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Folate Metabolism and Curli Synthesis in *Escherichia coli* K-12

Mahsa Naghavi

A Thesis in the Department of Biology

**Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science (Biology) at
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ABSTRACT

Folate Metabolism and Curli Synthesis in *Escherichia coli* K-12

Mahsa Naghavi

The genes *folA* and *thyA* have a key role in cell growth, as they are involved in the biosynthesis of folate cofactors directly and/or indirectly. The study of the relationship between these two genes will improve our understanding of cell growth.

The effects of *folA* and *thyA* on the growth of *Escherichia coli* cells were studied and the results showed that the absence of the *folA* gene affects the growth of cell. Without *folA* cells are not able to grow or grow very poorly; however, *folA* has no effect on phenotype of strains harboring *thyA*⁺ or *thyA*⁻ mutation. Also, different *thyA* alleles (*thyA*⁻ and *thyA*^{ts}) do not affect the growth of Δ *folA*::*Kan3* strains.

In addition, thin aggregative fimbriae (curli) protein by some of our *E. coli* K-12 lab strains resulted in improved growth in minimal medium compared to their parent. I developed assay for the presence of the CsgA subunit of curli protein in these strains via a rapid purification method (Collinson et al, 1992) and SDS-agarose gel electrophoresis (Horsten, 2003; Bagriantsev et al. 2006). This is a very fast and affective method to detect the presence of curli protein compared to other time consuming methods, such as western blotting and SDS-PAGE, and protein binding assays. Using this “rapid curli assay” I was able to detect the curli protein in the wells of SDS-agarose gels and identify the CsgA protein band of the recovered materials from this gel in a SDS-PAGE gel.

DEDICATION

*To my supervisor Dr. Muriel B. Herrington
for her great support, supervision and kindness*

*To my dear parents, Naghib Naghavi and Fatemeh Motamedi
for their endless love, encouragement and support*

*To my lovely daughter, Saena,
who always gives me hope*

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1. Introduction:

Folate, as a water-soluble B vitamin (B9), is important for normal growth of cells. Dihydrofolate reductase (DHFR) converts dihydrofolate (DHF) to tetrahydrofolate (THF), which is required for the synthesis of the other folate cofactors and therefore the synthesis of RNA, DNA and protein (Figure 1). As shown in Figure 1, thymidylate synthetase ((EC 2.1.1.45; TSase) (product of *thyA* gene), dihydrofolate reductase (EC 1.5.1.3; DHFR) (product of *folA* gene), and serine hydroxymethyltransferase (EC 2.1.2.1; SHMT) (product of *glyA* gene) create a three-enzyme cycle in the folate pathway. The folate cofactors in this three-enzyme cycle are representing major metabolic pathways that are important for cell growth. In *Escherichia coli* DHF is made *de novo* from GTP; it is then reduced to THF by dihydrofolate reductase (DHFR). One-carbon groups (methylene, methyl, and formyl) can be added to THF to make some THF derivatives. These conversions are applied by three different gene products: SHMT, FOLD (methenyl tetrahydrofolate cyclohydrolase) and MetF (methylene-THF reductase) (Figure 1). These THF derivatives (cofactors) are necessary in the biosynthesis of purines, methionine, fmet-tRNA, pantothenate, serine and glycine (Howell, Foster, and Foster, 1988; Matthews, 1996; Green, Nichols, and Matthews, 1996).

1.1. Interconversions of folate cofactors

Methylene-THF is synthesized from THF by two routes. It is formed during the *de novo* synthesis of glycine from serine in the reaction catalysed by SHMT (Figure 1). When glycine is abundant it can be formed by glycine cleavage (GCV) (Green, Nichols, and Matthews, 1996). Methylene-THF is used in four reactions. It is converted to

methenyl-THF by methylene-THF dehydrogenase (FolD) and to methyl-THF by methylene tetrahydrofolate reductase (MetF) (Matthews, 1996).

In pantothenate synthesis, the synthesis of pantoic acid (pantothenate) needs the transfer of the methylene group from methylene-THF to α -ketoisovaleric acid by α -ketopantoate hydroxymethyltransferase (PanB) (Jackowski, 1996; Jones, Judith, and Buck, 1993).

Methylene-THF is also used in the synthesis of thymidylate. In this reaction, catalyzed by thymidylate synthase, it both methylates and reduces the substrate dUMP and thus is converted to DHF (Matthews, 1996).

Methyl-THF, which is synthesized from methylene-THF, converts to THF by methylene-THF reductase (MetF) (Sheppard, 1999). In this reaction methionine is produced from homocysteine (HC) by methionine synthase (MetE and MetH) (Matthews, 1996; Chirwa and Herrington, 2004). The enzymes MetE and MetH transfer the methyl group from methyl-THF to homocysteine to form methionine; however, they have different preferences for the number of glutamate residues on the cofactor, and MetH requires vitamin B12 for activity. The enzymes are cobalamin-dependent methionine synthase (MetH) and cobalamin-independent methionine synthase (MetE) (Greene, 1996) (Green, Nichols, and Matthews, 1996; Greene, 1996; Matthews, 1996; Stauffer, 1996).

