR$^-$ strains but leads to a 50% reduction in *metR*$^+$ (Steiert *et al.*, 1990).

Two binding sites, one with low affinity, for MetR were identified in the *glyA* control region of *E .coli* and *S .typhimurium.* The MetR site 1 is localized from -155 to-135, and the low affinity MetR site 2 is found from -109 to -135 region upstream of the transcriptional start site (Lorenz and Stauffer, 1995). Binding of MetR to the glyA control region causes DNA bending. Interaction between MetR and RNA polymerase could only be possible with DNA bending since the binding sites are quite far upstream of the transcription start site. It has been suggested that there is cooperative binding to the different sites. Homocysteine increases moderately the binding to the metR site1 and significantly to site 2, but does not influence the DNA bending. Mutations of site 1 and 2 have illustrated that both sites are necessary for normal regulation of *glyA*. It has, therefore, been proposed that homocysteine activates *glyA* gene expression via the MetR protein. Internal methionine pools also modulate SHMT synthesis by decreasing MetR levels through the action of MetJ and its co-repressor SAM (Plamann and Stauffer, 1989).

The *pur* regulon regulatory protein PurR, with hypoxanthine and guanine as co-repressors, mediates repression of *glyA*. Repression of *glyA* transcription by purines occurs in both metR$^-$ and metR$^+$ backgrounds. SHMT synthesis is decreased to 40% of wild-type level in media supplemented with purines. Although *purR* mutants have 1.8 to 2 fold higher levels of SHMT activity than wild type , purine repression is still observed in these mutants. Moreover, the highest decrease in SHMT activity levels (3 fold) during purine supplementation was observed in a *metR* mutant strain. These results suggest that MetR-mediated activation of the *glyA* gene limits the extent of purine repression by PurR.

PurR binds to the *glyA* promotor in a region ranging from 15 to 38 bp upstream of the -35 promoter sequence of the *glyA* (Steiert *et al.*, 1990). The DNA sequence of this region matches the consensus sequence of operator sequences from *pur* genes, but in *pur* genes it is normally located between the –10 and –35 promoter regions and thus directly interferes with RNA polymerase binding. Having the consensus sequences a further distance in the *glyA* promoter region weakens the interaction with RNA polymerase thus allowing a narrow range of purine repression, which in turn would permit sufficient levels of methylene-THF to be produced even in the presence of repressing concentrations of purines (Figure 6) (Stauffer, 1996).

Mutations mapped to 85.5 minutes of the *E.coli* chromosome , have suggested that, components other than MetR or PurR are involved in the control of *glyA* expression (Lorenz, Plamann and Stauffer, 1996).

1.7 Regulation of HMP

Interestingly, regulation of the *glyA* also affects *hmp* expression. The two genes are adjacent to each other and are divergently transcribed. *Hmp* encodes a soluble flavohaemoglobin (HMP) with DHPR activity although it is distinct from a previously purified DHPR (Vasudevan *et al.*, 1991). *E. coli* DHPR has been shown to possess DHFR activity *in vitro* (Vasudevan, Paal and Armarego, 1992), but DHFR activity of HMP has not been reported.

In higher organisms globins are known for their role as facilitator of oxygen transport and storage. The role of microbial hemoglobin is now becoming clearer. HMP is made up of haem domain homologous to classical globins and a ferredoxin-$NADP^+$

reductase domain with multiple reductase activities such as nitric oxide reductase (although of minor significance), nitroglutathione reductase and nitrite reductase activities. It has also been demonstrated that HMP has substantial nitric oxide dioxygenase activity (Gardner and Gardner, 2002). These activities offer the cell some protection against oxidative and nitrosative stress agents which interfere with enzyme activities in the glycolytic and citric cycle pathways and can be damaging to the cell (Membrillo-Hernandez *et al.*, 1997; Membrillo-Hernandez *et al.*, 1999).

The expression of *hmp* is modulated by *rpoS* (Membrillo-Hernandez, Cook and Poole, 1997) and strongly induced in a SoxRS-independent manner by nitrite and nitric oxide. *Hmp* expression is also induced by S-nitrosoglutathione (GSNO, a NO releaser) and sodium nitroprusside (SNP, a NO^+ donor). This activation requires the MetR protein, the key regulator in methionine and glycine biosynthesis (Membrillo-Hernandez *et al.*, 1998) . In contrast to glyA regulation where both MetR binding sites are occupied when homocysteine levels are high, *hmp* expression requires binding to only one MetR binding site (site1, proximal to hmp). The latter interaction is favored when homocysteine levels are low. Moreover conditions that are known to activate (homocysteine) or inhibit (purine) the expression of the *glyA* gene modify *hmp* expression in an opposite manner (Membrillo-Hernandez *et al.*, 1998). Iron limiting conditions also activates transcription of the *hmp* gene by 40 fold. Expression of *hmp* is repressed under anaerobic conditions by the Fnr protein, a regulator of anaerobic gene expression (Poole *et al.*, 1996). Expression of *hmp* is increased 3 to 4 fold when a *fnr* null mutant strain is grown anaerobically but there is no effect of the mutation when the strain is grown aerobically. An Fnr binding site has been identified in the *glyA-hmp* intergenic region (Figure 6).

1.8 Biofilms and curli

A gene involved in biofilm formation was isolated during the screening of suppressors of the growth defect caused by the deletion of the *folA* gene. This section will therefore review in this section what is currently known about curlin biogenesis.

Biofilms are matrix encased communities of microorganisms that are tightly interacting with each other. Bacteria like *E.coli* must adapt to different ecological niches in order to survive when growth conditions such as temperature, osmolarity and availability of nutrients are less than ideal. Normally, *E.coli* colonizes the gastro-intestinal tract of humans and other animals, but it can also survive in *extra-intestinal* environments. Its survival in extreme conditions is facilitated by the expression of adhesive organelles (pili or fimbriae) allowing for auto-aggregation of individual microbes to enhance metabolic breakdown and colonization of organic and inorganic matter (Olsen *et al.*, 1993). *Escherichia coli*, *Salmonella enterica serovar Enteritidis* and *S. enterica serovar Typhimurium* produce surface bound, long, thin, flexible filaments. These filaments are called curli in *E.coli* and thin aggregative fimbriae or SEF 17 in *Salmonella*. They are normally produced in response to limiting nutrients, at temperatures below $32^{O}C$, in a low osmolarity medium and under stationary phase conditions. These fibres promote bacterial autoaggregation and mediate binding to the dye Congo red, and to a variety of extracellular matrix and serum proteins such as plasminogen, plasminogen activator protein, soluble fibronectins, laminins and major histocompatibility complex class I (MHC-I) molecules (Olsen *et al.*, 1998). Since nutrient trapping and protection of the population by providing an extracellular layer against mechanical stress are

characteristics of biofilms (Romling *et al.*, 1998), it is therefore not surprising that a gene involved in biofilm formation, is being implicated in one carbon metabolism possibly in response to the limitation in nutrients.

1.8.1 What are curli?

A curli fibre is a highly insoluble polymer composed of curlin (CsgA), a 15.3 kDa protein. The fibres are resistant to heat or denaturing agents and require boiling in acid to break the subunit interactions (Hammar *et al.*, 1995). CsgA is highly homologous to the AgfA subunit of SEF 17 fimbriae, encoded by the *agfA* gene. CsgA has an unusually high percentage of glycine residues relative to the average for *E. coli* proteins. The glycine residues clustered through- out the protein (Olsen *et al.*, 1993) (Figure 7).

1.8.2 The *csg* operon

Two divergently transcribed operons, *csgBA and csgDEFG* located at 23.8 minutes on the chromosome, are required to produce curli in *E. coli* (Figure 8). The regulation of the genes coding for curli fibers is complex and involves several control elements, such as H-NS, IHF (integration host factor), RpoS, OmpR ,CpxR and MlrA (Gerstel and Romling, 2003). The *csgBA* promotor requires an AT-rich upstream activating sequence which is recognized by both σ^s and σ^{70} sigma factors and is repressed by H-NS. This prevents the formation of transcription initiation complexes with σ^{70} under conditions where σ^s is not expressed, for example at temperatures above 26^0C,

1 mkllkvaaia aivfsgsala↑ gvvpqy**gggg** nh**ggggnnsg** pnselniyqy **ggg**nsalalq
61 tdarnsdlti tqh**gggngad** vgq**gsddssi** dltqr**gfgns** atldqwn**gkn** semtvkq**fgg**
121 **gngaavdqta** snssvnvtqv **gfgnnatahq** y

Figure 7: Amino acid sequence of curlin CsgA from *E.coli* Swissprotein accession P28307 (arrow indicates the putative cleavage site for signal peptidase I)

Figure 8: Transcription of the *csg* operon.

Gene Products

CsgA: major curlin fiber unit.

CsgB: nucleator protein.

CsgD: transcriptional regulator

CsgG: outermembrane lipoprotein.

CsgE: fibronectin and Congo red binding.

CsgF: required for nucleation of CsgA and CsgB.

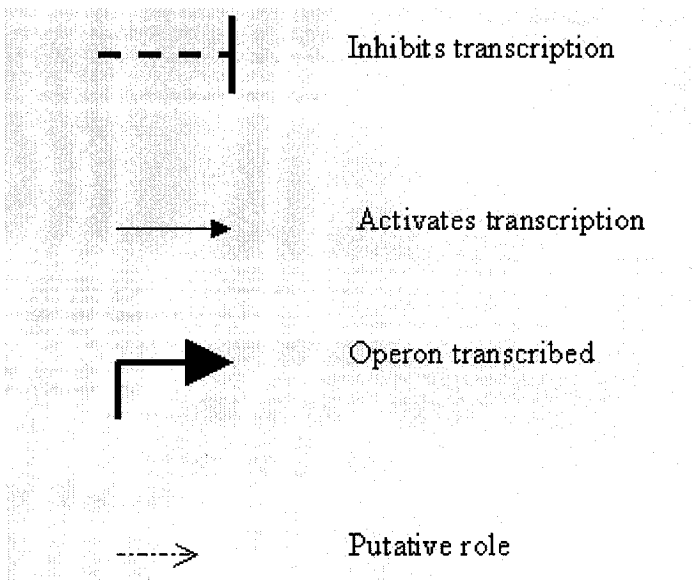
MlrA: Transcriptional regulator required for curli production
and extracellular matrix formation

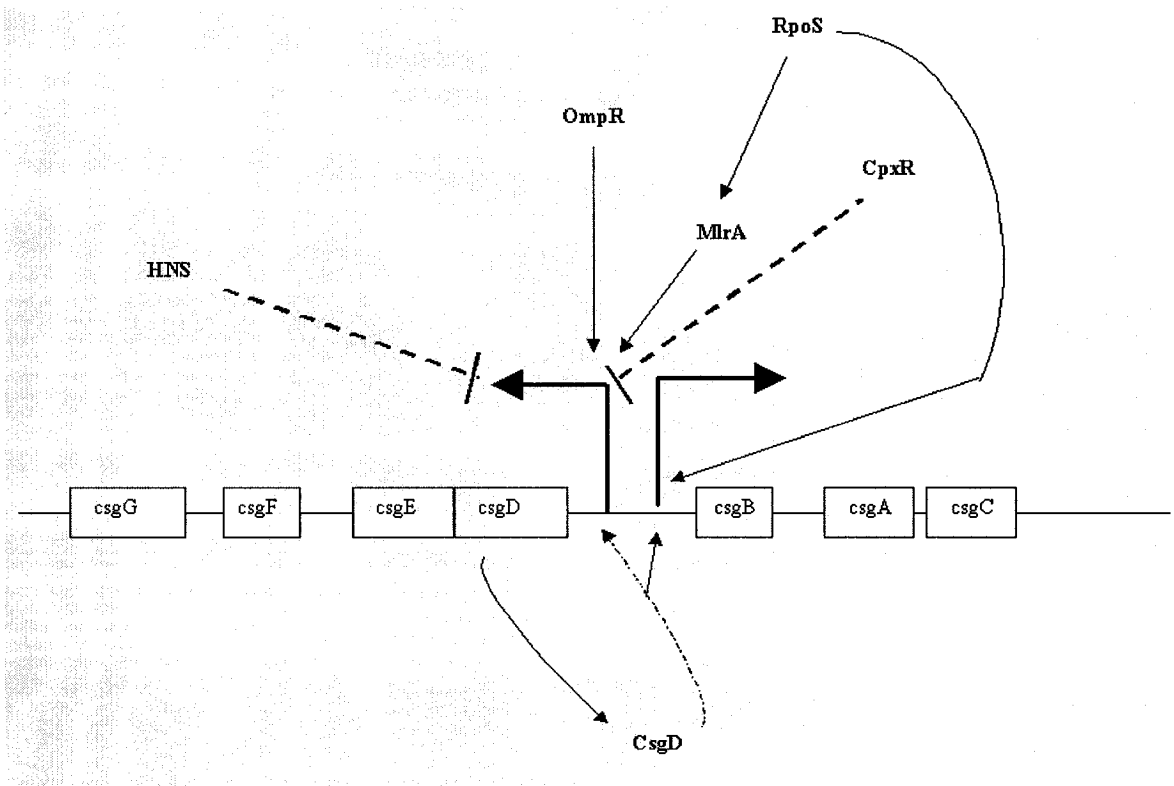
OmpR: Response regulator of the porins OmpF and OmpC,

CpxR: Response regulator mediates transcription of stress-combative genes.

RpoS: Stationary phase Sigma factor (σ^S)

Legend:




RpoS
OmpR
CpxR
MlrA
HNS
csgG
csgF
csgE
csgD
csgB
csgA
csgC
CsgD

at high osmolarity or during logarithmic growth (Collinson *et al.*, 1996). The second operon *csgDEFG* is composed of the putative transcriptional activator of curli biosynthesis, *csgD* and three genes encoding curli assembly factors (Chapman *et al.*, 2002). *CsgG* encodes a lipoprotein located in the outer membrane, as deduced from biochemical evidence and from the predicted amino acid sequence , which contains a lipoprotein leader peptide. Lipoproteins are implicated in secretory pathways, pilus assembly and protection against limited proteolysis in the periplasm.

Insertions in *csgG* prevent curli formation, fibronectin –binding activity and Congo red staining. High intracellular levels of CsgG result in higher levels of CsgA and CsgB being detected suggesting that CsgG stabilizes a curli assembly complex at the outer membrane by protecting CsgA and CsgB against rapid proteolytic degradation. It is not excluded that CsgG may act upon the other two genes *csgE* and *csgF* (Loferer, Hammar and Normark, 1997). *CsgE* is required for the fibronectin and Congo red binding properties of curli fibers but it does not affect polymerization of the fiber subunit. *CsgF* is thought to be involved in the polymerization process, as nucleation is impaired in a *csgF* mutant. Also non polar mutation in *csgF* gene resulted in abnormal congo red binding properties (Chapman *et al.*, 2002). Curli fibers are highly conserved between *Salmonella typhimurium* and *E.coli* with respect to operon structure and regulation. Divergence at the DNA sequence level of the region is about 22.4%, but there is a high level of conservation at the protein level ranging from 86% to 99% amino acid homology (Romling *et al.*, 1998).

1.8.3 Regulation of curli biosynthesis

Transcription from the *csgDEFG* promoter requires the stationary phase-specific/starvation sigma factor σ^s (RpoS) and transcription from the *csgBA* promotor requires CsgD. Even though all *E.coli* K-12 strains carry the *csg genes,* only a subset of them can transcribe them. The explanation behind this difference in behavior is not yet fully understood, although it has been suggested in some cases that an amber mutation in the *rpoS* gene might be responsible for this inhibition. In *E.coli* K-12 strains that are $rpoS^-$, *csgA* is transcriptionally activated by a mutation of the *hns* gene, suggesting that RpoS or another protein positively regulated by RpoS, relieves the transcriptional repression mediated by the histone- like protein product of the *hns* gene. *Hns* $^-$ *rpoS* $^-$ double mutants are still under temperature and osmolarity control suggesting that other factors may also play a role (Olsen *et al.*, 1993). This is also suggested by transcription of curli genes being abolished under high osmolarity conditions, even when RpoS levels are increased.

Classical laboratory strains can be rendered curli proficient by single mutation in the *ompR* gene (Vidal et al., 1998) , or mutations in the *csgD* promoter (Romling *et al.*, 1998 and Uhlich, Keen, and Elder, 2001). OmpR is a regulatory protein, member of the two component regulatory system OmpR/EnvZ that modulates the expression of the *ompC* and *ompF* coding for two major outer membrane proteins in response to surrounding osmolarity sensed by the EnvZ. An OmpR binding site centered at position – 49.5 relative to the start site of *csgD* has been identified (Romling, *et al.*, 1998; Prigent-Combaret *et al.*, 2001). Also, no transcriptional signal is detected for *csgD* or *csgA* in

ompR null mutants in *E.coli*. This suggests that OmpR regulates curli synthesis via *csgD*, which may act upon *csgBA* promotor to initiate transcription.

IHF, a histone-like heterodimeric protein encoded by *ihf*, has also been implicated in the regulation of curlin synthesis (Gerstel and Romling, 2003). Under microaerophilic conditions and in a temperature independent manner, expression of *csgD* was reduced 3 fold in *S. typhimurium* carrying an *ihf* mutation. This decrease was not observed when the strain was grow under aerobic conditions. An IHF binding site in *csgDEFG –csgBA* intergenic region has been identified. IHF is thought to activate *csgD* transcription under microaerophilic conditions by competing with OmpR for binding. A second IHF binding site has been located downstream of the *csgD* transcriptional start. Binding of IHF to this second site had no effect on *csgD* transcriptional activity. The precise role of IHF in modulating *csgD* expression is still under investigation (Gerstel and Romling, 2003).

Another two component regulatory system CpxA/CpxR, activated in response to damage of envelope proteins and changes in inner membrane lipid composition and controlled by RpoS, has also been implicated in the regulation of *csg* gene expression. It has been demonstrated that CpxR binds to both *csgD* and *csgBA* promoters and mediates repression on the operon in response to high osmolarity, curlin overproduction or a combination of these two factors (Prigent-Combaret *et al.*, 2001).

A novel regulator, MlrA, has been recently identified and shown to be required for curli production and extracellular matrix production. The *mlrA* gene was positively regulated by RpoS. Both *csgD* and *csgBA* transcription were abolished in a *mlrA* mutant grown under conditions that promote curli production. The N-terminus region of MlrA contains a putative DNA binding domain with the helix-turn-helix motif and is

homologous to members of the stress response regulators such as mercury resistance regulator (MeR) family of proteins. The C-terminal, however only shares homology to the putative regulators of unknown functions from *E.coli, Salmonella enterica* and *Vibrio cholerae* (Brown *et al.*, 2001).

1.8.4 Is CsgD a transcriptional regulator?

Even though a direct interaction between CsgD and the *csg* promoter regions has not been established, there are several lines of evidence that support a transcriptional regulatory role for CsgD in curli biogenesis. First, the C-terminus of CsgD is highly homologous to the DNA-binding motif (helix-turn-helix) found in members of the FixJ/UhpA/LuxR family of transcriptional regulators. Some transcriptional activators of the LuxR family are known to respond to stationary phase conditions, as a tool for quorum sensing. Quorum sensing allows bacteria intercellular communication to regulate transcription of multiple target genes and control of different functions. They respond by binding autogeneously produced metabolites that are derivatives of homoserine lactones. The N-terminal half of the CsgD sequence, which could act as a response domain, does not show any significant homologies to known homoserine lactone –responding proteins or to any other proteins that bind small molecule effectors in data banks. Secondly, transposition insertion in *csgD* completely abolishes transcription of both *csg* operons. Single transposition insertion in the other genes of the *csgDEFG* operon prevented formation of curli polymers but the *csgBA* operon is still transcribed (Hammar *et al.*, 1995). Morever, mutations in the promoter region of *csgD* result in constitutive curli

expression in an *rpoS* and temperature independent manner (Uhlich, Keen and Elder, 2001; Romling *et al.*,1998(b)). Overexpression of *csgD* from a plasmid also induces curli formation in non curli proficient cells (Pringet-Combaret, 1999; Chirwa and Herrington, 2003).

The *csgD* gene has also been implicated in other pathways such as the regulatory circuit controlling formation of the matrix in biofilms through induction of *adrA* or *yaiC* (*E.coli* homologue), encoding a putative transmembrane protein involved in cellulose production (Zogaj *et al.*, 2001; Brombacher *et al.*, 2003). Bacteria produce cellulose as an extracellular component for mechanical and chemical protection and the co-expression of cellulose and curli leads to the formation of a highly hydrophobic network with tightly packed cells aligned in parallel in a rigid matrix.

Curli proficient strains are able to use arginine or pyruvate or both as metabolic substrates suggesting that *csgD* may influence gene expression beyond those involved in curli production (Uhlich, Keen, and Elder, 2001). There are major changes in gene expression observed within *E.coli* biofilms consistent with new protein synthesis (Prigent-Combaret *et al.*, 1999). Therefore it is not unlikely that one carbon metabolism will be influenced in order to meet the new demands imposed by biofilm formation.

Other indirect evidence suggesting that CsgD regulates other genes come from transcription profiling micro-array studies. When compared to a wild-type *E. coli* laboratory strain, 10 genes were differently expressed in a strain carrying an *ompR234* mutation which activates *csgD* expression. As expected, transcription of the *csgB* and *csgA* genes was up-regulated by $\geq$ 2.5 fold. Other genes that were significantly upregulated include *recT* (coding for a DNA binding protein involved in renaturation of

homologous DNA), *yhiE*, *yjbR* and *ydjC*. Transcription of the following genes was down-regulated by 3-4.8 fold: *thyA* (thymidine synthetase involved in C1-metabolism), *yagS* (a putative xanthinine dehydrogenase), *glnS* (glutaminyl-tRNA synthetase) and *pepD* (carnitine synthetase). Interestingly, the *pepD* and *yagS* genes possess a 11 bp sequence (GGGKGAKNKA) that is also conserved in the promoter region of *yaiC* gene and *csgBA* operon. This sequence was proposed to be a putative binding site for *csgD* although there is still lack of direct evidence for DNA binding (Brombacher *et al.*, 2003)

2. MATERIAL AND METHODS

2.1 Bacterial Strains, Phages and Plasmids. *E. coli* K-12 strains, plasmids and phages are listed in Table 1.

2.2 Media and Growth Conditions. Minimal medium A with glucose (GM), R medium and LB containing 50 μg/ml thymidine (LB-thy) were used routinely (Miller 1992). Media were solidified with 15 g/l agar. When required, GM was supplemented with amino acids and thymidine at 50 μg/ml, adenine at 30 μg/ml and pantothenate at 1 μg/ml. Media containing subsets of FEP were identified by the missing FEP. For example FEP-met contained glycine, histidine, adenine, pantothenate and thymidine but not methionine. Ampicillin (100 μg/ml) was always added when growing ampicillin resistant transformants. Chloramphenicol (25 μg/ml), kanamycin (30 μg/ml), spectinomycin (100 μg/ml) and tetracycline (25 μg/ml) were added when required. 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 and Chirwa, 1999).

2.3 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.
Normally, cells were rendered competent for transformation using $CaCl_2$ (Sambrook, et al., 1989). To avoid heat shock, the one step PEG method (Chung et al., 1989) was used to make λ-lysogens competent.

2.4 Strain Construction. P1vir –mediated transduction was used to transfer the *purR6::Tn10* mutation from strain SØ5052 to strains MH829, GS162λ*glyA-lacZ* and RKP2178. Transductants were selected on LB-thy containing tetracycline and then purified. Similarly the metR::spec mutation was moved from strain RKPL4550 to the above mentioned reporter strains. *MetR* transductants were selected on LB-thy containing spectinomycin. The *metF159* mutation was moved from strain CAG18447 by cotransducing it with *zij501::Tn10* into strains MH828 and GS162λ*glyA-lacZ* and screening tetracycline resistant transductants for mutant phenotype. The MH828*metF*⁻ strain was subsequently made Δ*folA* by P1vir transduction. We constructed strains MH910 and MH911 by respectively transducing *csgA2::Tn105* and *csgG1::Tn105* mutations into strain MH829 with P1vir lysates made from strains MHR204 and MHR210. Strains MH912 and MH913 were constructed by moving *csgA2::Tn105* and *csgG1::Tn105* mutations via P1vir transduction into RKPL2178.

Table 1: E.coli K-12 strains and plasmids . * F. Blattner, University of Wisconsin, Madison, WI, USA; M. Belfort, Wadsworth Center, Albany, N.Y.USA; C.G. Cupples, Concordia Univesity, Montreal, Canada; B.Glick, University of Waterloo, Waterloo, Canada; E.B. Newman, Concordia Univesity,Montreal, Canada; S. Normark, Karolinska Institute, Stockholm, Sweden; C. Prigent-Cmbaret, INSA, Lyon, France; U. Römling, Karolinska Institute, Stockholm, Sweden; M Singer, University of California at Davis, USA; G. Stauffer, University of Iowa, Iowa City, USA; Per Nygaard, Department of microbiology, Technical University of Denmark; CGSC is Coli Genetic Stock Center, Yale University, Princeton N.J. Mary Berlyn, curator (strain was obtained from B. Bachmann). $^{\Psi}$Sequencing show a single base change at position –11 in the promoter region of *purR*.

	Description	Sources* or reference
Strains		
CAG18477	*zij501::Tn10 metF159*	Singer; Singer et al.1989
FB10186	*csgD::Tn5* <Kan-I-SceI >	F.Blattner
GS162λglyA-lacZ	*ΔlacU169 pheA905 araD129 rpsL thi*	G.Stauffer; Lorenz&Stauffer(1995)
GS162λglyT-lacZ	*ΔlacU169 pheA905 araD129 rpsL thi*	G.Stauffer; Jourdan&Stauffer (1998)
JC10289	*recA::Tn10* P1CM	M. Belfort; Csonka & Clark (1979)
MG1655	Wild type	M. Singer
MH618	*Δ(gpt-lac)5 nadB51 relA1 spoT1 thi-1*	Herrington
MH828	*thyA (ts) argE3 rna* λ	Herrington & Chirwa (1999)
MH829	*ΔfolA::kan3 thyA (ts) argE3 rna* λ	Herrington & Chirwa (1999)
MH859	*ΔlacU169 pheA905 araD129 rpsL thi purR6:: Tn10 λglyA-lacZ*	Chirwa and Herrington 2003
MH894	*ΔfolA::kan3 thyA (ts) argE3 rna purR6::Tn10* λ	Chirwa and Herrington 2003
MH901	*ΔlacU169 pheA905 araD129 rpsL thi csgA::Tn10 λglyA-lacZ*	Chirwa and Herrington 2003
MH902	*ΔlacU169 pheA905 araD129 rpsL thi csgG::Tn10 λglyA-lacZ*	Chirwa and Herrington 2003
MH903	*ΔlacU169 pheA905 araD129 rpsL thi purU::kan λglyA-lacZ*	This study
MH905	*thyA (ts) argE3 rna zij501 metF159* λ	This study
MH906	*ΔfolA::kan3 thyA (ts) argE3 rna zij501metF159* λ	This study
MH907	*ΔlacU169 pheA905 araD129 rpsL thi zij501::Tn10 metF159 λglyA-lacZ*	This study
MH910	*ΔfolA::kan3 thyA (ts) argE3 rna csgA2::Tn10* λ	Chirwa and Herrington 2003
MH911	*ΔfolA::kan3 thyA (ts) argE3 rna csgG1::Tn10* λ	Chirwa and Herrington 2003
MH912	*Δ(argF-lacZ)U169 csgA2::Tn10 Φ(hmp-lacZ)*	This study
MH913	*Δ(argF-lacZ)U169 csgG1::Tn10 Φ(hmp-lacZ)*	This study
MH914	*Δ(argF-lacZ)*U169 *purR6::Tn10 Φ(hmp-lacZ)*	This study
MH915	*ΔlacU169 pheA905 araD129 rpsL thi metR::spec λglyA-lacZ*	This study
MH917	*ΔfolA::aadA thyA (ts) argE3 rna* λ	Chirwa and Herrington 2003