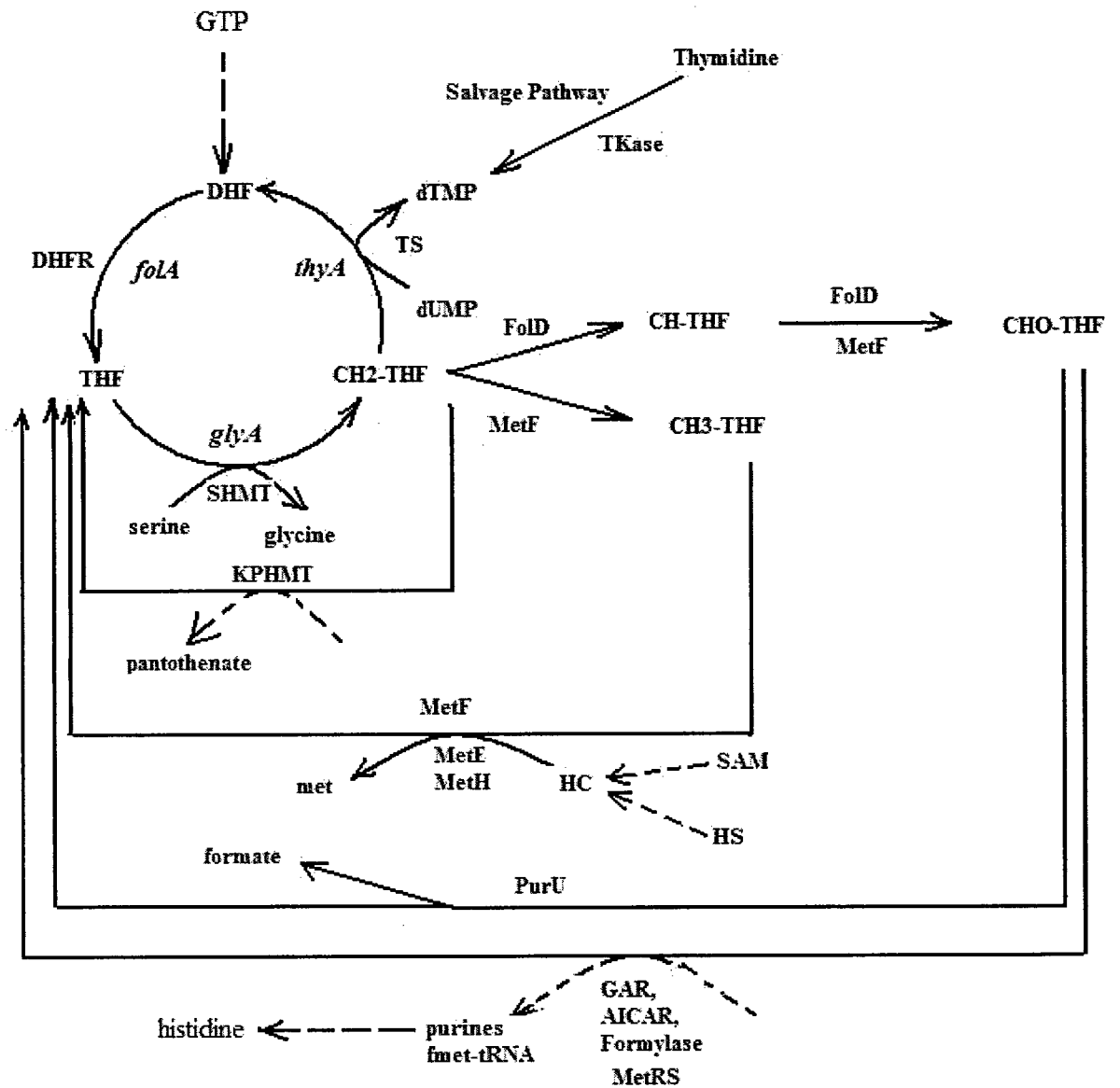


Figure 1. Formation of folate cofactors. Enzymes are indicated as follows: Fola, dihydrofolate reductase; TS, thymidylate synthetase and SHMT, serin hydroxymethyltransferase; MetF, methylene tetrahydrofolate reductase; TKase, thymidine kinase; FOLD, methenyl tetrahydrofolate cyclohydrolase; Met F, methylene-THF reductase; MetE, cobalamin independent; MetH, cobalamin dependent; MetRS, methionyl-tRNA synthetase; Formylase, Met-tRNA^{Met} formyltransferase; PurU, formyl-THF hydrolase; GAR, glycineamid ribonucleotide transformylase; AICAR, 5-aminoimidazole-4-carboxamid ribonucleotide transformylase; KPHMT, ketopantoate hydroxylethyltransferase. The dashed arrows are indicating several steps in a conversion. The abbreviations indicate THF (tetrahydrofolate), CH₂-THF (methylene-THF), CH-THF (methenyl-THF), CH₃-THF (methyl-THF), CHO-THF (formyl-THF), TKase (thymidine kinase), SAM (S-adenosyl-methionine), HS (homoserine), HC (homocysteine), met (methionine), MetF (methylene THF reductase).

F-Met-tRNA^{fmet} is formed by two enzymes. Methionyl-tRNA synthetase (MetRS) enzyme adds methionine to tRNA^{fmet}. Met-tRNA^{fmet} is the substrate for methionyl-tRNA transformylase (formylase) and formyl-THF is the cofactor (Matthews, 1996; Greene, 1996; Schmitt, Panvert, and Blanquet, 1998; Chirwa and Herrington, 2004).

Conversion of formyl-THF to THF involves *de novo* biosynthesis of purines catalyzed by glycineamid ribonucleotide (GAR) transformylase and 5-aminoimidazole-4-carboxamid ribonucleotide (AICAR) transformylase (Nixon, Warren, and Benkovic, 1997; Matthews, 1996). In the reaction of formyltetrahydrofolate deformylase (PurU), formyl-THF converts to THF and formic acid (Nixon, Warren, and Benkovic, 1997).

1.2. Synthesis of thymidylate

Thymidylate (dTMP) is an essential metabolite for DNA synthesis. *E. coli* can grow quite happily with no thymidylate synthase activity at all, as long as cells are provided with either thymine or thymidine. *E. coli* can either make thymidylate *de novo* or can take up exogenous thymine or thymidine and convert them to dTMP (Krishnan, and Berg, 1993; Kisliuk, 2006).

Thymine can be converted to thymidine by thymidine phosphorylase (Neuhard and Kelln, 1996). Also, dTMP can be recovered from thymidine by the intracellular thymidine kinases activity (Figure 1). Thymidylate kinase is the product of the *tdk* gene and the position of this gene is at 27.8min on the *E. coli* genome (Neuhard and Kelln, 1996).

The *de novo* synthesis of thymidylate from dUMP is catalyzed by thymidylate synthetase (TS) the product of the *thyA* gene. In eukaryotes and many bacteria, *de novo*

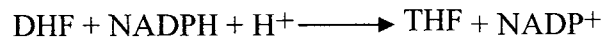
thymidylate synthesis utilize methylene-THF produced via the dTMP cycle (Figure 1), which involves TSase (ThyA), dihydrofolate reductase (DHFR) (FolA) and serine hydroxymethyltransferase (SHMT) (GlyA), as shown below (Krishnan, and Berg, 1993; Kisluk, 2006). In this pathway methylene-THF has bi-functional action as a one-carbon donor and also as a reductant:

•

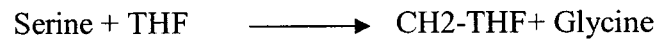
Thymidylate synthase (ThyA):



•Dihydrofolate reductase (FolA):



•Serine hydroxymethyltransferase (SHMT):



The enzyme, DHFR, is also able to reduce folic acid to DHF very slowly in many organisms including some strains of *E. coli* (Green, Nichols, and Matthews, 1996). But, as transportation of folate cofactors is not done by *E. coli* strains, Green, Nichols, and Matthews (1996) assumed this reaction does not occur in vivo. Actually, this enzyme in *E. coli* is only required for the reduction of recently synthesized DHF to THF (Green, Nichols, and Matthews, 1996).

Another *de novo* pathway has recently been found in many bacteria, in which thymidylate is formed from dUMP and methylene-THF by ThyX. ThyX is a novel flavin-

dependent TS that has no amino acid sequence homology with *thyA* coded TS. ThyX produces THF instead of DHF because the methylene-THF serves only as the one carbon donor and reduced flavin nucleotide serves as the reductant (Kisliuk, 2006).

1.3. The *lgt-thyA* operon and its expression

It has not been possible to delete the complete *thyA* gene from the *E. coli* chromosome, because of the presence of an essential gene within or close to the *thyA* gene (Chung and Greenberg, 1973). Later, Williams and his group (1989) discovered a structural gene for an essential membrane protein (unidentified membrane protein, *umpA*), which is located on a 1.5 Kbp DNA fragment immediately 5' to the *thyA* gene. The product of this gene is a membrane-associated polypeptide and approximately 25 kilodaltons in size (Chung and Greenberg, 1973; Belfort, Maley and Maley, 1983; Williams, Fortson, Dykastra, 1989; Gan, Sankaran, Williams, 1995).

The nucleotide sequence of the *umpA* gene and its amino acid sequence show very high degrees of identity with the *lgt* gene of *Salmonella typhimurium* and its gene product, respectively. Gan, Sankaran, and Williams (1995) showed that the *umpA* gene in *E. coli* and *lgt* in *S. typhimurium* are homologous and that phosphatidylglycerol:prolipoprotein diacylglyceryl transferase is an essential enzyme in enteric bacteria for the viability of the respective organisms. They sequenced the *lgt* and *thyA* genes and reported the transcription start sites, promoters and ribosome binding sites (RBS) for these two genes (Gan, Sankaran, and Williams, 1995).

Williams, Fortson, and Dykastra (1989) believed that, however, the function of UmpA protein was unknown, but it seemed to apply a direct or indirect effect upon cell

division and the synthesis of TSase (Williams, Fortson, and Dykastra, 1989). Gan, Sankaran, and Williams (1995) showed that in *E. coli*, where *lgt* and *thyA* form an operon, TSase levels, which are very low in bacteria (250 molecules per cell in *E. coli*), are regulated by transcription from the *lgt* promoter and by translational coupling (Gan, Sankaran, and Williams, 1995).

The existence of a 2.0-kb *thyA* transcript that presumably also contained the 5'-flanking *lgt* mRNA was observed (Belfort, Maley, and Pedersen-Lane, 1983; Gan, Sankaran, and Williams, 1995). Gan, Sankaran, and Williams (1995) determined the transcription start site of the *lgt* gene. They showed that there was a strong consensus -10 sequence (TATTAT) immediately upstream of the transcriptional start site but no apparent -35 consensus site. It would thus appear that the majority of the *thyA* transcripts arise from the *lgt* promoter, since the -10 sequence associated with the *thyA* promoter (TACATC) was predicted to be very weak (Gan, Sankaran, and Williams, 1995).

Belfort, Maley and Pedersen-Lane (1983) determined the DNA sequence of the *thyA* gene and found part of an open reading frame (ORF) that overlaps the *thyA* gene. This ORF situates immediately 5' upstream of *thyA* structural gene and its 3' translational terminator (UGA) of this upstream structural gene overlaps the *thyA* Shine-Dalgarno (SD) (GAGGA) sequence (Belfort, Maley and Pedersen-Lane, 1983; Williams, Fortson, and Dykastra, 1989; Bell-Pedersen, Salvo and Belfort, 1991).

As, the *lgt* open reading frame (ORF) overlaps the ribosome-binding site of *thyA*, the possibility of translational coupling between Lgt (25 kilodaltones) and ThyA (33 kilodaltones) proteins (Williams, Fortson, and Dykastra, 1989) was examined by Gan, Sankaran, and Williams (1995). When the majority of the Lgt coding sequence, including

its translation initiation codon and ribosome-binding site, was deleted but its promoter was left intact, ThyA levels increased two- to three-fold. Thus it suggested that translation of Lgt might serve to reduce the levels of ThyA by preventing ribosome loading (Gan, Sankaran, and Williams, 1995).