Strains		
MH918	Δ(*argF-lacZ)U169 metR ::spec* Φ(*hmp-lacZ*)	Chirwa and Herrington 2003
MH920	*ΔfolA::kan3 thyA* (*ts*) *argE3 rna metR::spec* λ	This study
MH921	*thyA (ts) argE3 rna purU* λ	This Study
MH922	*ΔfolA::aadA thyA (ts) argE3 rna purU* λ	This study
MH923	*thyA (ts) argE3 rna hmp*$^-$ λ	This study
MH924	*ΔfolA::aadA thyA (ts) argE3 rna hmp*$^-$ λ	This study
MH925	*Δ(gpt-lac)5 nadB51 relA1 spoT1 thi-1 λmetR-lacZ*	This study
MH926	*Δ(gpt-lac)5 nadB51 relA1 spoT1 thi-1 λpurR-lacZ*	This study$^{\Psi}$
MH929	*Δ(gpt-lac)5 nadB51 relA1 spoT1 thi-1 λpurU-lacZ*	This study
MH928	*Δ(gpt-lac)5 nadB51 relA1 spoT1 thi-1 λmetE-lacZ*	This study
MH936	*ΔfolA::aadA thyA (ts) argE3 rna csgD::Tn5* <Kan-I-SceI > λ	This study
MH937	thyA (ts) argE3 rna csgD::Tn5 <Kan-I-SceI > λ	This study
MH938	*ΔlacU169 pheA905 araD129 rpsL thi csgD::Tn5* <Kan-I-SceI > *λglyA-lacZ*	This study
MH939	*Δ(argF-lacZ)U169 csgD::Tn5* <Kan-I-SceI > Φ(*hmp-lacZ*)	This study
MHR204	*araΔ139 Δ(argF-lac) U169 rpsL150 relA1 flbB deoC ptsF25 csgA2::Tn105*	S. Normark; Hammar et al (1995)
MHR210	*araD139* Δ(argF-lac) U169 rpsL150 relA1 flbB deoC ptsF25 csgG1::Tn105	S. Normark; Hammar et al (1995)
PLN100	*araD139 Δ(argF-lac) U169 thi rpsL150 relA1 flbB deoC ptsF25 purU::kan*	Zalkin
RKP2178	Δ(*argF-lacZ)U169* Φ(*hmp-lacZ*)	Poole RK, Poole et al, 1996
RKP4550	Δ(*argF-lacZ)U169 metR::spec* Φ(*hmp-lacZ*)	Poole RK, Poole et al, 1996
Sϕ4021	*purD-lacZ*	Zalkin
Sϕ5052	*lacZ608(Am) purR6::Tn10 rpsL thi*	E.coli Genetic Stock center; Kilstrupet al (1989)
χPh43	*Mu cts Mu dII4042*, F- *Δ(argF lacIPOZYA) U169 trp Δ(brnQ phoA proC phoB phoR)24*	E.B Newman; Groisman & Casadaban (1984)

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. Romling; Romling et al. (1998 a)
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)
pSD6P	210 bp *HindIII csgD* fragment in pUC18	Chirwa & Herrington 2003
pUC18	Vector	C.G Cupples; Messing, 1983, Yanisch-Perron (1985)
pRS415	Vector	Simons; Simons et al. (1987)
Phages		
λRS45	Phage vector	Simons; Simons et al. (1987)
P1CM	Thermoinducible phage	M. Belfort; Csonka & Clark (1979)
P1vir	Phage	C.G Cupples; Miller (1992)

Strain MH901 and MH902 were obtained by transferring respectively *csgA2::Tn105* and *csgG1::Tn105* mutations to strain GS162λ*glyA-lacZ.* Chloramphenicol resistant colonies were selected. Strains MH936, MH937, MH938 and MH939 were obtained by respectively transducing the *csgD::Tn5<KAN-I-SceI>* mutation from strain FB10186 into strains MH917, MH828, GS162λ*glyA-lacZ* and RKPL2178. Kanamycin resistant colonies were selected and tested for their ability to form biofilm on Congo Red medium. We also used kanamycin selection to isolate P1vir mediated transductants constructed by moving a *purU::kan* mutation from strain PLN100 into strains GS162λ*glyA-lacZ* and MH917.

2.4.1 Construction of *lacZ* operon fusions

To construct a strain containing a *purU* (MH929), *purR* (MH926), *metE* (MH928) or *metR* (MH925) –driven *lacZ* gene on the chromosome, the method developed by Simons et al (Simons, Houman and Kleckner, 1987) was essentially followed. We used plasmid pRS415 a vector designed for the construction of lacZ fusions that are easily transferred to the phage vector λRS45, thus allowing formation of single copy chromosomal fusions.

The promoters of *metE, metR, purR* and *purU* were amplified by the polymerase chain reaction (PCR). The amplification primers had *EcorI* and *BamHI* restrictions sites added to force the direction of the cloning (table 2). The amplified promoter fragments were cleaned using the QIAquick PCR Purification Kit from QIAGEN and digested with *EcorI* and *BamHI* restriction enzymes . These were then ligated into pRS415 digested with the same restriction enzymes. The construct was confirmed by restriction digest

analysis and sequencing (done by the York University Core Molecular Facility, Toronto, Canada). Strain MC4100 was transformed with ligated plasmid and then infected with λRS45. Recombinant phage containing the desired promotor formed blue plaques on Xgal R plates whereas λRS45 formed white or very light blue plaques. These were then purified and used to infect strains MH618 and blue colonies were identified on LB-thy Xgal. The putative lysogens were tested for the presence of a single copy of the recombinant phage located in the bacterial chromosome using the PCR method described by Powell *et al.(*1994).

2.5 Isolation and Identification of multicopy suppressors. Strain xPH43 is a double lysogen for Mu *cts* and the mini-Mu replicon MudII4042 which confers chloramphenicol resistance on the host strain (Groisman, Castilho and Casadaban, 1984). 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°. Chloramphenicol resistant colonies were selected and were then replica plated to screen for growth on various media. Suppressing plasmids were digested with *Hind*III and the resulting fragments were ligated into the *Hind*III site of pUC18. DH5α was transformed with the ligation mixture. Recombinant plasmids were tested for their effect on growth of strain MH829. Inserts in some of the suppressor plasmids were then sequenced by the York University Core Molecular Facility. To identify the cloned gene(s) the sequencing results were searched against the *E.coli* genomic nucleotide sequences data base.

TABLE 2: Sequence of the synthetic oligonucleotides used for constructing LacZ-promotor fusions.

	Region Amplified	PCR Primer's Sequence
metEpls[a]	7421-7439 AE[b] 000458	**GACGGAATTCT**TCGACTACGCTGCACCGGA
metEmns	8343-8324 AE 000458	**ATACGGATCC**GGAGTTCCCCGCCCAATAAC
metRpls	7421-7439 AE 000458	**ATATGGATCC**AGCCGCAGTTCCGCAACGCT
metRmns	8774-8754 AE 000458	**GACGGAATTC**CTTTCACTTTCCCCAGCCAC
purRpls	1745-1764 AE 00026	GACG**GAATTC**CGGAAAGTACGTTGCCGAGC
purRmns	2625-2601 AE 00026	**GTGCGGATCC**CGTGTGACACAGTTGTAGTG
purUpls	9025-9041 AE 000221	TATG**GAATTC**ACCTGGCATCCCTCTTGTGG
purUmns	9403-9384 AE 000221	ATA**GGATCC**A**CGC**AACTTTACGTTGGAGTG

a. The primers are named according to the gene whose promotor is under investigation.

b. GenBank accession number. Nucleotides that were replaced to introduce restriction sites are indicated by boldface letters, restriction sites are underlined. Some nucleotides were also replaced outside of the restriction sites to avoid the generation of primer dimers.

2.6 Incorporation of pABA

The uptake of pABA was measured as previously described (Herrington, 1994). Briefly, cultures were grown to saturation in appropriate media containing 10 000 cpm/ml of [carboxyl-^{14}C] pABA (specific activity 2.04 GBq/mmol (55mCi/mmol)), the cells were harvested by filtration and the amount of label retained was determined by liquid scintillation counting.

2.7 Polymerase Chain Reactions (PCR)

Chromosomal DNA was prepared by suspending freshly grown cells from an LB-thy plate in distilled water, freezing for 30 minutes and then incubating at 37° for 45 minutes. 5 µl of the extract was used for the reaction using Ready-To-Go PCR Beads from Amersham Pharmacia Biotech. Reactions were incubated in Perkin-Elmer Gene Amp System 2400 . The following thermal cycling program was used 95° for 5 min; 55° for 1 min; 72° for 2 min ; followed by 30 X [55° for 1 min; 72° for 2 min, 95° for 1 min]; then 55° for 1 min; 72° for 5 min .

2.8 Enzyme assays.

Threonine dehydrogenase was measured by monitoring threonine dependent NADH formation in toluene permeabilized cells (Ravnikar and Somerville, 1987). Activity was expressed as nmoles of NADH produced per mg of protein per minute.

The serine hydroxymethyltransferase (SHMT) and glycine cleavage (GCV) activities were assayed in crude extracts prepared by sonicating cells. The protein content of extracts was determined by the commercial Pierce BCA protein assay using serum albumin as the standard. The SHMT was assayed as described by Taylor and Weissbach (1965) except that reactions contained 0.20 mCi/mmol of 3-[^{14}C] –serine. The

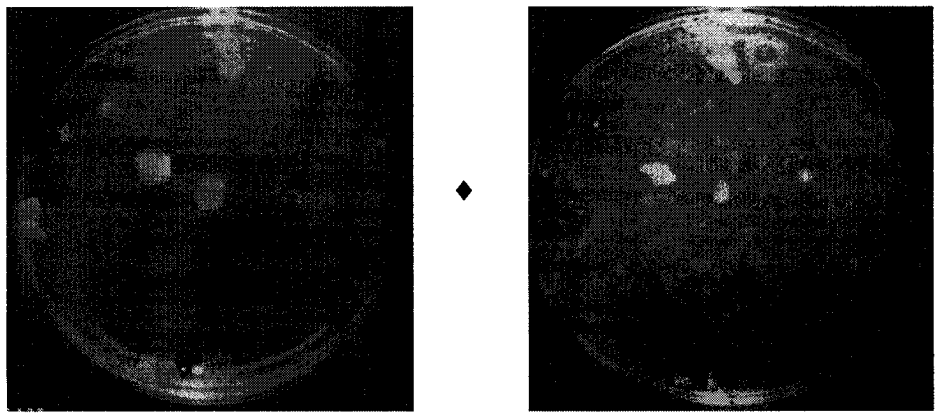
radiolabeled C1-THF produced readily equilibrates with formaldehyde. Addition of dimedone into the reaction mixture traps the labelled formaldehyde into a complex which is then extracted into toluene. The radioactive material present in the upper phase is counted by liquid scintillation. The activity is expressed as nmoles of HCHO generated per milligram of protein per minute. The GCV was assayed by a modification of the SHMT assay (Nagarajan and Storms, 1997). The reaction mix contained 50 mM potassium phosphate pH 7.4, 5 mM dithiothreitol, 1.14 mM THF, 1mM pyridoxal phosphate and 2-[^{14}C] –glycine (0.16 mCI/mmol). Reactions were incubated at 32°. Reactions were stopped and analyzed as for SHMT. β-Galactosidase activity in reporter strains (lacZ fusions) was assayed as described by Miller (1992) using the sodium dodecyl sulfate/chloroform lysis method.

2.9 Curli formation. Curli proficiency was assayed on Congo Red (Hammar,M. *et al.*, 1995) plates supplemented with thymidine, by the ability of cells to bind to polystyrene (Vidal *et al.*, 1998) and by binding of Congo Red in solution (Gophna et al., 2001).

3. RESULTS

3.1 The csgD gene is a multicopy suppressor. We used the in vivo mini Mu cloning system to generate a population of plasmids with random inserts. Phage mini Mu is a temperate phage that integrates the host genome in a random manner. It also contains a high-copy number plasmid replicon that allows cloning of host DNA sequences when they are flanked by 2 copies of mini Mu. The flanked DNA sequences are able to circuliarize by homologous recombination and form a plasmid (Groisman, Castilho and Casadaban, 1984). A mini Mu lysate prepared on the wild -type strain MG1655 was used to transduce strain MH829/Mu *cts* (the prophage Mu carried a temperature sensitive mutation in the *c* gene to prevent induction of the incoming mini Mu elements) to chloroamphenicol resistance.

A total of 59,195 chloramphenicol resistant colonies were screened for the ability to suppress the requirement for glycine or methionine in minimal media containing FEP-gly, FEP-met, FEP-his-met and FEP-his-gly. The majority of colonies did not grow on minimal media supplemented with FEP-gly or FEP-met, and grew slowly on FEP-his-gly and FEP-his-met. Thirty colonies grew faster on FEP-his-gly and FEP-his-met. Five of these grew on FEP-gly and on FEP-met, sixteen grew on FEP-gly but not on FEP-met, and the remaining nine did not grow on either FEP-gly or FEP-met. Growth or faster growth of MH829 /Mu cts transductants on the screened media could result from inactivation of a gene caused by integration of the Mu phage in the chromosome of strain MH829. Alternatively, suppression of the growth requirement of the *folA* null strain could be explained by overexpressing a gene that is normally weakly expressed from the



FEP-his-gly ♦ FEP –his-met

FIGURE 9: Isolation of multicopy suppressors. MH829/Mu *cts* transductants were screened on GM+ supplements. ♦ These are replicates of the same plate.

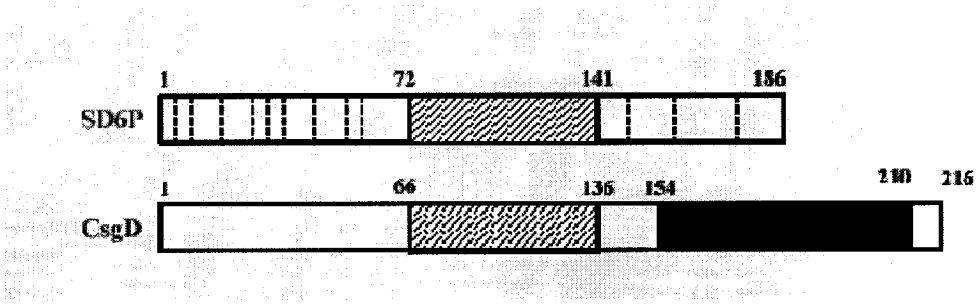


Figure 10: Comparison of CsgD and the putative protein coded by pSD6P. The amino acid sequences of CsgD and the chimeric protein coded by pSD6P were aligned with MultAlin (Corpet, 1988). The shaded area represents regions with 100% identities. The area in black is the helix-turn-helix characteristic of DNA of DNA binding domains. Dashed lines indicate positions in the chimeric protein where the amino acid is identical to that in CsgD. (Chirwa and Herrington 2003)

This predicted ORF could be transcribed from a weak promotor located at the complement of nucleotide 746 of pUC18.

chromosome under the tested conditions. To ensure that the observed phenotype resulted from overexpression of a gene, plasmids isolated from the chloramphenicol resistant colonies were used to transform strain M829. Growth of transformants was tested on the appropriate media.

Mini Mu plasmids can carry insertions of up to 22.3 kilobases (Groisman, 1991; Groisman and Casadaban, 1984). Therefore, *HindIII* fragments of suppressing plasmids were subcloned into the high-copy number vector pUC18. The resultant recombinant plasmids were tested for their ability to alleviate the growth requirement for glycine or methionine of strain MH829.

We isolated two recombinant plasmids from strain MH829 that were able to suppress the glycine auxotrophy. One recombinant plasmid, pSD6P, contained a 210 bp insert. Its sequence was used in a BLAST (Altschung *et al.*, 1997) search of *E .coli* genomic sequence data base. It matched part of the *csgD* gene (AE000205). The other recombinant plasmid contained *yicG* sequences encoding a hypothetical membrane protein. We were unable to isolate any suppressors of the methionine auxotrotrophy.

When pSD6P was analyzed for open reading frames, two were observed. One corresponded to the *bla* gene of pUC18 (Genbank AC# L08752). The other initiated at the complement of nucleotide 499 of pUC18, spanned the insert and terminated at complement of nucleotide 143 of pUC18. The predicted chimeric protein contains 70 amino acids corresponding to the central region of CsgD (Figure 10).

The following observation supports the expression of a chimeric protein. 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

GM+Supplements	pUC18		pSD6P		pKK233-2		pCP994	
	T^1	C^2	T^1	C^2	T^1	C^2	T^1	C^2
Thy	2	s	2	s	2	s	2	n
FEP-his-met	5	s	4	s	5	s	2.5	n
FEP-his-gly	2	s	2	s	2	s	1.5	n
FEP-gly	5	-	4	s	5	v	4	n

Table 3: Suppression of the glycine auxotrophy in strain MH829 by CsgD containing plasmids. [1] Growth is expressed as the days required for colony formation with the highest dilution of cells spotted and - indicates that no colonies were observed at day 5 of incubation. [2] Colony size observed after 5 days of incubation: n indicates normal growth, s small and v very small colonies. All strains formed normal sized colonies on LB-thy at day 1, at day 2 on FEP (but those were not as large as *folA*$^+$ derivatives) and no colonies on FEP-met.

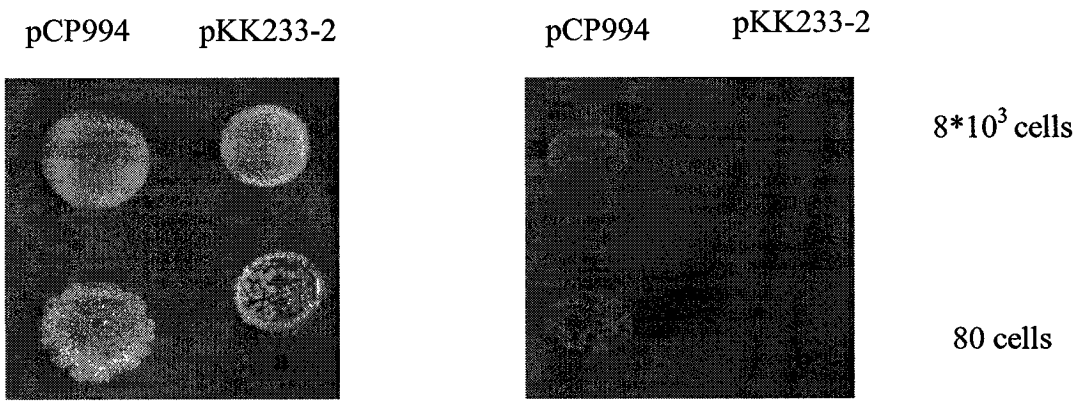


Figure11: Growth of transformants of strain MH829. Transformants were grown overnight in LB-thy ampicillin and aliquots (10 µl) were spotted on plates that were incubated for 5 days at 37° C. (a, b) Upper row, samples were diluted 10^{-3}; lower row, samples were diluted 10^{-5}. (Chirwa and Herrington 2003)

Table 4: Growth of strain MH829 and derivatives. * Growth is expressed as days required for colony formation with the highest dilution of cells spotted on minimal media supplemented with FEP-gly , ¤colonies were barely visible and – indicates that no colonies were observed at 5 days. In general strains carrying control plasmids gave extremely small sized colonies at day 5 compared to strains transformed with plasmids containing *csgD* sequences. All strains formed colonies on LB-thy at day 1. † Herrington and Chirwa, 1999; • Chirwa and Herrington, 2003.

Strain	Relevant genotype	Growth* on FEP-gly				
		Test plasmid	Control plasmid	No plasmid	Test plasmid	Control plasmid
MH829		pGS29	pBR322		4•	-
MH917	ΔfolA::aadA	None	None	5		
		PSD6P	pUC18		5•	-
		pCP994	pKK233-2		4•	5II
MH936	Δ folA::aadA csgD::kan	PSD6P	pUC18		4•	-
		pCP994	pKK233-2		4	-
MH894	ΔfolA::kan3 purR6::Tn10	None	None	4•		
		pCP994	pKK233-2		4•	4
MH906	ΔfolA::kan3 MetF159	pCP994	pKK233-2		-	-
MH920	ΔfolA::kan3 metR::spec	pCP994	pKK233-2		5II	5II
MH910	ΔfolA::kan3 csgA2::Tn105	pCP994	pKK233-2		4•	5II
MH911	ΔfolA::kan3 csgG1:: Tn105	pCP994	pKK233-2		4•	5II
MH921	FolA$^+$purU::kan	pCP994	pKK233-2		2	-
MH922	ΔfolA::aadA purU::kan	pCP994	pKK233-2		5II	5II
MH924	ΔfolA::kan3 hmp$^-$	PCP994	pKK233-2		5	-

sequenced and shown to have the same insert in the opposite orientation to pSD6P, suggesting that changing the orientation of the insert eliminates the suppression of the glycine auxotrophy . Furthermore, suppression of the glycine requirement on FEP-gly minimal media in presence of pSD6P is still observed in strain MH936 where the coding sequence of *csgD* has been disrupted, suggesting that pSD6P does not activate the chromosomal *csgD* .

3.2 Plasmids containing intact csgD suppress. Colony formation was monitored in strain MH829 transformed with pSD6P, pCP994 which contains the intact E.coli *csgD*, pCSGD which contains the homologous gene (*agfD*) from *Salmonella enterica servovar typhimurium* and control plasmids pUC18 and pKK233-2. All transformants formed small colonies within 24 hours on LB-thy (Figure 11 a). On FEP-gly, transformants with control plasmids made no colonies or very small colonies after 5 days. Transformants with pSD6P formed small colonies after 4 days and those with pCP994 or pCSGD formed larger colonies after 3-4 days (Figure 11b, Table 3). Similar results were obtained when plasmids were transformed into strains MH917 and MH936 (Table 4). In strain MH917 the deletion in *folA* is marked with the *aadA* (spectinomycin resistant) cassette rather than the kanamycin cassette. Both the *folA* and *csgD* genes are disrupted in the chromosome of strain MH936. This indicates that the suppression does not require an intact chromosomal copy of *csgD*. In contrast to the Δ*folA*, the $folA^+$ strain MH828 formed large colonies in presence or absence of plasmids on all media by 2 days.

Strain MH829 transformed with plasmids was grown in liquid FEP-gly containing ampicillin with varying amounts of glycine. In the absence of glycine or at low

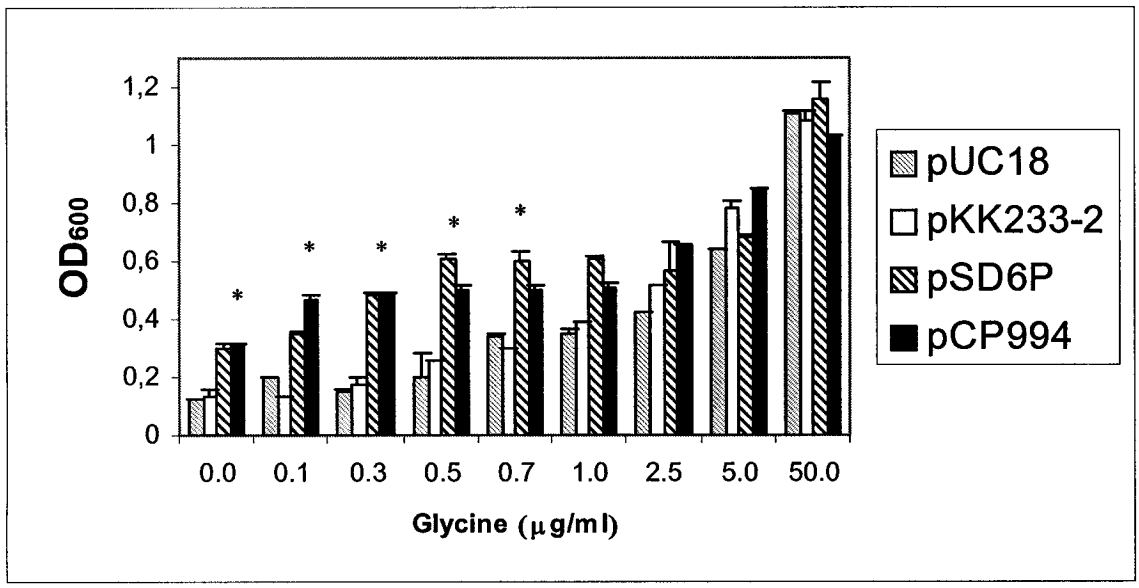


Figure 12: Growth with varying amounts of glycine. Strain MH829 transformed with different plasmids was grown in minimal media containing FEP with the glycine concentration ranging from 0 to 50 µg ml^{-1}. One milliliter cultures were inoculated with 10^6 cells and the OD_{600} was measured after 5 days of incubation at 37° C. Results represents the mean of two assays in which each sample was done in duplicate. Values for pUC18 and pKK233-2 transformants were respectively compared against pSD6P and pCP994 using Student's t test; *, conditions in which pSD6P and pCP994 significantly improved growth.

	^{14}C pABA retained (cpm/OD_{600})					
	folA^{+}		ΔfolA:kan3		*ΔfolA:kan3 pab^{-}	
	pUC18	pSD6P	pUC18	pSD6P	pUC18	pSD6P
Min thy	2500	2200	600	1050	9450	13400
FEP-his-gly	2300	2500	800	1050	7100	8000
FEP	1500	2000	1000	900	ND	ND
LB thy	430	450	400	300	150	150

Table 5: Cells were grown to saturation in appropriate medium containing 10,000cpm/ml (11ng/ml) of ^{14}C pABA at 30 ^{O}C, cells were harvested by filtration and the amount of label retained was determined by liquid scintillation. The values are an average of 10 trials and the standard deviation was less than 2%. *Strains required 8ng/ml of pABA for optimal growth.

concentration, the yield of cells transformed with pCP994 (entire *csgD*) was higher than those transformed with pKK233-2 (control). Similar results were obtained with pSD6P (chimaeric *csgD*) transformants (Figure12). Altogether, our results indicate that multicopies of the intact gene *csgD* gene suppress the glycine auxotrophy.

3.3 Is there an increase in folate synthesis?

Since *folA* null mutant strains have limited pools of folates, we wished to investigate whether the *csgD* containing plasmid had an effect on total folate synthesis. We used ^{14}C pABA uptake to monitor the levels of folate biosynthesis. The data (Table 5) indicate the presence or absence of a *csgD* containing plasmid has no effect on the levels of uptake in both *folA*$^+$ and Δ*folA* strains, although the levels of uptake were lower in *folA* null strains compared to wild-type strains. We confirmed these results using a similar system in a *pab*$^-$ background. Strains carrying a *pab*$^-$ mutation rely sorely on exogenously supplied pABA. Our results followed the same pattern as in a *pab*$^+$ background . The results are consistent with no drastic changes in de novo folate biosynthesis in the presence of *csgD* containing plasmids.

3.4 Serine hydroxymethyltransferase 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

tetrahydrofolate from tetrahydrofolate. To test whether *csgD* sequences modified the expression of SHMT, we assayed SHMT activity in transformants of strains MH828 (*folA*$^{+}$ *thyA* (*ts*)), MH829 (Δ*folA::kan3 thyA* (*ts*)) and GS162 λ*glyA-lacZ* (*folA* $^{+}$ *thyA* $^{+}$). SHMT activity was significantly and reproducibly 1.5 to 3 fold higher in pSD6P and pCP994 transformants compared to control transformants when MH829 and GS162 λ*glyA-lacZ* transformants were grown in GM + thy and when MH828 and GS162 λ*glyA-lacZ* transformants were grown in FEP-gly (Table 6). There were no reproducible differences in activity when MH828 transformants were grown in GM +thy. SHMT activity was higher in the Δ*folA* strain MH829 than in the *folA* + strains. Similar results were observed in a wild type strain when DHFR was inhibited by trimethoprim (Stauffer, 1996 (a)). 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 and Newman, 1974; Greene and Radovich, 1975). We observed repression on FEP-gly with strain GS162 λ*glyA-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 *thy*A(Ts) allele normally express 5% of wild-type level of thymidylate synthase activity (Herrington and Chirwa, 1999). The reduced demand for methylene tetrahydrofolate for thymidylate synthesis could be perceived by the cell as a signal to provide more formyl-tetrahydrofolate for protein synthesis, hence the upregulation of SHMT.

3.5 Expression of β-galactosidase from a *glyA-lacZ* fusion is also increased.

Since our results indicate suppression of the glycine requirement of *folA* null strains and an increase in SHMT activity when strains are transformed with plasmids containing *csgD* sequences, we examined the ability of plasmids pSD6P (truncated *csgD*) and pCP994 (entire csgD) to induce β-galactosidase activity in a reporter strain. Strain GS162 λ*glyA-lacZ* is lysogenized with the *glyA* reporter, λ*glyA-lacZ*. This phage contains the entire *hmp-glyA* intergenic region and the sequence coding for the first 50 amino acids of SHMT fused in frame with the *lacZ* gene (Lorenz and Stauffer, 1995). β-galactosidase activity in strain GS162 λ*glyA-lacZ* transformed with pKK233-2 and pCP994 paralleled SHMT activity (Table 7). This indicates that overexpressing CsgD protein has a stimulatory effect on SHMT expression.