The open reading frame of *thyA* sequence is 792 nucleotides, corresponding to the enzyme of 264 amino acids, which initiates from the methionine start codon. The molecular weight of the enzyme subunit based on the predicted protein sequence is calculated to be 30,441 Da (Belfort, Maley and Maley, 1983).

A deletion at the 5' end of the *umpA* (*lgt*) gene reduces the level of TSase synthesis (Williams, Fortson, and Dykastra, 1989). Williams, Fortson, and Dykastra (1989) observed a 10- to 20-fold reduction in TSase synthesis when the *lgt* promoter and the first 70 amino acids of the coding sequence were deleted. Since the lack of *umpA* expression causes a decrease in the TSase expression (Bell-Pedersen, Salvo and Belfort, 1991), this can be another possibility in the regulation of *thyA* expression.

In a growing *E. coli* cell, the amount of enzyme TSases is low at about 250 molecules (Bell-Pedersen, Salvo and Belfort, 1991). Both deficiency and excess of thymidylate are mutagenic in bacteria and eukaryotic cells. The lack of thymidylate causes insertion of the high amount of dUMP into DNA during replication of cells. Thymidylate excess causes damage of DNA replication, since increasing thymine affects the balance of the nucleotide pools at the replication fork. Hence, the precise regulation of *thyA* expression is essential to keep a balance level of TMP (Bell-Pedersen, Salvo and Belfort, 1991).

The expression of the *thyA* gene in *E. coli* is affected by some characteristics of its nucleotide sequence (Belfort, Maley and Maley, 1983; Bell-Pedersen, Salvo and Belfort, 1991). The position of Shine-Dalgarno (SD) sequence (GAGGA) situates only 3 nucleotides (nt.) upstream of the start codon AUG. As the actual space of the SD sequence is 6 to 8 nucleotides, this situation in the *E. coli* genome is rare and it could be one possibility for proficiently controlling the translation of *thyA* mRNA (Bell-Pedersen, Salvo and Belfort, 1991).

1.4. Effects of deleting folA gene on E.coli

DHFR activity is essential for the recycling of DHF produced during thymidylate synthesis as well as for reducing newly synthesized DHF. This suggests that the gene coding for DHFR (*folA*) would be essential for providing the THF that allows the cells to grow (Howell, Foster, and Foster, 1998; Ahrweiler and Frieden, 1988; Hamm-Alvarez, SancarYl, and Rajagopalan, 1990; Krishnan and Berg, 1993; Myllykallio, Ledu, and Filee, 2003; Herrington and Chirwa, 1999).

DHFR has been successfully deleted from the *E. coli* chromosome (Howell, Foster, and Foster, 1998; Ahrweiler and Frieden, 1988; Hamm-Alvarez, SancarYl, and Rajagopalan, 1990; Krishnan and Berg, 1993; Myllykallio, Ledu, and Filee, 2003; Herrington and Chirwa, 1999). The relationship between expression of DHFR and TS activities was additionally investigated when Howell, Foster, and Foster (1988) found their $\Delta fol::kan$ mutation was not viable in a *thyA*⁺ background. They found that Fol⁻ cells are not viable unless TS activity is, at the same time, eliminated. *thyA* is the structural gene for TS. The mutants of *thyA* are blocked in the conversion of dUMP to dTMP,

which consumes methylene-THF (Green, Nichols, and Matthews, 1996; Krishnan and Berg, 1992). However, Ahrweiler and Frieden (1988) showed that some of their *fol*⁻ strains could reduce *thyA*⁺ cells. Krishnan and Berg (1993) successfully deleted the *folA* gene and they found that construction of haploid $\Delta folA$ derivatives was easy and did not need a prior inactivation of *thyA* gene or the introduction of other auxotrophic mutations. Also, deletion of *folA* was successfully done in wild type cells using a $\Delta folA::Kan3$ allele to transduce a *thyA*⁺ strains (Herrington and Chirwa, 1999; This study).

Reduced folates were found in *folA*⁻ strains of *E. coli* and cells were able to grow (Hamm-Alvarez, SancarYI, and Rajagopalan, 1990; Myllykallio, Ledu, and Filee, 2003). Hamm-Alvarez, SancarYI, and Rajagopalan, (1990) found there were other THF derivatives present in the strains with deletion of the *folA* gene. They confirmed that production of some THF in *E. coli* does not need the DHFR activity coded by *folA*, since they could extract formyl-THF, THF, methyl-THF and traces of folic acid (derivatives of THF) from their $\Delta folA$ strains. Hence, folate pool reduction by TS in the absence of DHFR could explain the lethal effects on *folA*⁻ *thyA*⁺ cells (Hamm-Alvarez, SancarYI, and Rajagopalan, 1990). Myllykallio, Ledu, and Filee (2003) reported those cells lacking *folA* were still alive and it was suggested that they contained reduced folates for synthesis of RNA and proteins. Shiman (1985) previously proposed that nonenzymatic oxidation of THF produces a quinonoid-DHF cofactor that might be regenerated to THF by dihydropteridine reductase (DHPTR). Hamm-Alvarez, SancarYI, and Rajagopalan (1990) suggested that in the absence of TS, DHPTR activity could catalyze the stable reduction of DHF to THF slowly. This enzyme also was identified in *folA*⁻ strains of *E. coli*, by Green, Nichols, and Matthews (1996). The presence of this substitute for DHFR in *folA*⁻

strains was proposed by Myllykallio, Ledu, and Filee (2003) and was originally explained as a dihydropteridine reductase (DHPTR; accession # gi:78392) enzyme in *E. coli*, which could reduce DHF to THF *in vitro*. Since DHPTR a known enzyme for reducing DHF *in vitro*, could be responsible for synthesis of THF in the absence of DHFR, deletion of *folA* in $\Delta folA::Kan$ strains was not lethal and these strains could produce THF (Herrington and Chirwa, 1999).

Cells lacking DHFR activity are able to grow on minimal medium as long as it is supplemented with all or some of the following folate end product (FEP) combinations: methionine, thymine, histidine, adenine, glycine, and pantothenate (Howell, Foster, and Foster, 1988; Hamm-Alvarez, SancarYI, and Rajagopalan, 1990; Krishnan and Berg, 1993; Green, Nichols, and Matthews, 1996; Herrington and Chirwa, 1999). Strains with the DHFR gene (*folA*) deleted grow slowly on rich or supplemented minimal medium (Herrington and Chirwa, 1999). These kinds of mutated strains are auxotrophic for FEP supplements. The auxotrophic strain is not able to produce sufficient reduced forms of THF for one-carbon transfer reactions (Green, Nichols and Matthews, 1996). Depending on the strain, the supplements are different, but most strains can grow on minimal medium with thymine (MinA+Thy) or combination of folate end products (Herrington and Chirwa, 1999).

1.5. CsgD is a multicopy suppressor of $\Delta folA$ growth defects

Chirwa and Herrington (2003) discovered that in strains lacking DHFR activity ($\Delta folA$), the gene *csgD* serves as a multicopy suppressor of the glycine auxotrophy. Since the protein coded by *csgD* gene resembles proteins of the FixJ/UhpA/LuxR family, it is

suggested as a regulatory protein. Inactivation of *csgD* prevents the transcription of the *csgBA* operon, which is required for the synthesis of curli (Hammar, Arnqvist, and Bian, 1995).

Chirwa and Herrington (2003) showed that plasmids containing *csgD* sequences enabled faster growth of *ΔfolA::Kan* mutants in the absence of glycine in a folate end product (FEP) supplemented minimal medium. These results came from an increased expression of *glyA* gene, encoding SHMT (EC 2. 1. 2. 1) (Chirwa and Herrington 2003). They found CsgD protein could upregulate *glyA* gene expression in these *folA* mutants.

It was reported that up to 11.5% of the amino acid residues in total *E. coli* protein are glycine (Chirwa and Herrington, 2003). Curlin, the major protein of curli (CsgA), includes 1.7 times as many glycine residues. As a hypothesis, in those cells that make a lot of curli, the glycine requirement is particularly high and the producing of curli is more facile. But it is not yet known (Chirwa and Herrington, 2003).

1.6. Curli biosynthesis

Curli are the fimbrial component of a complex extra-cellular matrix expressed by *E. coli* on the cell surface. Similar structures expressed by *S. enteritidis* have thin aggregative fimbriae. Fimbriae are thinner and generally shorter and more numerous than flagella (100 to 1000 fimbriae per cell), also they are not involved in cellular motility (Low, Braaten and Woude, 1996; Pawar, 2005).