In all media tested, the presence of pCP994 (entire csgD) elicited 1.5 to 3 times more β -galactosidase activity than the control plasmid pKK233-2. In absence of csgD containing plasmids, the lowest observed activity was when cells were grown in GM supplemented with FEP where the combination of methionine, adenine, thymidine and glycine would be expected to repress *glyA* expression. Similar but slightly smaller increases were observed with pSD6P transformants compared to pUC18 transformants (Table 9). We therefore conclude that the increase in SHMT activity was a specific response to increased levels of CsgD or the chimaeric protein. We only observed suppression with some recombinant mini-Mu plasmids although many would be expected to overexpress protein. Moreover, deliberate high level expression of luciferase in *E. coli* actually reduces *glyA* expression (Oh and Liao, 2000).

Medium	Strain	SHMT Activity * pUC18	pSD6P	pKK233-2	pCP994
GM + thy	MH828	7 ± 0.3	14 ± 1.7	9 ± 0.2	10 ± 0.15
GM + thy	MH829	55 ± 3	81 ± 1*	26 ± 1	98 ± 3*
FEP-gly	MH828	20 ± 0.8	32 ± 4*	28 ± 0.9	80 ± 6*
GM	GS162λ*glyA-lacZ*	ND	ND	18 ± 1.5	28 ± 2*
FEP-gly	GS162λ*glyA-lacZ*	ND	ND	12 ± 0.8	25 ± 1.8*

Table 6 : SHMT Activity in Transformants. * SHMT activities (nmoles min $^{-1}$ mg $^{-1}$) are means ± standard deviation 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 GS162 λ*glyA-lacZ* transformants were from a separate experiment. Repeated experiments gave similar results. Values for the control and *csgD* containing plasmids were compared using the Student's t-test and * indicates conditions where the *csgD* plasmid significantly increased the activity.

	SHMT specific activity [b]		β-galactosidase activity[c]	
Supplements[a.]	pKK233-2	pCP994	pKK233-2	pCP994
None	18	28	7100	15600
FEP-GLY	12	25	4600	7200

Table 7: SHMT and β-galactosidase activity of GS162 λglyA-lacZ. [a.]Phenylalanine (50μg/ml) and thiamine (1μg/ml) were added to meet the strain requirement . [b.]Expressed as nanomoles of HCHO generated per milligram of protein per minute. [c.]Units of activity are Miller Units, The standard deviation is less than 10% in all samples.

Table 8: Expression of β-Galactosidase from the λ*glyA-lacZ* fusion. Transformants were grown in the indicated media. β-Galactosidase activities were normalized against the activity of control plasmid pKK233-2 transformants grown without FEP. 100% activity for strain GS162λglyA-lacZ was 7100 Miller units (Miller 1992); for strain MH859,14600; for strain MH901, 8750; for strain MH902, 12,000 and strain MH915, 3700. The standard deviation was less than 15% in all samples. Data are from two representative experiments in which two cultures of each transformants were assayed in duplicate. Strain GS162λglyA-lacZ data are all from one experiment, strains MH901 and MH902 from another, and strains MH859 and MH915 from another. Experiments were reproduced at least twice. * Minimal A glucose media with a higher concentration of FEP contains 200μg ml^{-1} serine, 300 μg ml^{-1} glycine, 200 μg ml^{-1} histidine, 200 μg ml^{-1} methionine, 50 μg ml^{-1} adenine, 50 μg ml^{-1} guanine and 10 μg ml^{-1} thymidine.

Strain	Supplements	Normalized β-Galactosidase activity	
		pKK233-2	pCP994
GS162λ*glyA-lacZ*	NONE	100	250
	THY GLY	100	197
	FEP-HIS-MET	100	130
	FEP-HIS-GLY	100	140
	FEP-GLY	65	100
	FEP-MET	60	135
	FEP	55	70
MH901 (*csgA*⁻)	NONE	100	170
	FEP-GLY	82	160
MH902 (*csgG*⁻)	NONE	100	140
	FEP-GLY	75	200
MH859 (*purR*⁻)	NONE	100	60
	FEP-GLY	75	40
	C_1FEP*	95	75
MH915 (*metR*⁻)	NONE	100	100
	FEP-GLY	40	35
	FEP	30	30

Normalized β-Galactosidase activity				
Supplements	pUC18	pSD6P	pKK233-2	pCP994
MIN A	100	160 (1.5-1.6)*	100	250 (1.8-3)
FEP -GLY	52	69 (1.4-1.6)	55	100 (1.5-3)

Table 9: Comparison of β-Galactosidase activity induction in strain GS162 λ*glyA-lacZ* transformed with plasmids containing *csgD* sequences. Units of activity are Miller units and are normalized against units of activity of lysogen transformed with appropriate control plasmid and grown in Glucose minimal media. Activities for pUC18 and pKK233-2 transformants are respectively 5562 and 1537 units. The standard deviation in all samples was less than 10%. * Numbers in parentheses give the range of the ratios of activity of the csgD containing plasmid compared to the control plasmid observed in three independent experiments.

3.6 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-tetrahydrofolate to grow on FEP-gly. SHMT activity or expression could be increased by 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 8). Strain MH829 grows on FEP-his-gly (Herrington and Chirwa, 1999), and grew on FEP-gly when transformed with pGS29 or made *purR* $^{-}$ (Table 4) indicating that the increase in SHMT activity was sufficient to compensate for the lack of glycine in the medium.

3.7.1 Is the response maintained in strains carrying null mutation of known regulatory genes?

We have determined that a 1.2 to 2 fold increase in SHMT levels could account for the alleviation of the glycine requirement of Δ*folA* strains when grown in FEP-gly minimal media. Two proteins MetR and PurR are known to control the expression of SHMT. Table 8 shows the effect of CsgD on β-galactosidase expression from a λ*glyA-lacZ* fusion in a *metR* and *purR* background. In the *purR*$^{-}$ strain, in the absence of *csgD* containing plasmid, the β-galactosidase activity is derepressed (2 to 3 fold higher) compared to the levels in wild type strain transformed with control plasmid, even at high purine concentrations. Surprisingly in *purR*$^{-}$ background, the β-galactosidase activities are lower in the presence of PCP99 (entire *csgD*) than in the presence of the control plasmid. In contrast, in a wild type (*purR*$^{+}$) background, the presence of multicopy *csgD*

stimulates an increase in β-galactosidase activity. Although *csgD* decreased the expression of β-galactosidase in the *purR*⁻ strain, the activity is 1.2 –1.3 fold higher than the values in purR$^+$ strains grown in absence of CsgD. MH894, the p*urR*⁻*ΔfolA* strain could grow on FEP-gly and CsgD did not seem to improve the growth (Table 4). These results suggest that the CsgD mediated SHMT activity increase requires a functional PurR protein and that in absence of PurR, overexpressing *csgD* is inhibitory to SHMT expression.

In a *metR* background, the presence of *csgD* multicopy plasmid did not result in increased β -galactosidase activity, suggesting that MetR is also required for the *csgD* mediated activation of SHMT. As expected, the levels of β -galactosidase activity were reduced in *metR* mutants when compared to a wild type strainand were subject to purine repression (Steiert *et al.*, 1990).

We have also monitored growth of strain MH920 (*ΔfolA::kan3 metR)* (Table 4), and we did not observe any effect of the *csgD* containing plasmid on growth on any of the media tested including FEP-gly where we normally see a striking difference in growth between transformants with *csgD* plasmids and those without. This corroborates the requirement of an increase in SHMT activity to observe growth.

3.7 .2 What is the effect of CsgD on *metR* and *purR* expression?

Since the CsgD mediated SHMT increase required the presence of PurR and MetR, we hypothesized that an increase in MetR or a decrease in PurR could explain the increase in SHMT activity that we observed in strains carrying a multicopy *csgD* plasmid. To test this, we constructed reporter strains lyzogenised with λ phage containing a *lacZ* gene

β-galactosidase Activity in reporter strains			
Promoter fusion	Supplements	pKK233-2	pCP994
MetR-lacZ	NONE	805 ± 100	710 ± 41
	FEP-GLY	340 ± 44	235 ± 21
	FEP	315 ± 15	220 ± 10
PurR-lacZ	NONE	990 ± 26	1140 ± 103
	FEP-GLY	550 ± 93	645 ± 216
	FEP	700 ± 62	590 ± 44

Table 10: β-galactosidase Activity as a measure of MetR and purR expression in response to the presence of *csgD* on a multicopy plasmid. Units are Miller units (Miller 1992). Values are an average of three experiments in which two cultures of each transformants were assayed in duplicated. Strains were grown in GM supplemented with the indicated FEPs.

fused to the promoter regions of *purR* and *metR*. To our surprise, in presence of multicopy *csgD* gene, the levels of *metR* transcription were reduced significantly on FEP-gly and FEP (1.4 fold) but were not affected on unsupplemented media (Table 10). We observe no significant differences in the levels of *purR* transcription as measured by β-galactosidase activity. We conclude that CsgD has no direct effect on the levels of expression of *purR* gene but reduces *metR* expression under some conditions.

3.7.3 Is there any direct effect on other folate dependent genes ?

In order to elucidate whether csgD plasmid has any effect on the regulation of other genes involved in one carbon metabolism, we have measured the β-galactosidase activities in reporter strains lysogenized with λ phage containing the following promotor fusions: *metE-lacZ, purD-lacZ* and *purU-lacZ.*

The promotor fusion *metE-lacZ* monitors the expression of the *metE* gene coding for a B-12 independent methionine synthetase which transfers the methyl group from methyl-THF to homocysteine to form methionine. Expression of *metE* was not affected by the csgD plasmid (Table 11).

The *purD* gene encodes 5'-phosphoribosylglycinamide synthetase, which is involved in the de novo synthesis of purine (Figure 5). As with other *pur* genes, the expression of *purD* is repressed by purines (Aiba and Mizobuchi, 1989). Our data (Table 11) show no effect of CsgD in the different media tested, but we confirmed that purines repress *purD* transcription.

We also tested the effect the multicopy csgD plasmid on the expression of formyltetrahydrofolate hydrolase, encoded by the *purU* gene. PurU is proposed to balance THF and C1-THF pools in response to glycine and methionine concentration

and to provide formate for GAR transformylase encoded by *purT*. Our data indicate that addition of FEPs to the growth media has no effect on *purU* expession but there is a small increase (1.3 fold) in presence of the *csgD* containing plasmid in all the media tested suggesting an increase in the regeneration of THF. We did not observe activation by methionine or repression by glycine. If THF is rate limiting in glycine synthesis, an increase in *purU* could increase the rate of glycine synthesis.

3.7.4 What is the effect of lowering the demand for reduced folates by other folate dependent pathways?

Inactivation of *metF* blocks the formation of methyl-THF and inactivation of *purU* reduces generation of THF from formyl-THF. These mutants presumably change the distribution of folate in cells. We tested the effect of these mutants, in the presence or absence of the csgD containing plasmid pCP994, on growth of wild type and Δ*folA* strains and on β-galactosidase activity in the reporter strain carrying the *glyA-lacZ* fusion. We scored growth of strain MH906 (Δ*folA metF159)*. The results indicate that making the strain *metF*$^{-}$ does not alleviate the glycine requirement on FEP-gly (Table 4). These results are corroborated by the β-galactosidase experiment (Table 12) where the CsgD effect on FEP-gly is lost although the increase in the level of *glyA* transcription, as measured by the β-galactosidase activity, in presence of *csgD* containing plasmids is still maintained in the other two media tested. Substantial amounts of 5-methyl-THF and 5-formyl-THF have been detected in rat liver (Stover and Schirch, 1990 a). Both compounds are potent inhibitors of SHMT activity. Rabbit liver cytosolic SHMT and *E.*

	β-galactosidase Activity in reporter strains		
Promoter fusion	Supplements	pKK233-2	pCP994
metE-lacZ	NONE	785 ± 150	735 ± 55
	FEP-GLY	175 ± 65	110 ± 12
	FEP	200 ± 10	180 ± 16
purD-lacZ	NONE	40 ± 7	26 ± 4
	FEP-GLY	15 ± 3	16 ± 2
	FEP	15 ± 2	18 ± 2
purU-lacZ	NONE	23 ± 2	30 ± 3*
	FEP-GLY	19 ± 2	25 ± 3*
	FEP	21 ± 0	30 ± 6

Table 11: Effect of CsgD on expression of genes involved in one carbon metabolism. Units are Miller units (Miller 1992). Values are an average of three experiments in which two cultures of each transformants were assayed in duplicate. * indicates conditions in which the csgD containing plasmid significantly (using Student's t-test) increased the β-galactosidase activity when compared to reporter strains transformed with control plasmid.

coli SHMT have been reported to convert 5,10 methylene-THF to 5-formyl-THF in the presence of glycine (Stover and Schirch, 1990 b) and SHMT-glycine-5-formyl-THF complexes have been isolated from *E.coli* (Scarsdale *et al.*, 2000). We speculate that an accumulation of methylene-THF in *metF* mutants could influence the levels of 5-formyl-THF and consequently inhibit SHMT activity rather its expression. *PurU* strains do not grow on media supplemented with adenine and histidine or methionine unless glycine is added (Nagy et al., 1995). Strain MH921 (*purU folA*$^{+}$) did not grow on FEP-gly either (Table 4). When transformed by pCP994 it did grow. Interestingly, strain MH922 (Δ*folA purU*) grew very slowly on FEP-gly in the presence or absence of pCP994. The *purU* mutation did not affect the upregulation of *glyA* by pCP994 (Table 12).

We have shown (Table 8) that there is a histidine component to SHMT repression on media supplemented with FEPs. Glycine starvation in *purU* mutants could result from the combination of limited glycine synthesis because of low SHMT levels in the presence of histidine and adenine and the lack of conversion of excess formyl-THF to THF.This auxotrophy is overcome by csgD-mediated induction of SHMT expression. In strain MH922, even though SHMT expression is increased in the presence of pCP994, glycine synthesis is low because it is not only limited by the amounts of THF available but also by the lack of recycling of THF through PurU. Taken together, these results seem to suggest that the PurU protein plays an important role for the growth of a Δ*folA* strain on FEP media lacking glycine but is not a major contributor in the CsgD response to glycine deprivation when THF is not limiting.

		β-galactosidase Activity in mutants	
Strain	Supplements	pKK233-2	pCP994
m*etF* λ*glyA-lacZ*	MET	11 250 ± 800	14 200* ± 650
	FEP-GLY	9 000 ± 900	8 800 ± 650
	FEP	7 700 ± 950	11 500* ± 1650
purU λ*glyA-lacZ*	NONE	12 000 ± 122	15 100* ± 1714
	FEP	8 700 ± 550	12 300* ± 227

Table 12: Effect of reducing the demand of C1-THF on other folate dependent reactions. Units of activity are Miller units . Values are from a single representative experiment, where each sample is an average of two independent colonies assayed in triplicates. Experiments were reproduced. *Indicates values that are statistically different when comparing pKK233-2(control) and pCP994 (entire *csgD*) using the Student's t test.

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

Since glycine can also be produced from threonine via threonine dehydrogenase (Fraser and Newman, 1975; Ravnikar and Somerville, 1987) or be spared by reducing glycine cleavage (Stauffer, 1996), we investigated threonine dehydrogenase activity and glycine cleavage activity in transformants of MH829 and β -galactosidase activity in transformants of GS162 λ *gly*T-*lacZ*. In minimal media or minimal media supplemented with FEP's, threonine dehydrogenase activity was undetectable in strains MH828 and MH829 in the presence or absence of pCP994. The activity was detectable when cells were grown in LB-thy or inducing medium (Ravnikar and Somerville, 1987), but the presence or absence of pCP994 had no effect on the measured levels (data not shown).

The β-galactosidase activities in GS162 λgcvT-lacZ and the specific activities of GCV measured in strains MH828 and MH829 were generally very low (Tables 13 (a) and 13 (b)). There were no significant differences in the levels of β-galactosidase activity measured between strains transformed with csgD containing plasmids and their respective control plasmids. The differences in specific activity between control and pSD6P transformants in media supplemented with FEP-gly or FEP were not reproducible in a repeat experiment.

We have also scored growth of a strain carrying a *glyA* null mutation. Our results show that the presence of CsgD does not alleviate the glycine requirement for growth in the strain (data not shown). We conclude that alternative pathways of synthesizing glycine do not play a critical role when cells are grown in FEPs.

		Specific activity of GCV	
		Plasmids	
Strain	Medium	pUC18	pSD6P
MH828	Thy	0.65 ± 0.2	0.60± 0.15
	FEP-gly	0.12 ± 0.01	0.45 ± 0.10
MH829	Thy	0.29 ± 0.009	0.26 ± 0.001
	FEP	3.30± 0.14	6.00 ± 0.31

Table 13 (a): Values are from a single experiment where assays were done in duplicate. Experiments was repeated three times and the differences observed in this experiment were not reproducible. Values are expressed as nanomoles of HCHO generated per milligram of protein per minute.

	β-galactosidase activity in GS162 λ*gcvT-lacZ* Plasmid			
Medium	pUC18	pSD6P	pKK233-2	pCP994
Thy	100± 18	90± 14	230± 18	330± 75
Thy gly[a]	450 ± 75	540± 40	550± 60	700 ± 100
FEP	280± 60	250± 30	340± 45	400± 28
LB thy	350 ± 100	490± 21	420 ± 10	450± 24

Table 13(b): [a] Minimal A glucose media is supplemented with 200 μg ml^{-1} of glycine. Units of activity are Miller Units. Values are from a representative experiment in which two cultures of each transformants were assayed in duplicate.

3.9 Does CsgD affect *hmp* expression?

The flavohaemoglobin HMP protein has been implicated in the nitrosative stress response. Since its gene is immediately adjacent to the *glyA* gene and its expression is also modulated by the MetR protein, we were interested in determining whether its expression was affected by CsgD. We tested this by monitoring β-galactosidase activities in a reporter strain lysogenized with a *hmp-lacZ* promotor fusion (Poole *et al.*,1996).We observe a weak repression of *hmp* expression as measured by β-galactosidase activity when cells are grown in FEP-gly and FEP (Table 14 and 15). In presence of the chimaeric SD6P plasmid or the *csgD* containing plasmid, *hmp* expression was increased 1.2 to 2 fold in cells grown in GM or FEP-gly. Expression of *hmp* in cells grown in FEP was consistently and reproducibly elevated in cells transformed with the chimaeric plasmid SD6P but with pCP994 it was elevated two out of three experiments (Table 14). There was no difference in the magnitude of induction between the chimaeric plasmid and the CsgD containing plasmid in media tested, except in GM media where the induction by *csgD* was stronger. We conclude that CsgD also has an effect on *hmp* expression.

Since binding of MetR to the *glyA-hmp* intergenic region was shown to modulate *hmp* transcription, we investigated the effect of *csgD* on a multicopy plasmid on the level of transcription of *hmp* in a *metR*$^-$ background (Membrillo-Hernandez *et al.*, 1998). Membrillo-Hernandez *et al.*(1998) reported a 1.5 fold decrease in *hmp* expression in absence of MetR when cells were grown in MOPS-glucose media. In contrast, we observe a 1.8 to 2.3 fold increase in expression. Three factors could explain this

Normalized β-Galactosidase activity				
Supplements	pUC18	pSD6P	pKK233-2	pCP994
MIN A	100	130 (1.2-1.3)*	100	215 (1.6-2)*
FEP -GLY	85	110 (1.3-1.6)*	80	100 (1.2)*
FEP	70	120 (1.3-1.7)*	70	100 (1-1.4)*

Table 14: Comparison of β-Galactosidase activity induction in strain RKP2178 Φ *hmp-lacZ* transformed with plasmids containing *csgD* sequences. Units of activity are Miller units and are normalized against units of activity of lysogen transformed with the appropriate control plasmid and grown in Glucose minimal media. Activities for pUC18 and pKK233-2 transformants are both 70 units. The standard deviation in all samples was less than 10%. * Numbers in parentheses give the range of the ratios of activity of the csgD containing plasmid compared to the control plasmid observed in repetitive independent experiments.

Table 15: Effect CsgD on hmp expression. Units of activity are Miller units. Values are from a single representative experiment, where each sample is an average of two independent colonies assayed in duplicates. Experiment was repeated at least twice.

β-galactosidase Activity in a hmp-lacZ reporter strain			
Strain	Supplements	pKK233-2	pCP994
hmp-lacZ	NONE	70 +/- 8	150 *+/- 20
	FEP-GLY	55 +/- 7	70 *+/- 5
	FEP	50 +/- 4	70 +/- 11
hmp-lacZ metR$^-$	NONE	120 ± 7	170 *± 18
	FEP-GLY	120 ± 7	165* ± 6
	FEP	110± 6	155 * ± 3
hmp-lacZ purR$^-$	NONE	90 +/- 8	140 *+/- 22
	FEP-GLY	60 +/- 4	85 *+/- 16
	FEP	50 +/- 6	85 *+/- 14
hmp-lacZ csgA$^-$	NONE	95 ± 10	165* ± 30
	FEP-GLY	90 ± 6	135* ± 14
	FEP	55 ± 6	135* ± 14
hmp-lacZ csgG$^-$	NONE	100 ± 5	170* ± 28
	FEP-GLY	90 ± 16	125 * ± 15
	FEP	75 ± 15	125 * ± 29

discrepancy.Firstly, we measured the levels of gene expression in reporter strains grown in GM as opposed to the MOPS-glucose media used by Membrillo-Hernandez *et al.* Secondly, our data was obtained from strains transformed with plasmids unlike Membrillo-Hernandez *et al*'s report. Lastly, it is also plausible that the discrepancy observed is linked to a difference in background. Strain RKP4550 (Φhmp-lacZ metR::spec) from Poole's group (Membrillo-Hernandez *et al.* 1998) grew poorly on LB-thy and was not viable in GM with or without supplements which is uncharacteristic of *metR* mutants. We therefore reconstructed the strain MH918 (Φhmp-lacZ metR::spec) by moving the *metR::spec* into strain RKPL2178 (Φhmp-lacZ) by transduction. Interestingly the weak repression of *hmp* expression observed, when cells are grown in FEP-gly and FEP, is not seen a $metR^-$ background. PCP994 (entire *csgD*) induces *hmp* transcription to the same extent (1.4 fold) in all media tested. These results suggest that MetR protein is not implicated in the induction of *hmp* expression elicited by CsgD.

We have also tested the role of PurR on *hmp* expression by monitoring β-galactosidase activity in a $purR^-$ background. In GM, the levels of expression are increased by 1.4 compared to a $purR^+$ background. *Hmp* expression is inhibited when the cells are treated with FEPs but in all media tested expression was higher in pCP994 transformants than in control transformants (Table 15). Inactivating PurR had no effect in the *csgD*-mediated induction of *hmp* expression. These results suggest that PurR is not necessary for the CsgD mediated induction of *hmp* expression and is probably not involved in the repression by FEP. Induction of *hmp* expression when cells are treated with purines has been reported by Membrillo-Hernandez *et al.* (1998). Our results

indicate that *hmp* expression is repressed in FEP and FEP-gly suggesting that the presence of other compounds interfere with purine induction.

We also a tested the effect of a *hmp* mutation on growth in a Δ*folA* and *folA*$^+$ background. There was no effect of the mutation in a *folA*$^+$ strain, but in a Δ*folA hmp*$^-$ strain growth was altered although the response to *csgD* containing plasmid was maintained. On media supplemented with thymidine, MH829 transformed with pCP994 or the control plasmid formed colonies within 2 days. The *hmp*$^-$ derivatives required 4 days of incubation. Similarly the growth on FEP-gly of the *hmp*$^-$ mutant transformed with pCP994 (Table 4) was delayed to 5 days whereas the control transformants did not grow. These results suggest that *hmp* gene is not an essential gene for survival.

3.10 Are Curli produced in strains transformed with *csgD*- containing plasmids ?

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 strain MH829 transformed with pSD6P or pCP994 were red whereas transformants with the control plasmids pKK233-2 and pUC18 were white (Figure 13). Similar results were obtained with strains MH828 (*folA*$^+$) and MH937 (*csgD* $^-$). Strain MH828 transformed with control plasmids bound less Congo red than *csgD* transformants (Table 16). The *csgD* plasmids also promoted binding to plastic whereas the control plasmid did not (data

not shown). These results indicate that our strains made curli when the csgD plasmids were present.

3.11 Does a Δ*folA* strain have to produce curli to grow on FEP-gly?

To determine whether the ability to make curli was necessary for growth on FEP-gly or increasing *glyA* and *hmp* expression we constructed *csgA*⁻ and *csgG*⁻ derivatives of strains MH829, GS162 λ*glyA-lacZ* and RKP2178 Φ *hmp-lacZ* . Both genes are essential for curli biogenesis.

Transformants of strains MH910 (Δ*folA csgA*⁻) and MH911 (Δ*folA csgG*⁻) were tested for growth on FEP-gly (Table 4). The csg mutations did not affect growth. We measured β -galactosidase activities in the glyA-lacZ reporter strains (MH901(*csgA*⁻) and MH902 (*csgG*⁻)) (Table 8) and the hmp-lacZ reporter strains MH912 (*csgA*⁻) and MH913 (*csgG*⁻) (Table 15). The β -galactosidase activities were consistently higher in pCP994 (entire *csgD*) transformants than in control transformants. We conclude that formation of curli fibers was not necessary for the CsgD mediated suppression of glycine auxotrophy or elevation of *glyA* and *hmp* expression.

3.12 Is chromosomally encoded CsgD involved?

To determine if the chromosomal csgD gene was required for suppression by the plasmid pSD6P and pCP994 and for the increased expression of *glyA* and *hmp*, we constructed *csgD*⁻ derivatives of strains MH829, GS162λglyA-lacZ, and RKP2178 and transformed the resultant strains with pSD6P, pCP994 and their respective control plasmids. The pCP994 transformants of strain MH936 (Δ*folA::aadA csgD*⁻) grew as well

Plasmid	Congo Red Binding*
pUC18	49 ± 6.5
pSD6P	67 ± 7.0
pKK233-2	47 ± 0.1
pCP994	96 ± 32

Table 16. Congo Red Binding by Transformants of Strain MH828. * Congo Red binding was 1000 (ΔA_{500}/ OD_{600}) where A_{500} was the difference between the A_{500} of the congo red solution without cells and the A_{500} measured after cells were incubated in the solution and then removed. Data were from a representative experiment and were averages of two determinations on each transformants. The binding varied from day to day, but followed the same pattern.

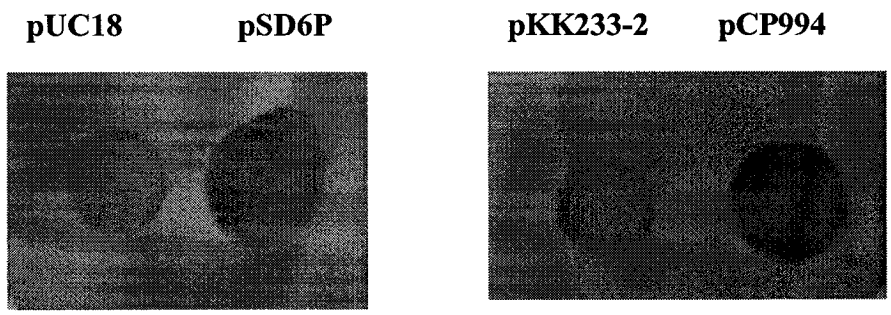


Figure 13: Congo red spots on undiluted cultures of MH829 transformed with plasmid and incubated at 23^{O}C. Dark grey spots are red and light gray are pink.

as transformants of MH829 (Table 4) and were red on congo red plates (data not shown). Similarly growth of strains MH829 and MH936 transformed with pSD6P were comparable. The activity of β-galactosidase in pCP994 transformants of the csgD$^-$ reporter strain MH938 was higher than in controls (Table 17) and the magnitude of increase was similar to that observed in the csgD$^+$ strain GS162λglyA-lacZ. In contrast, pSD6P enhanced the activity only moderately in two of the three growth conditions tested. Interestingly, in strain MH939 (*hmp-lacZ csgD*$^-$), the levels of beta-galactosidase activity induction in presence of pSD6P or pCP994 are comparable and sometimes higher for pSD6P transformants in two of the media tested (Table 17).