Curli fibers are involved in adhesion to surfaces such as proteins, glass and polystyrene. Also, binding of these fibers in cell aggregation is very important for biofilm formation. Curli also mediates host cell adhesion and attack, and they are strong inducers

of the host inflammatory response. Structurally and biochemically, curli belongs to a growing class of fibers known as amyloids. Amyloid fiber formation is responsible for several human diseases including Alzheimer's, Huntington's, and prion diseases, although the process of *in vivo* amyloid formation is not well understood (Bian and Normark, 1997; Barnhart and Chapman, 2006).

In *E. coli*, synthesis of curli fibers involves expression of six genes, the *csg* (*agf* in *Salmonella enterica*) group, that are organized in two divergently transcribed adjacent operons, *csgBA* and *csgDEFG*. The *csgBA* operon contains the gene for a nucleator protein (*csgB*) and the gene for curlin (*csgA*), the major protein component of the curli fibre (CsgA) in *E. coli*. The *csgDEFG* operon contains four genes that are involved in curli synthesis and secretion (Hammar, Bian, and Normark, 1996; Low Braaten and Woude, 1996; Bian and Normark, 1997; Chirwa and Herrington, 2003 and 2004). Since the sequence of the CsgD (AgfD) protein reassembles the FixJ/UhpA/LuxR family, and inactivation of *csgD* prevents the transcription of the *csgBA* operon, it is suggested that the *csgD* gene serves as a regulatory protein (Hammar, Bian, and Normark, 1996; Chirwa and Herrington, 2003).

1.7. Mutant suppressors of $\Delta folA$ growth defects

Mutant suppressors were isolated in two ways. A mutagenized $\Delta folA$ strain was plated on various minimal media. Colonies growing faster than the majority were selected and further characterized. Some of these were curli proficient. As well, the unmutagenized strain was plated on Congo red plates on which curli proficient cells form red colonies. It was possible to select tiny red colonies in a white lawn of cells. These

curli proficient strains grew faster on some supplemented minimal media (Chirwa, N.T., MacRae, T., Zambrana, C. and Herrington, M.B. unpublished results).

In this project the focus was on the suppressor mutants defective in the *folA* gene. Our $\Delta folA$ strain is a derivative of a strain that has a temperature sensitive *thyA* mutation and requires thymidine only at high temperatures. However, both the $\Delta folA$ strain and its faster growing derivatives are unable to grow on minimal medium lacking thymidine at both high and low temperatures (Herrington and Chirwa, 1999; Chirwa, N.T., MacRae, T., Zambrana, C. and Herrington, M.B. unpublished results). Surprisingly, when the suppressor strains were made *FolA*⁺ they required thymidine for growth at both low and high temperatures, whereas when the parent strain was made *FolA*⁺ it grew well without thymidine at the low temperature (Metallic, T. and Herrington, M.B, unpublished; This study).

It was therefore interesting to examine whether the *thyA* alleles affected the growth of $\Delta folA$ strains. I also compared the sequences of the *thyA-lgt* operon in suppressor and non-suppressor strains to determine if a change in sequence accounted for the *Thy*⁻ phenotype.

Also, production of curli (thin aggregative fibers) protein was studied using a novel technique, since some of the *Fol*⁻ derivatives expressed high levels of curli protein, while their parents did not proficiently make curli fibers. The derivative *FolA*⁻ strains that made high levels of curli protein also produced red colonies on Congo-red plates and showed faster growth on minimal medium compared to the *folA*⁺ parent.

2. Materials and Methods:

2.1. Bacteria, Phage and Plasmids

All bacterial strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 1. Also, phages and plasmids used are listed in Table 1.

Liquid cultures were prepared from colonies raised in agar plates inoculated with strains stored in 25% (v/v) glycerol at -80°C.

2.2. Media and growth conditions

2.2.1. Luria-Bertani medium (LB or rich medium)

Generally, 25 g dehydrated LB from Fisher or Bioshop was dissolved in 1 liter of distilled water. Occasionally, LB was prepared by dissolving ten grams of Bacto-tryptone, 5 g of Bacto-yeast extract and 10 g of sodium chloride in 1 liter of distilled water. After pH was adjusted for 7.0, the medium was sterilized by autoclaving, and then stored at room temperature (Miller, 1992). To solidify the media, 15 g of agar was added to the LB liquid medium before autoclaving.

2.2.2. LB+thymidine (LB+Thy) medium

The amount of 50 µg/ml thymidine was added to LB Broth prepared by Fisher or Bioshop. Agar (15g/L) was used to solidify the media.

Strains	Description	Source or reference
MC4100	<i>araD139 Δ(argF-lac)UI69 rpsL150 relA1 flbB deoC ptsF25</i> <i>rbsR</i>	(1)
MHR204	<i>araD139 Δ(argF-lac)UI69 rpsL150 relA1 flbB deoC ptsF25</i> <i>rbsR csgA2::Tn105</i>	(1)
MH428	<i>thyA-722 metB rna</i>	(2)
MH429	<i>thyA-723 metB rna</i>	(2)
MH430	<i>thyA-724 metB rna</i>	(2)
MH431	<i>thyA-725 (ts) metB rna</i>	(2)
MH432	<i>thyA (ts) metB rna</i>	(3)
MH828	<i>thyA (ts) argE3 rna</i>	(3)
MH829	<i>thyA (ts) argE3 rna ΔfolA::kan3 c+</i>	(3)
MH840	<i>thyA (ts) argE3 rna ΔfolA::kan3 c+</i>	(4) (a)
MH841	<i>thyA (ts) argE3 rna ΔfolA::kan3 c+</i>	(4) (a)
MH842	<i>thyA (ts) argE3 rna ΔfolA::kan3 c+</i>	(4) (a)
MH950	<i>thyA (ts) argE3 rna ΔfolA::kan3 fg</i>	(5) (b)
MH951	<i>thyA (ts) argE3 rna ΔfolA::kan3 fg</i>	(5) (b)
MH952	<i>thyA (ts) argE3 rna ΔfolA::kan3 fg</i>	(5) (b)
MH953	<i>thyA (ts) argE3 rna ΔfolA::kan3 fg</i>	(5) (b)
MH956	<i>thyA (ts) argE3 thy (ts) ma</i>	(6) (c)
MH958	<i>thyA (ts) argE3 thy (ts) ma</i>	(6) (c)
MH981	<i>thyA (ts) argE3 rna</i>	This study (d)

MH982	<i>thyA (ts) argE3 rna</i>	This study (d)
MH983	<i>thyA (ts) argE3 rna</i>	This study (d)
MH986	<i>thyA (ts) argE3 rna</i>	This study (d)
MH984	<i>thyA (ts) argE3 rna</i>	This study (e)
MH985	<i>thyA (ts) argE3 rna</i>	This study (e)
MH989	<i>thyA 722 metB rna ΔfolA::Kan3</i>	This study (f)
MH990	<i>thyA 723 metB rna ΔfolA::Kan3</i>	This study (f)
MH991	<i>thyA 724 metB rna ΔfolA::Kan3</i>	This study (f)
MH992	<i>thyA 725 (ts) metB rna ΔfolA::Kan3</i>	This study (f)
MH993	<i>thyA (ts) metB rna ΔfolA::Kan3</i>	This study (f)
Hfr strains		
Plasmids		
pUC18	Vector	(7)
pMGSA5	Carrying the <i>folA</i> gene, Amp ^R	(8)
pACBSR	Carrying the I- <i>SceI</i> and λ Red genes, Cm ^R	(9)
Phages		
P1vir	Generalized transducing Phage	(10)

Table 1. E. coli strains, plasmids and phages. (a) These strains were isolated as curli proficient (c+) derivatives of strain MH829; (b) These strains were isolated as faster growing (fg) derivatives of strain MH829; (c) MH956 and MH958 are *folA*⁺ derivatives of strains MH841 and MH950, respectively; (d) These strains are *folA*⁺ derivatives of strain MH950 by gene gorging; these strains are phenotypically Thy⁻. (e) These strains are *folA*⁺ derivatives of strain MH829 by gene gorging; these strains are phenotypically Thy (Ts). (f) These strains are $\Delta folA::Kan3$ transduced derivatives of strains MH428 to MH432, respectively. The source and references indicate: (1) Hammar, Arnqvist, and Bian, Mol. Micro. 18:661 (1995); (2) Faraci, M and M.B. Herrington (unpublished); (3) Herrington and Chirwa (1999); (4) Herrington M. B. and T. MacRae (unpublished); (5) Herrington M. B., N. T. Chirwa and C. Zambrana (unpublished); (6) Herrington M. B. and T. Metallic (unpublished); (7) C.G. Cupples; Messing, (1983), Yanisch-Perron (1985); (8) Herrington (2003); (9) Herring, Glasner, and Blattner (2003); (10) C.G. Cupples and Miller (1992).