Taken together, these result suggest that the chimaeric protein coded by pSD6P is not sufficient for optimum suppression, curli formation and glyA expression, but it is adequate to increase *hmp* expression. The results also suggested that it influences the expression or the activity of the chromosomally encoded CsgD protein (Chirwa and Herrington 2003).

		Normalized β-galactosidase activity in strains transformed with :			
Strain	Supplement	pUC18	pSD6P	pKK233-2	pCP994
MH938 *λglyA-lacZ csgD::kan*	None	100	113	100	200
	FEP-gly	44	61	65	110
	FEP	74	74	60	84
MH939 *hmp-lacZ csgD::kan*	None	100	175	100	130
	FEP-gly	80	130	105	160
	FEP	80	140	110	135

Table 17: β-Galactosidase activities in strain MH938 and MH939. Transformants were grown in media indicated. β-Galactosidase activities were normalized against the activity of control plasmid transformants grown without FEP. One hundred percent activity for strain MH938 (pUC18) was 3460 Miller Units; for strain MH938 (pKK233-2), 3750 Miller Units; for strain MH939 (pUC18) 100 Miller Units and for strain MH939 (pKK233-2) 120 Miller Units. The SD was less than 15% in all samples. Data are from one representative experiment for each strain, in which two cultures of each transformants were assayed in duplicate. The experiment was reproduced.

4. Discussion

4.1 Isolation of multicopy suppressor of the glycine auxotrophy in *folA* null mutant

We isolated part of the *csgD* gene (pSD6P), while cloning multicopy suppressors that allowed MH829 to grow on FEP-gly and demonstrated that both pSD6P, which appears to express a chimaeric protein and plasmid pCP994, expressing intact CsgD, could also suppress. Both plasmids increased the expression of the *glyA, hmp* and purU genes and made strains curli proficient. The plasmid pCP994 reduced transcription of the *metR* gene in FEP-gly and FEP.

Increased expression of *glyA* relieves the glycine auxotrophy of strains lacking dihydrofolate reductase whether this increase is achieved by expressing *csgD* or *glyA* from multicopy plasmids, or by inactivating the PurR protein which prevents *glyA* transcription expression by binding to a site overlapping the RNA polymerase binding site (Chirwa and Herrington, 2003; This study). It can also be achieved by removing histidine from the growth media (Chirwa and Herrington, 2003). The synthesis of histidine is interconnected with purine biosynthesis. A molecule of ATP and phosphoribosylpyrophosate (PRPP) are precursors for the formation of histidine and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) an intermediate in the synthesis ATP and GTP. Therefore the availability of histidine could influence the intracellular concentration of guanine and hypoxanthine. These are corepressors of the purine repressor PurR protein and changes in their levels could modulate g*lyA* expression as well as genes involved in the purine pathway. The PurU protein has been implicated in balancing the demand for glycine and pools of C1-THF/THF. Little is known about the

regulation of the *purU* gene but its inactivation leads to glycine auxotrophy when cells are grown in presence of adenine and either methionine or histidine. (Nagy *et al.*, 1993). The presence of adenine with either methionine or histidine in the growth media represses *glyA* expression. Furthermore purine and/or methionine biosyntheses are inhibited. This limits C1-THF flux through these 2 pathways which enhances *glyA* repression. Other studies have shown that intracellular levels of MetR and its co-regulator homocysteine also influence *glyA* expression. The expression is induced when MetR levels are high and repressed when the levels are low. *MetR* transcription is inhibited via autoregulation or through the MetJ repressor and its co-repressor methionine (Urbanowski and Stauffer, 1986). Coincidentally MetR, also regulates *hmp,* a gene adjacent to *glyA* that codes for a flavohaemoglobin that possesses number of reductase activities. High levels of homocysteine inhibited *hmp* expression by 1.5 fold whereas inactivation of homocysteine by S-nitrosothiols increase its expression by 3.75 (Membrillo-Hernandez *et al.*, 1998). Some other conditions (iron limitation, growth in presence of nitrite, a *fnr* mutant grown anaerobically) can induce to much (6-40 fold) higher levels. Interestingly, the purine inosine induces hmp expression, suggesting that *glyA* and *hmp* expression are inversely regulated (Poole *et al.*, 1996).

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 (MacRae, TJ., C. Zamabrana, N.T Chirwa and M.B Herrington unpublished results). This suggests, that part of the cell's response to signals that induce curli formation, is to increase glycine

synthesis. 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.

4.2 Sources of THF in Δ*folA* strains

In spite of the lack of DHFR, reduced folates can still be detected in a *ΔfolA thyA* strain (Hamm-Alvarez *et al.*, 1990). Reduction of DHF to THF was reported to be 11 times lower in *ΔfolA* compared to *folA*$^+$ strains (Vasudevan *et al.*, 1992). This may explain the requirement for FEP's for optimal growth in *ΔfolA* strains in order to supplement the limited THF available.

DHPR and FolM shown to possess DHFR activities could possibly act as alternate pathways for the reduction of DHF to THF (Hamm-Alvarez *et al.*, 1990; Vasudevan *et al.*, 1992; Giladi *et al.*, 2003). The DHPR gene has not been identified. FolM, when overexpressed from a multicopy plasmid was shown to complement a *folA* null mutation (Giladi *et al.*, 2003). On one hand, gene expression experiments (Brombacher *et al.*, 2003), suggests that *folM* gene is not induced by CsgD. On the other hand, since *Δ (folA folM)* double mutants are not viable (Giladi *et al.*, 2003), one can speculate that it contributes to the growth of Δ*folA* strains although the physiological role and the regulation of *folM* are still unclear.

We propose that an increase in non-specific DHF to THF reduction by cellular reductases leads to an increase in SHMT activity. We have measured the levels of transcription of the *hmp* (to be discussed on later pages) gene whose product is known to

have numerous reductase activities. Our results (Table 15) show that overexpressing *csgD* from a multicopy plasmid weakly induces hmp expression. The growth response to CsgD is maintained in a *hmp-* strain although it is delayed. These results suggest that HMP reductase properties do not play a role in DHF reduction and that other reductases or pathways must be involved.

4.3 Effect of medium on THF

Reduced folates and one-carbon derivatives form a very complex mixture in *E.coli,* because of their varied glutamation levels and different one-carbon units. The demand for specific folates could influence the composition of the mixture and thereby control the rate of FEP synthesis and initiation of protein synthesis (Herrington and Chirwa, 1999). The amounts of specific folates derivatives are not well characterized in a Δ*folA* strain but they are lower than in a $folA^+$ (Hamm-Alvarez *et al.*, 1990). This decrease of total folate pools in a Δ*folA* strain could alter the normal distribution of folates or modify the response to exogeneous nutrients to modulate the growth rate or prevent growth. The inability to grow in a particular medium could result either because the cell is unable to initiate protein synthesis or because it is unable to synthesize a FEP.

Δ*folA* strains require glycine and methionine for growth on media containing adenine and histidine. Adenine and histidine act to limit the turnover of CHO-THF to THF via *purN* and *purH.* First, adenine represses and inhibits purine biosynthesis thus interfering with a major source of recycled THF. Secondly, histidine represses the histidine pathway therefore reducing the demand for ATP and the production of AICAR so that there is little THF generated by PurH (Figure 5). Regeneration of THF is of

tremendous importance in Δ*folA* strains because they have limited resources of THF. Furthermore, on FEP-met, glycine inhibits the activity of PurU (Nagy *et al.*, 1995) thereby also contributing to the reduction of THF turnover from CHO-THF. The lack of available THF limits the formation of CH_3-THF and therefore methionine synthesis is prevented. This could also have repercussions on the available amounts of Fmet-tRNA and thus protein synthesis would be impaired. On FEP-gly, methionine represses the transcription of the genes in the met regulon (Green, 1996) which in turn reduces the amounts of THF that flow through this pathway. Hamm-Alvarez *et al.* (1990) have shown that 10-CHO-THF, required for the de novo purine synthesis and formylation of methionyl-tRNA, is the predominant species in both Δ*folA* and *folA*$^+$ strains grown in LB medium. We speculate that in LB media, the limited 10-CHO-THF available in a *folA* null strain is committed to purine synthesis rather than Fmet-tRNA synthesis. This could explain the slow growth of the *folA* null strain on this media. We also hypothesize that, with the methionine, histidine and purine pathways being repressed when cells are grown on FEP-gly, most of the reduced folate available will be sequestered as 10-CHO-THF. As a result a *folA* null strain could be so limited for THF that it is unable to meet the cell's demand for sufficient glycine (Herrington and Chirwa, 1999).

4.4 Does the Δ*folA* cell have to increase the turnover of C1-THF to THF in order to grow?

We have established that expression of *csgD* from a multicopy plasmid alleviates the glycine auxotrophy on FEP-gly by increasing *glyA* expression. An increase in the amount of SHMT could promote more efficient turnover of the small amounts of THF in

the *folA* null mutants. Turnover of CH_3-THF and CHO-THF could also be enhanced if *csgD* or enhanced *glyA* expression increased the expression of genes in the methionine or purine pathways. Alternatively, formation of glycine could stimulate synthesis of purines or methionine thereby increasing the availability of THF.

To test whether CsgD alters the expression of genes in folate dependent pathways, we constructed promoter fusions using a lac-based reporter system and tested transcriptional levels of several genes (Table 11).

Transcription of two genes, *purD* and *metE* was unaffected by *csgD* suggesting that upregulation of purine and methionine biosynthesis genes does not contribute to suppression of the glycine auxotrophy. Transcription of *purU* was slightly elevated on 2 out of the 3 media tested. PurU generates the formate that is to be used by the alternative and less efficient (135 fold lower) GAR-transformylase and is reported to balance the cellular need for glycine, methionine and THF (Nagy *et al.*, 1995). In *purU*$^-$ strains, CsgD can suppress glycine auxotrophy in a *folA*$^+$ background but not in a Δ*folA* one. This suggests that the observed small increase in *purU* transcription contributes towards the available THF in *folA* null strains. P*urU*$^-$ *folA*$^+$ strains can make THF via DHFR suggesting that, in media containing adenine and histidine or adenine and methionine, SHMT is either inhibited or can not get THF with sufficient glutamate on it. An increase in the glutamate chain length has been thought to increase the affinity for SHMT by 2-fold (Fu *et al.*, 2003).

Δ*folA* strains carrying a *metF*$^-$ mutation are not able to grow on FEP-gly in the presence or absence of CsgD (Table 4). *GlyA* expression in a *metF*$^-$ strain (Table 12) is upregulated when cells are grown on media supplemented with methionine or FEP but

not FEP-gly. On media supplemented with methionine, folate dependent pathways (with the exception of methionine) are not repressed and thus participate fully in the flow through of THF leading to an increase in SHMT synthesis. The results on the two other media tested are unexpected and it is not clear why there is a lack of induction on FEP-gly . Taken together, the lack of growth of Δ*folA metF*$^{-}$ and the lack of induction of *glyA* when a *metF*$^{-}$ strain is grown on FEP-gly, suggest that CH_3-THF used in the *metF* catalyzed synthesis of methionine provides some of the THF needed for growth.

Flow through PurU conversion, purine and methionine pathways, all seem to contribute to the growth of a Δ*folA* strain on FEP-gly media by providing THF to SHMT. It would be interesting to investigate the effect of increasing the THF flow through PurU conversion or the methionine biosynthesis, by using strains transformed with multicopy plasmids carrying *purU*, *metE*, or *metF* genes. It would be equally interesting to measure β-galactosidase activity of *glyA*- l*acZ* reporter strains carrying *metF purU* double mutation and of a *metE- lacZ* reporter strain carrying a *purU* mutation.

4.5 CsgD weakly induces *hmp* expression

The *hmp* gene, whose product is known to have a numerous reductase activities such as nitric oxide, nitroglutathione and nitrite reductase activities (Gardner and Gardner, 2002) and DHPR activity (Vasudevan *et al.*, 1991), was cloned while attempting to identify the gene coding for DHPR. Our data (Table 15) show that CsgD weakly induces *hmp* expression. The physiological significance of stimulating *hmp* transcription, when *csgD* is expressed, is not yet clear. In response to bacterial infection by curliated *E.coli*, mammalian cells induce the expression of nitric oxide synthase coded

by *NOS2* gene thereby increasing the levels of nitric oxide. This induces the innate immune system where immune cells engulf and kill invading pathogens. Nitric oxide radicals are known to be potent antimicrobial agents (Bian *et al.*, 2001). It is therefore not surprising thatCsgD, regulator of curli biofilms and possibly pathogenesis (Gophna *et al.*, 2001), has an effect on the expression of HMP. HMP protein detoxifies NO by converting it to N_2O. Thus, increasing the levels of HMP in curliated bacteria would have a beneficial role, as it would adequately prepare or at least facilitate the bacterial cells survival against future damage in the host's organs.

4.6 How does CsgD regulate *glyA* and *hmp* expression?

We have proposed elsewhere (Chirwa and Herrington 2003), that upregulation of the *glyA* gene is an integral response to signals eliciting curli formation. Curli fibers are polymerized in abundance on the surface of cells that are curli proficient. The curlin subunit, normally synthesized during stationary phase, is rich in glycine content, suggesting that the demand for glycine is particularly high at a time when the availability of nutrients is scarce. Also, it has been shown elsewhere (Schembri, Kjærgaard and Klemm, 2003) that transition to the biofilm state requires new protein synthesis. Therefore increasing the cell's ability to make glycine by elevating SHMT levels prior to and during stationary phase could facilitate the production of curli and therefore the transition into biofilm mode.