2.2.3. Minimal medium A (minA)

This medium contained 10.5 g of K_2HPO_4 , 4.5 g of KH_2PO_4 , 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate.2H₂O dissolved in 1 liter distilled water. After sterilization by autoclaving, 10 ml of 20% glucose and 1 ml of 20% Mg_2SO_4 were added to the medium. Medium was solidified with 15 g per liter of agar.

In our experiments we made 5X minA and before sterilizing, 1X minA was prepared by diluting 200 ml of 5X in 800 ml of distilled water. After autoclaving, the medium was stored in the volumes of 100 ml at room temperature.

2.2.4. Folate end product medium (FEP)

Glycine (50µg/ml), thymidine (50µg/ml), methionine (50µg/ml), histidine (50µg/ml), adenine (30µg/ml) and pantothenic acid (1µg/ml) were added to minA medium to make FEP (folate end product) medium.

FEP-G medium had the same additions except that glycine was not added as one of the supplements. Any other required media were supplied with one or a combination of some of these nutrients.

As many strains were auxotroph for arginine, arginine (50µg/ml) was added to these media.

2.2.5. Congo-Red and YESCA

The Congo-Red (CR) medium per liter contained Bacto-yeast extract (1 g), Bacto-Casamino acids (10 g), agar (20 g), 50 µg/ml of Congo red and 25 µg/ml of Coomassie Brilliant Blue G.

The YESCA medium contained the same ingredients with Congo-red and Coomassie Brilliant blue omitted.

2.2.6. Antibiotics

Final concentrations used for antibiotics were kanamycin (30 µg/ml), ampicillin (100 µg/ml), and chloramphenicol (25 µg/ml). These antibiotics were added to the LB+Thy medium, when required for selecting antibiotic resistant strain(s). Stock solutions of ampicillin and kanamycin in water were filter sterilized using 0.2 µm filters. Stock solutions of chloramphenicol was prepared in ethanol and methanol respectively. All antibiotics were added to autoclaved media.

2.2.7. Stock solution of 1X minimal medium A (1X min A) used in gene gorging

The solution contained 1 ml of 20% MgSO₄, 10 ml of 20% glycerol, 10 ml of each arginine (50µg/ml) and thymidine (50µg/ml) and 10 ml of 0.5% Cassamino acids dissolved in 1 liter distilled water.

2.2.8. Stock solutions used in SDS-Agarose method

- 4X Tris-Glycine contained 0.5 M Tris and 2 M glycine. The pH was adjusted to 1.8.
- 1 M Tris-HCl (pH 6.8)
- 10% SDS (sodium dodecyl sulfate)
- 10 N NaOH

2.2.9. Lysis buffer used in SDS-Agarose method

The buffer was prepared of 0.25 volumes of 4X Tris-Glycine, 0.40 volumes of 10% SDS, 0.20 volumes of 100% glycerol, and 0.15 volumes of distilled H₂O.

2.2.10. Soft agar used in RNase assay

This medium contained 7 g of agar, 30 g of yeast RNA dissolved per liter of 0.1 M of EDTA (pH 7.0) (Miller, 1992).

2.2.11. R-top agar used in P1 vir Transduction

This medium contained 10 g of tryptone, 1 g of yeast extract, 8 g of Difco agar, and 8 g of NaCl.

Sterile CaCl₂ (1M) and Glucose (20% w/v) were added to the medium of the final concentration of 2 mM and 0.1% separately after autoclaving (Miller, 1992).

2.2.12. Preparation of competent cells

The cells were prepared using the CaCl₂ method (Sambrook and Russell, 2001). Briefly, the strains were streaked for single colonies on an LB+Thy plate and incubated overnight at 37°C. A single colony of this overnight culture was incubated in 25 ml of LB+Thy and grow at 37°C until it was visibly turbid but not overgrown. Then cells were centrifuged at 13,000 × g for 10 minutes and the supernatants were discarded. The pellets were suspended in 10 ml of ice cold 0.1 M CaCl₂ and again centrifuged at 13,000 × g for 10 minute. Finally the pellet was suspended in 1 ml of ice cold 0.1M CaCl₂ and stored in the refridgerator.

2.3. Strain construction

2.3.1. Transductions

To make the deletion of *folA* gene, the transduction method (Miller, 1972 and 1992) was used to replace *folA*⁺ allele with Δ *folA::kan3* allele in strains that had different alleles of *thyA* gene.

Briefly, the Δ *folA::kan3* allele from strain MH829 was transduced into the thymidine requiring strains ((MH428, MH429, MH430, MH431 and MH432) using a P1vir lysate prepared on strain MH829.

2.3.1.1. RNase assay to confirm the new transduced strains

As there was a mutation of *rna* in strains MH428, MH429, MH430, MH431, and MH432, the RNase assay (Gesteland, 1966) was used to confirm that new derivatives (MH989, MH990, MH991, MH992, and MH993) had the same phenotype of RNase activity. Due to mutation, the RNase enzyme was inactive in desired strains. As this nuclease catalyses the degradation of RNA into smaller components, I checked the activity of this enzyme in desired derivative strains by this assay. If RNA in used medium was digested by RNase made from strain, the small pieces would exist around the colony. The precipitated RNA by HCl caused light halo around the colony.

One single colony of desired strains (MH428, MH429, MH430, MH431, and MH432) and controls, MC4100 as a positive (RNase⁺) and MH828 and MH829 as negative (RNase⁻) were grown overnight in 1 ml of LB+Thy medium at 37°C. 5 µl of each strain spotted on LB+Thy plate and incubated overnight at 37°C. The day after 2.5 ml of soft agar (contained 7 g of agar, 30 g of yeast RNA, and 0.1 M of EDTA (pH, 7.0)

dissolved in 1 liter of distilled water) were poured on top of the grown strains. After 2-4 hours of incubation at 43°C, 1N HCl was added to precipitate RNA.

2.3.2. Gene gorging and Transformation

Gene gorging (Herring, Glasner, and Blattner, 2003) is a method for allele replacement in which cells are transformed with a donor plasmid and the mutagenesis plasmid, pACBSR.

The mutagenesis plasmid, pACBSR, carries four genes, *I-SceI* and lambda Red genes, which are necessary for mutagenesis in desired cells. The product of *I-SceI* endonuclease gene cuts the *I-SceI* site on the donor plasmid that making the donor plasmid linear. Lambda Red genes stimulate the recombination between homologous sequences on the plasmid and chromosome. The *I-SceI* endonuclease gene and the lambda Red genes are under inducible control of the arabinose promoter (Herring, Glasner, and Blattner, 2003). The donor plasmid, in this study was pMGSA5 (A. Beyde and M. B. Herrington, unpublished), which transports the gene of interest, *folA*, (Figure 2). The donor and mutagenesis plasmids are compatible replicons.

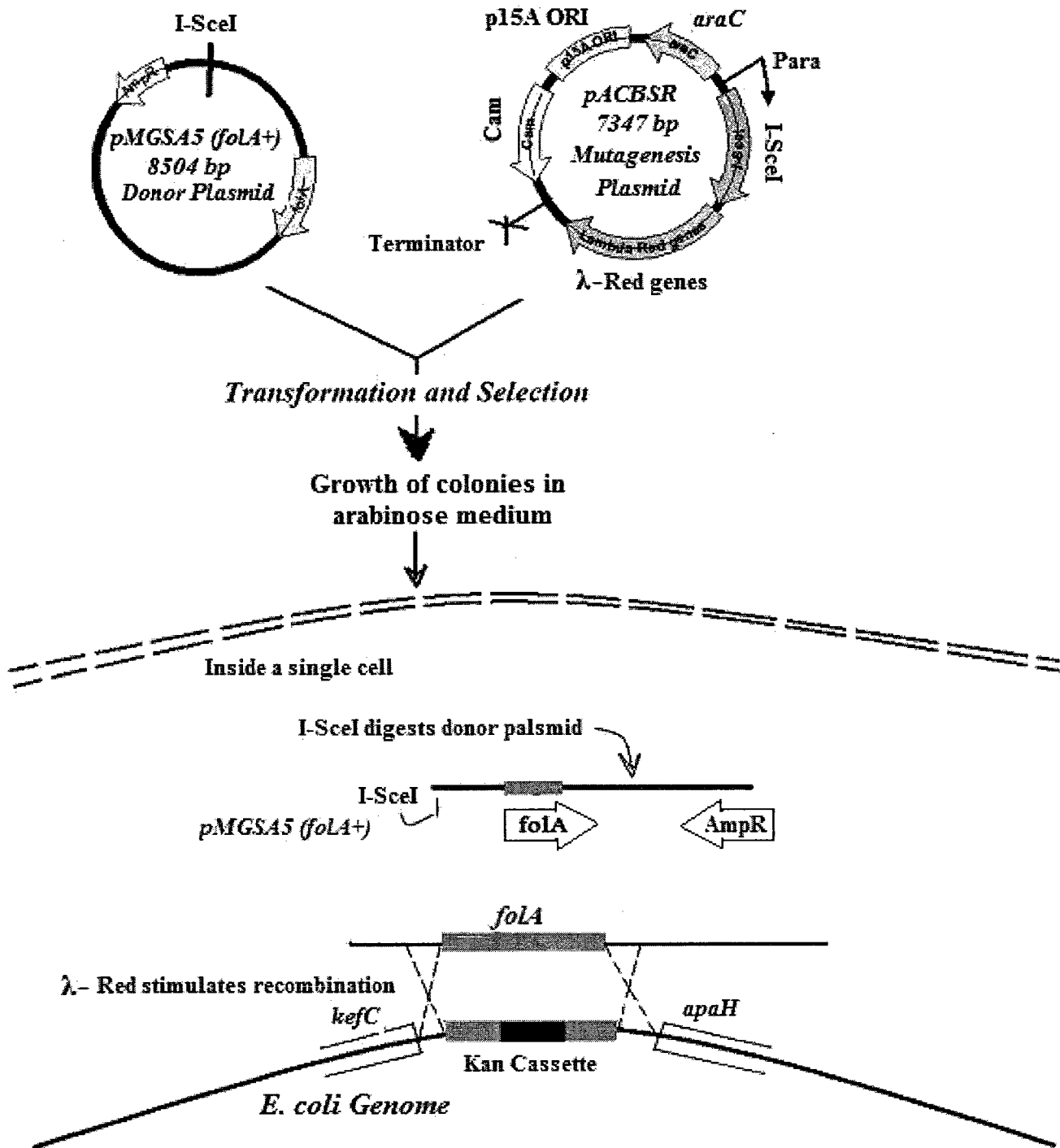


Figure 2. Gene gorging diagram. The mutagenesis plasmid, pACBSR, contains chloramphenicol resistance gene as marker. The donor plasmid, pMGS45, carries the *foLA* gene on a 5.8 Kb inserts derived from the *E. coli* chromosome and the restriction enzyme site of *I-SceI*. Also, it contains the marker gene of ampicilline resistance.

2.3.2.1. Transformation

In order to transfer desired plasmids (donor and mutagenesis) into each MH829 and MH950 strains individually, the transformation method (Sambrook, Fritch, and Maniatis, 1982) was employed. The 100 μl of desired competent cells (MH829 and MH950) were added to the required number of Eppendorf tubes chilled in ice. Then 2 μl of DNA (final concentration of 0.1 $\mu\text{g}/\mu\text{l}$) were added. Cells without any DNA (plasmid) were used as the negative control. The plasmid, pUC18, was used as positive control. Then tubes were incubated on ice for 30 minutes. The procedure was followed with “heat shock“the samples by placing them at 42° for exactly 90 sec, then were placed on ice for approximately 2 minutes. Then 400 μl of LB+Thy liquid was added and incubated at 37° for 45 minutes. Then 200 μl was plated on different LB+Thy plates containing desired antibiotics; ampicillin and chloramphenicol.

There were single and double transformations. In order to check the capability of cells for transformation, each cell of MH829 and MH950 strains received either pACBSR or pMGSA5 plasmid in single transformation. In double transformation, each cell of strains was introduced with plasmids 1 μl of both plasmids pACBSR and pMGSA5, and then was purified.

Plasmids were used tat the final concentration of 0.1 $\mu\text{g}/\mu\text{l}$ to keep the efficiency of transformation.

2.3.2.2. Gene gorging procedure

The overnight grown colonies on AMP-CM plates were suspended in 1 ml of 1x minA-stock solution. Immediately, appropriate dilutions were spotted at time zero (t=0) on AMP, CM, AMP-CM and LB-Thy plates and then incubated at 37°C.

Then 20 µl of 20% L-arabinose was added to each of the undiluted samples and incubated with shaking at 37°C for 6 hours. In this step, after 30-60 minutes, 20 µl of L-chloramphenicol (2.5 mg/ml) was added. After incubation was done, 5-10 µl of appropriate dilutions were spotted on AMP, CM, AMP-CM and LB-Thy plates and incubated at 37°C (Herring, Glasner, and Blattner, 2003 with modifications by S. Little and M.B. Herrington).

2.3.3. Curli Preparation

Highly purified curli protein was prepared as described (Chapman, Robinson, Pinkner, 2002; Collinson, Emody, and Muller, 1991). In experiments using SDS-agarose gel analysis a crude preparation of protein was done as follows.

Overnight culture of desired strains in LB-Thy medium was diluted 100-fold in 1x minA. Then 100 µl of that solution was plated on YESCA and incubated at 30°C for 2 days. The cells were harvested by scraping using a glass rod with a rubber policeman, and then cells were suspended in 3 ml of 10 mM Tris-HCl (pH 6.8). The OD was measured by spectrophotometer to determine the concentration of cells and then added tris to adjust all the samples to an OD of 10.

An equal volume of cell suspension was mixed with lysis buffer. Then samples were incubated in a boiling water bath for 10 minutes, and cooled down in ice water.

After mixing well, immediately 1 ml aliquots were transferred to microfuge tubes. They were centrifuged at 13,000×g for 10 minutes at room temperature. The pellet was re-suspended in 1 ml water and washed once.

2.3.4. Quantification of Protein

The BCA assay (Sorensen and Brodbeck, 1986) was used to determine the concentration of protein. The glycine in the extraction buffer interferes with the assay; hence the protein was precipitated with trichloroacetic acid (TCA) (Pierce protocol, <http://www.piercenet.com/files/TR0008-TCA-acetone-precip.pdf>).

In order to eliminate the glycine interference these reagents were used:

- TCA reagent, 72% (w/v) trichloroacetic acid,
- Sodium deoxycholate reagents, 0.15% (w/v) sodium deoxycholate,
- SDS reagent, 5% (w/v) SDS in 0.1 N of NaOH.

The mixture, for TCA precipitation, contained 100 µl of crude extract, 450 µl of sterilized distilled water, 100 µl of sodium deoxycholate reagent, and 100 µl of the TCA reagent in microcentrifuge tube. Then this mixture was incubated for 10 minutes at room temperature (RT). After vortexing, it was spun down by centrifugation at maximum speed in a microcentrifuge. After discarding the supernatant, 50-100 µl of SDS reagent was added to completely dissolve the protein pellet.

2.3.5. BCA assay

Twenty-five μl of protein standard (including blank) and washed samples were pipetted into a 96 well microplate. Then 200 μl of working reagent (WR) were added and after 5 minutes shaking the plate was incubated at 37°C for 30 minutes. Finally, the absorbance was measured at 562 nm with a Bio-TEK power wave HT microplate reader.

Protein standards (Bovine Serum Albumin (BSA)) were prepared according to the BCA Protein Assay Reagent Kit (Product No. 23225). BCA working reagent (WR) was prepared from mixing 50 parts of reagent A and 1 part of reagent B, according to the instruction for the BCA Protein Assay Reagent Kit (Product No. 23225, 23227).

2.3.6. DNA extraction

A modified method of Sato and Miura (1963) was used to isolate DNA from *E. coli*. Briefly, the overnight grown cells in 5ml of LB+Thy medium were spun down. The cells pellet was lysed by suspending the cells in 15 μl of lysozyme solution (freshly dissolved at a concentration of 2 mg/ml in 0.15 M NaCl, 0.1 M EDTA, pH 8.0) and then incubation at 37°C for 10-20 minutes. After lysis, cells were frozen in liquid nitrogen, 125 μl of 1% SDS, 0.1 M NaCl, 0.1 M Tris-HCl, pH 9.0 were added to the frozen cells and stirred as the cells thawed. 150 μl of phenol chloroform were added to the cells, centrifuged for 3 min, at 13 X1000g. Upper aqueous phase containing DNA was transferred to a clean tube and, 300 μl of 95% ethanol was added, mixed and stored at -20°C for 30 min, centrifugation was done for 5 min and DNA pellet was resuspended in 100 μl of TE buffer. After adding 3 μl of RNase A (2 mg/ml) to the DNA and incubating for 30 min at 37°C, 100 μl of phenol chloroform was added. This mixture was vortexed vigorously; and

then was centrifuged ($13,000 \times g$) for 3 min. The aqueous layer was removed and precipitated DNA with ethanol (Sambrook, Fritch, and Maniatis, 1982).