CsgD could activate the *glyA* gene directly by binding to its regulatory region. CsgD is a homologue of the two component FixJ/UhpA/LuxR family of proteins characterised by a DNA binding (helix-turn –helix) domain in the C-terminal region and

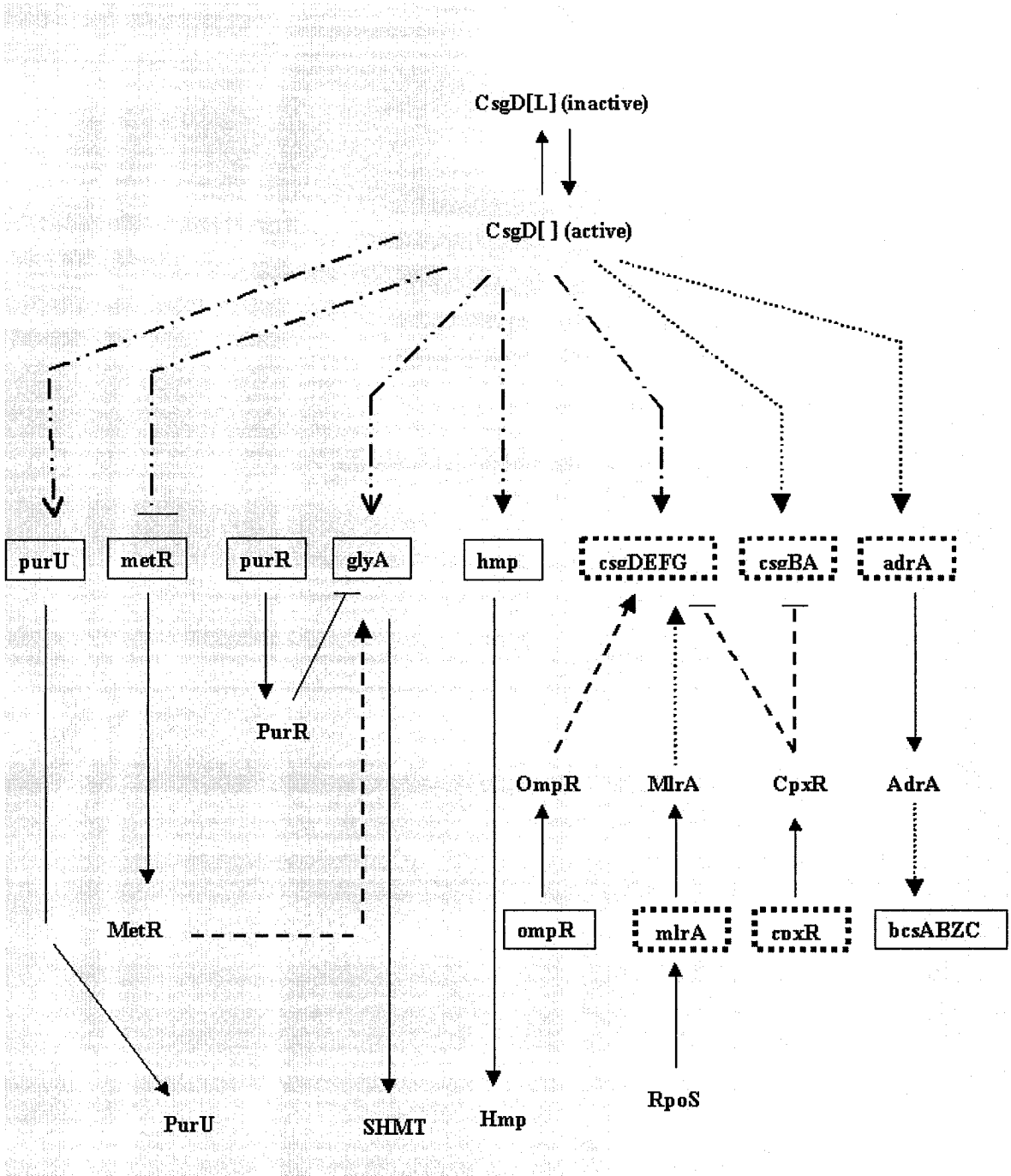
a receiver domain in the N-terminal region which contains a phosphorylation site or binds a small molecule. In the inactive form of the protein, the receiver domain interacts with C-terminal DNA-binding and transcriptional activating sites, thereby preventing the binding of the C-terminal region to DNA binding sites. Activation of the protein is mediated by phosphorylation (or binding of small molecules) of the receiver domain enabling a conformation change that allows the C-terminal to be free to interact with DNA. The chimaeric protein made by pSD6P does not contain the DNA binding domain, and a search against the protein database for patterns and profiles of protein families and domains returned no hits. This argues against it binding to the *glyA* gene or the *csgBA* operon to activate transcription. Furthermore, Brombacher *et al.* (2003) have proposed a putative CsgD binding sequence. We have search the *hmp-glyA* intergenic region for the putative binding site and our results returned no match.

Alternatively, CsgD activation could be mediated by titrating out small molecule effectors that inhibit *glyA* or *csgBA* expression. Activation could also be mediated by facilitating or preventing (in case of repressors) the binding of known regulators to the promoter regions through protein- protein interactions or by regulating their expression. Comparison analysis of CsgD with homologues, that are known to be phosphorylated, suggest that it does not contain a phosphorylation site similar to that of the homologues (Romling *et al.*, 2000). MetR and its co-activator homocysteine, activates the transcription of *glyA*. PurR and its co-repressor guanine or inosine represses transcription. A search of the Conserved Domain Database (Marchler-bauer, 2002) using the N-terminal receiver region of CsgD returned no hits indicating that the receiver domain does not resemble any proteins known to bind homocysteine, guanine, inosine or

related molecules. This suggests that CsgD does not titrate out known ligands involved in *glyA* expression but does not rule out the possibility that CsgD binds other small molecules or protein. In the cell, CsgD can presumably have ligand bound to it (CsgD[L]) or not (CsgD[ ]) (Figure 14). The response to SD6P is weaker in a *csgD* null mutant background compared to wild type background, suggesting that SD6P positively influences the synthesis of CsgD from the chromosomal DNA. We have proposed (Chirwa and Herrington 2003) that CsgD[ ] activates the transcription of target genes whereas (CsgD[L]) is the inactive form. CsgD is normally synthesized during stationary phase although very low levels of expression have been also detected in growing cells (Pringet-Combaret, 2001). Following our model (Figure 14), this would imply that during the logarithmic phase, if a small amount of CsgD is synthesized, most of it would have a ligand bound and thus transcription of target genes would be low. If there is an increase of *csgD* expression through its transcription activators, OmpR, MlrA or because the strain carries multicopy plasmid of *csgD* or pSD6P, then CsgD[ ] predominates and activates transcription.

We have asked whether derepression of the glyA gene by CsgD is influenced by the levels of transcription of genes known to regulate its expression. We have measured the levels of *purR* expression in the presence and absence of CsgD. Our results indicate that CsgD has no significant effect on the levels expression of the PurR protein (Table 10). We have also measured transcription levels of MetR. MetR transcription is autoregulated and is normally repressed by methionine (Urbanowski and Stauffer, 1987a; Green, 1996). As expected, metR transcription is repressed by at least 2 fold when the

Figure 14: 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. 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. Dashed-dotted lines represent hypothetical interaction, dotted lines and dashed represent regulatory circuits that have been identified respectively by mutants or mutants and DNA binding studies. (Chirwa and Herrington, 2003)


CsgD[L] (inactive)
CsgD[ ] (active)
purU
metR
purR
glyA
hmp
csgDEFG
csgBA
adrA
PurR
OmpR
MlrA
CpxR
AdrA
MetR
ompR
mlrA
cpxR
bcsABZC
RpoS
PurU
SHMT
Hmp

cells are grown in FEP-gly or FEP which both contain methionine. Interestingly, the presence of pCP994 repressed transcription by 1.4 fold on these two media but had no effect in unsupplemented media (Table 10) suggesting that the change in *metR* transcription levels in not critical for the CsgD mediated induction of targeted gene. The decrease in *metR* transcription is opposite to what is expected in order to observe activation of glyA.

We have proposed in the previous section that recycling of THF units through the methionine pathway, although minor, could contribute towards a higher flux of C1-THF when csgD is overexpressed. If this is true, then methionine synthesis would be slightly increased even in media containing exogenous methionine and as a result transcription of *metR* would be decreased by the action of the MetJ repressor and its co-repressor SAM.

We have shown (Table 8) that CsgD has no effect on *glyA* expression in metR$^-$ mutants suggesting that MetR is required for the CsgD-mediated induction. CsgD could induce an increase in SHMT synthesis by increasing the affinity of MetR for the two MetR binding sites in the *glyA* promoter (Figure 6). Overexpression of CsgD could also influence the bending of the DNA in the *glyA* promoter in order to facilitate the recruitment of RNA polymerase. Alternatively, CsgD could influence other unknown regulatory proteins of *glyA*. CsgD reduces the derepression of *glyA* transcription in *purR*$^-$ *MetR*$^+$ mutants (Table 8). *GlyA* expression is normally derepressed in strains with inactive PurR (Steiert *et al.*, 1990). Because of the increase in C1-THF's, folate dependent pathway including the methionine synthesis pathway could be upregulated to sustain adequate THF levels. An increase in methionine biosynthesis could lead to reduced intracellular levels of homocysteine (Figure 4) which would normally increase

the binding affinity of MetR to the two binding sites (Lorenz and Stauffer, 1995). Lower levels of homocysteine coupled to reduced synthesis of MetR resulting from an increase in methionine synthesis would weaken the interaction of the protein to MetR site 2 with the lower binding affinity. Thus in PurR$^-$ mutants, the presence of CsgD would lower *glyA* expression. Other studies (Steiert et al., 1990) have looked at levels of SHMT activities and reported that in absence of MetR and PurR, *glyA* expression is slightly derepressed and is subject to a moderately higher purine repression when compared to SHMT levels in *purR*$^-$ *metR*$^+$ mutants. Purine repression in *purR*$^-$ *metR*$^-$ strains suggests that other mechanisms must be involved in the regulation of *glyA* transcription and CsgD could possibly interact with them.

We have also asked whether MetR and PurR levels influence transcription of the *hmp* gene. We have looked at *hmp* transcription levels that are not influenced by MetR or PurR using a reporter strain carring Φ*hmp-lacZ* fusion. Our results (Table 15) show that the response to CsgD is maintained in both *purR*$^-$and *metR*$^-$ mutants, suggesting on one hand that these two regulatory proteins are not involved in the CsgD-mediated induction. On the other hand, MetR seems to play a role in repression of *hmp* by FEP. Membrillo-Hernandez *et al* (1998) have reported that *glyA* and *hmp* genes are regulated in an opposite manner. Our data show activation of both genes under the same growth conditions. CsgD (or a protein regulated by CsgD) could interact with another regulatory protein to enhance RNA polymerase recruitment in the *glyA-hmp* intergenic region.

4.7 Is CsgD a global regulator?

Martinez-Antonio and Collado-Vides (2003) have defined global regulation as the ability of a gene to affect the regulation of operons belonging to several metabolic pathways and to affect an organism by having multiple phenotypic effects. Although they have not classified CsgD as a global transcription factor, it is now becoming clear through indirect evidence that the CsgD effect is not limited to curlin production but also influences other pathways. Zogaj *et al.* (2001) have shown that CsgD regulates the *adrA* (*yaiC* in *E.coli*) whose product is required for the activation of the bacterial cellulose synthesis genes (*bcs*) genes. Cellulose and curli are components of the extracellular matrix that enables biofilm formation in these bacteria. Bacterial cells in a biofilm community are better protected against damaging agents, amoebas and bacteriophages in the surrounding environments. Curli polymers are involved in bacterial adherence to solid surfaces and invasion of host cells (Vidal *et al.*, 1998; Brombacher *et al.*, 2003; Zogaj *et al.*, 2001). Recently, gene array experiments probing global transcription have shown that *E.coli* K-12 expressing curli because of an *ompR234* mutation repress genes that negatively affect biofilm formation (Brombacher *et al.*, 2003). The genes implicated were *pepD,* coding for carnitine dipeptidase (normally induced during phosphate starvation), and *yagS,* coding for a putative xanthine dehydrogenase involved in nucleotide metabolism. Other genes that were down-regulated as a result of curlin synthesis were glutaminyl-tRNA synthetase coded by *glnS,* and interestingly, *thyA* coding for thymidylate synthetase involved in one carbon metabolism. We have shown previously (Chirwa and Herrington, 2003) and in this thesis that overexpression of CsgD in *E.coli* K-12 strains affects transcription of *glyA, purU* and

metR genes also involved in one carbon metabolism. The significance of repressing transcription of *thyA* while activating those of *glyA* and *purU* genes, in cells expressing CsgD could be to efficiently use the limited resources in stationary phase and not deplete the available THF. This would focus the cells' machinery towards the synthesis of glycine and formylated met-tRNA^{fmet} to be used in the biosynthesis of the curlin protein.

The range of genes induced by CsgD include *glyA* and *hmp* (this thesis), *recT* coding for a recombinase (which promotes homologous pairing and strand exchange of DNA) and 2 genes of unknown function (*yhiE* and *ydjC*) (Brombacher *et al.*, 2003). We speculate that range of effect of CsgD is not limited to the above mentioned genes and could be open to other genes that were beyond the scope of methods presented in this thesis or by Brombacher *et al.* (2003) . For instance the cut off value in the gene array experiments is 2.5 fold (induction or repression) which excludes lower levels of induction that might be significant as is the case with the *glyA* gene.

5. CONCLUSION

1. Δ*folA* mutation is not lethal but causes glycine or methionine auxotrophies on media supplemented with FEP lacking glycine or methionine.
2. The glycine growth requirement can be suppressed by increasing SHMT synthesis.
3. High levels of *glyA* transcription can be achieved in strains transformed with plasmids containing intact *csgD* gene or pSD6P, a multicopy plasmid containing part of the *csgD* gene. Increases in SHMT synthesis are also measured in strains carrying a *purR* mutation or transformed with a multicopy plasmid carrying the *glyA* gene.
4. Activation of *glyA* transcription requires functional MetR and PurR.
5. CsgD has no effect on the transcription of *purD* or *purR*, but represses transcription of *metR*.
6. We speculate that C1-THF/ THF are mainly recycled through the purine pathway and slightly through the methionine pathway. We have shown indirectly that CsgD moderately increases the turnover of C_1-THF through *purU*.
7. CsgD increases the transcription of HMP, which may lead to an increase in nonspecific DHF to THF reduction. Activation of transcription requires neither MetR nor PurR.
8. Synthesis of curlin is not required for the CsgD-mediated increase of *hmp* or *glyA* transcription.
9. We propose that CsgD is part of a global stress response with that induces multiple genes required to address different stress conditions such as nutrient limitation, nitric oxide toxicity and oxygen limitation.

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