For a faster preparation of the DNA template, one colony from an overnight culture was picked into 20 μ l of sterile ice cold water in an Eppendorf tube. The cells were suspended by pipetting the sample up and down. Then 5 μ l of this suspension was used for the PCR.

2.3.7. Primer Design

In order to study the mutations in the region of *thyA* and *lgt* genes, I used primers to amplify and sequence the region of the *E. coli* chromosome containing the *thyA* and *lgt* genes. All primers are listed in Table 2. I designed several primers that some of them used in this study (*thyA_reg_L*, *thyA_reg_R*, *pts_r_R*, *t_lgt_2*, *ppdA_r_L*) as listed in Table 2. The desired primers were made for the region containing *thyA* and *lgt* genes from complete genome of strain K-12 of *Escherichia coli*, sub-strain MG1655 (ref|NC_000913.2) using online software Primer3 (<http://www.ncbi.nlm.nih.gov/>). The resultant primers were checked against the complete genome of *E. coli* (ref|NC_000913.2) by basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov/>) in order to amplify and sequence only the required region of the genome (*thyA* and *lgt* genes). Then a DNA manipulation program (pDRAW) (<http://www.acaclone.com/>) helped to annotate desired sequences (*thyA* and *lgt*) and locate the primer binding sites on these sequences.

<i>Primer</i>	<i>Primer sequence</i>
thyA_Pm_R_X	GTGCTCGAGCAATGGGAGCTGTCTCAGG
thyA_reg_L	AAAAAGACGAGGGCCAGTTT
thyA_reg_R	CTGATTCGCGGAGGGTTAT
RT_thyA_L	GCCGTCTGCCACATAGAACT
RT_thyA_R	ATGTCACCATCTGGGACGAA
pts_r_R	TGCAATGTTACGAGCTCTGG
RT_lgt_R_test	CCACTCATTCCGTTTGGTCT
ppdA_r_L	AGTCCACTTGCGCTTAGCAT
thyA_R_t	TAGTCGAAGATGGATTCGGG
t_lgt_2	GTCCCAGACACGGAACAGAT
folamnskpn	TTTGGTCACTGGGTACCGCTGGAGG
folaplsnar	CGTTGGTTGGCGCCGTTGTTAGGGC

Table 2. Primers used for amplification and sequencing of the *lgt-thyA* region and sequence used to sequence *folA* gene from upstream and downstream of gene in strains with either *folA* or $\Delta folA::Kan3$ alleles.

2.3.8. Polymerase Chain Reaction (PCR)

Bioshop Taq polymerase was used for PCR. It was supplied with 10X buffer without MgCl₂.

The PCR reaction was carried out in a total volume of 20 µl. This consisted of 5 µl template and 15 µl of master mix. The master mix contained 0.5 µM concentration of each primer, 2 µl of 10X buffer, 0.6 µl of 50 mM MgCl₂, 0.4 µl of dNTPs mixture, 0.05 µl of Taq polymerase, and distilled water to get the total volume of 15 µl. The reaction was performed using the following program based on (Sambrook, Fritch, and Maniatis, 1982); 94°C for 3 min., 35 cycles with 94°C for 30 sec., 65°C for 1 min., 72°C for 2 min and 10 sec., and the final extension at 72°C for 10 min. Depending on the primers and the length of desired region to be amplified; the annealing temperature and the extension time in different PCR reactions were different.

GeneAmp, PCR system 9700 was used for amplification. After amplification, 5 µl of the products were analyzed on a 1% agarose gel.

2.3.9. Sequencing

The PCR reaction used for sequencing was carried out in a total volume of 50 µl containing 5 µl of template and 45 µl of master mix.

The PCR products and the appropriate primers (Table 2) were sent to the Genome Québec Innovation Centre for sequencing at McGill University.

2.3.10. DNA and Protein separations

PCR products were analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide (Sambrook, Fritch, and Maniatis, 1982).

Depending on the fragment size, the percentages of gels were made different. The chromosomal DNA samples were analyzed by a 1.2% (w/v) agarose gel dissolved in 1×Tris-Acetate-EDTA (1×TAE) and λ Hind III as marker. A 1.6 % or 0.8% (w/v) agarose gel dissolved in 1×TAE was prepared for analyzing PCR products. 5 μ l of samples or marker was mixed with 1 μ l of Bromophenol blue dye and then was loaded to the wells of gel. Running was done at 72 volt for 1 hour. A λ HindIII, lambda marker and Gene Ruler 100-bp DNA Ladder-Plus, were used as DNA markers.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and SDS-agarose gel electrophoresis (Horsten, 2003; Luo, Wehr, and Levine, 2006) were used for protein separation analysis. The SDS-PAGE was stained with Coomassie Brilliant Blue R-250. The staining of SDS-agarose gels was done by EZ-blue buffer.

2.3.10.1. SDS-Agarose Gel electrophoresis

A final concentration of 1.2% agarose was used. After melting the agarose in TAE and cooling down to 50°C, 250 μ l of 10% SDS was added slowly and stirred with a stir bar.

Samples were suspended (10:20 μ l) in 3X sample buffer (3 ml 10X TAE, 12 ml 10% SDS, 3ml 100% glycerol, 15 mg bromophenol blue, and 2 ml H₂O). Agarose gel was run with the running buffer (20 ml 10X TAE, 2 ml 10% SDS, and 178 ml H₂O) at the voltage of 70 for 45 minutes. The gel was stained with EZ Blue TM Gel Staining

Reagent (Sigma), and after washing with water it was imaged on the Odyssey Infrared imager.

2.3.10.2- SDS-PAGE

A final concentration of 12% separating and 8% stacking SDS-PAGE (Laemmli, 1970) were prepared.

After recovering almost the total amount of materials from the wells of SDS-agarose gel, they were washed once by suspending in distilled water and centrifuging at 13,000×g for 2 minutes. Then protein was quantified by BCA assay and a known amount of samples were treated by 90% cold formic acid (1 µg protein /µl) for 10 minutes, and then was boiled with SDS-sample buffer in water bath for 10 minutes at 100°C. The gel was run at 72 volt for 45 minutes. Staining for 15 minutes, washing with distilled water and destaining (methanol, acetic acid) prepared gel for scanning.

3. Results:

3.1. Construction and confirmation of strains

In order to study the relationship between thymidylate synthetase (TS, the product of *thyA* gene) and dihydrofolate reductase (DHFR, the product of *folA* gene) in folate pathway the *folA* deletion allele, $\Delta folA::Kan3$ was transferred to some strains and the *folA*⁺ allele was transferred to others.

3.1.1. Comparisons of $\Delta folA::Kan3$ strains carried different *thyA* alleles

To test whether the *thyA* allele present affects the growth of strains unable to make dihydrofolate reductase, I constructed $\Delta folA::Kan3$ derivatives of five strains. These strains (MH428, MH429, MH430, MH431 and MH432) were auxotrophic for methionine and had different *thyA* alleles (Faraci, M and M.B. Herrington, unpublished; Herrington, Kohli, and Faraci, 1986). Three strains required thymidine at the three temperatures tested (30, 37 and 43°C) and two strains required it only at higher growth temperatures. The new $FolA^-$ strains resulted from transduction are listed in Table 1 and the relationships between strains are shown in Figure 3.

Strains were constructed by transducing $\Delta folA::Kan3$ allele from strain MH829 using P1vir. The resultant plaque forming units (pfu) of lysate showed successful lysis. Then five host strains were infected by this phage. The infected recipients were plated on LB+Thy+Kan plates to select for $\Delta folA::Kan3$ allele of the donor strain MH829. The kanamycin resistant colonies were picked up as $\Delta folA::Kan3$ derivatives to use for the rest of the experiments.

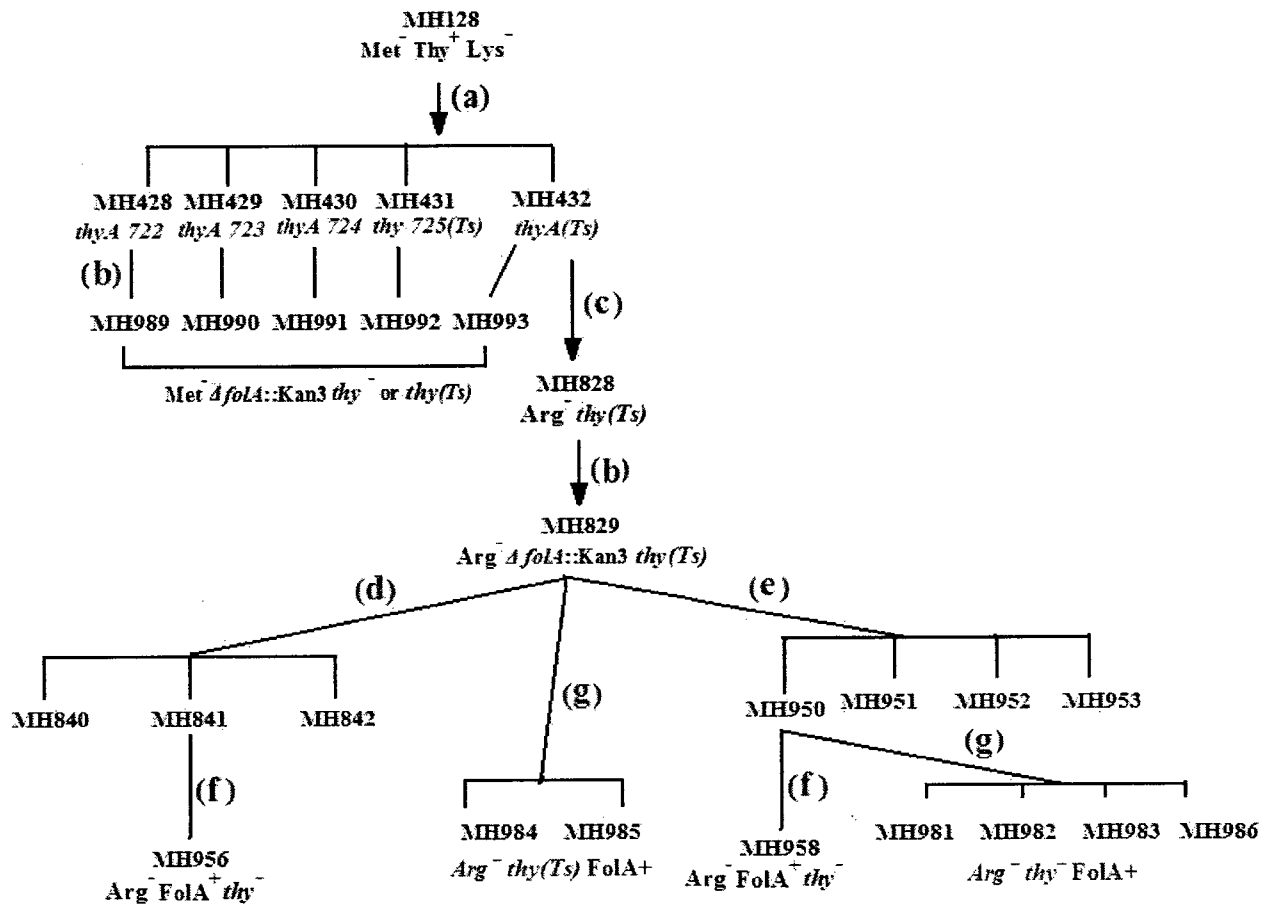


Figure 3. Strain relationships. (a) Thymine requiring derivatives of strain MH128 were constructed by P1CM transductions, using different donor strains and selecting for Lys⁺. Transductants were then tested for the thymine requirement. (b) The $\Delta folA::Kan^3$ mutation was transferred to strains MH428, MH429, MH430, MH431, and MH432 by P1vir transduction from strain MH953 (this study) and to strain MH828 by P1CM transduction (Herrington and Chirwa, 1999). (c) The *argE3* mutation was transferred from strain KL719 to strain MH432 by P1CM transduction to produce the Met⁺ Arg⁻ strain MH828 (Herrington and Chirwa, 1999). (d) Approximately 10⁸ MH829 cells were plated on Congo-red plates. A tiny red colony was observed in a lawn of white cells on some plates. These colonies were isolated and tested for growth on supplemented minimal medium. They grew better than strain MH829 under same conditions (M. B. Herrington and T. MacRae, unpublished). (e) Strain MH829 was plated on supplemented minimal medium. Colonies that grew faster than the majority were isolated and retested. Strains MH950-953 grew better than the parent strain. As well, strains MH950-952 were red on Congo red plates. (f) F_{olA}⁺ derivatives of strains MH841 and MH950 were constructed by transforming the parent strains with a plasmid containing the *folA* gene, and growing transformants without selection for the plasmid and screening for improved growth in the absence of the plasmid (M. B. Herrington and T. Metallic, unpublished). (g) F_{olA}⁺ derivatives were constructed by gene gorging (Herring, Glasner, and Blattner, 2003) using the *folA* donor plasmid pMGSA5 (this study).

Thy phenotype of parents (MH428, MH429, MH430, MH341 and MH432) was checked before transduction. The related strains MH828 and MH829 were also tested (Figure 3). As parent strains were auxotrophic for methionine and they had different *thyA* alleles, the strains were tested on minimal medium containing methionine and thymine (minA±Met±Thy) at room temperature (RT), 30°C, and 43°C. For related strains, arginine was added to the media, as well. Also, strains were tested on ArgFEP, ArgFEP-gly, and LB+Thy media and plates were tested at three temperatures (30, 37 and 43°C).

All strains could grow on LB+Thy (37°C) after one day of incubation. On minimal medium containing methionine and thymidine, parent strains (MH428, MH429, MH430, MH431 and MH432) grew into colonies formation 1-2 days at 30°C, and 43°C. On plates without thymidine only strains MH431 and MH432 grew at 30°C, but not at 43°C. There was therefore no observed growth of any of the strains on plates without added thymidine at 43°C. There was no growth observed at any temperature, when methionine was omitted. These results prove that parent strains were methionine auxotroph, as expected. These data are not shown.

The related strains, MH828 and MH829, could grow on minimal medium with arginine and thymidine in the absence of methionine. MH828 grew after one day at 30 and 43°C. The strain MH829 grew after 3 days at 30 °C and 5 days at 43°C (Table 3).

Both the parent strains and the related strain MH828 grew on minimal medium supplemented with arginine and the folate end products (ArgFEP) after 1-2 day incubation at 30, 37 and 43°C. In contrast, strain MH829, which is deleted for *folA*, grew after 3-4 days only at two temperatures (30 and 37°C) (Table 4).

	ArgFEP-Gly			ArgFEP			Arg+Thy (-Met)		LB+ Thy
	30	37	43	30	37	43	30	43	37
MH828	1	1	-	1	1	1	1	1	1
MH829	-	-	-	4	2	2	6*	-	1
MH428	4	3	-	1	1	1	-	-	1
MH429	3	2	-	1	1	1	-	-	1
MH430	3	2	-	1	1	1	-	-	1
MH431	3	2	-	1	1	1	-	-	1
MH432	5	4	-	2	2	2	-	-	1

Table 3. Growth phenotype of strains carrying different *thyA* allele. Strains MH428, MH429, MH430, MH431 and MH432 was tested by spotting 10µl aliquots of undiluted and diluted culture on folate end product (FEP) medium with and without methionine and glycine, as well on LB+Thy at different temperatures. Growth was monitored during 7 days. The first day that visible colonies were seen with the most dilute sample was noted.

* The colonies of strain were formed but where the growth of less dilute samples showed abnormalities such as a thin lawn.

	ArgFEP-Gly			ArgFEP			Arg+Thy (-Met)		LB+ Thy
	30	37	43	30	37	43	30	43	37
MH828	1	1	-	1	1	1	1	1	1
MH829	-	-	-	1	1	-	3	5	1
MH989	-	-	-	1	6	-	-	-	1
MH990	-	-	-	1	6	-	-	-	1
MH991	-	-	-	1	6	-	-	-	1
MH992	-	-	-	1	6	-	-	-	1
MH993	-	-	-	1	7	-	-	-	1

Table 4. Phenotypic growth of $\Delta folA::Kan3$ derivatives of methionine auxotroph strains carrying different *thyA* allele. From overnight culture of derivative strains resulted from transduction and the parents, 10 μ l of 10^{-6} dilution was spotted on different media and incubated at different temperatures (30, 37 and 43°C). Growth was noted during seven days of incubation and the mentioned day is the first that visible colonies were observed.

When glycine was omitted from ArgFEP medium (ArgFEP-Gly), all strains, except strain MH829, were able to grow at two lower temperatures (30 and 37°C) during two days of incubation, but did not grow at 43°C. Strain MH829 did not grow at any temperature on ArgFEP-Gly (Table 3).

The results of ArgFEP plates show that parent strains display about the same growth as related strain MH828. The strain MH828 is ThyA(Ts) and was able to grow on this supplemented media at the three temperatures. But when glycine was omitted it was not able to grow at higher temperature (45°C). The parent strains, which had different *thyA* alleles, showed the same growth on these conditions and media at higher temperature. But they grew slower than related strains (MH828 and MH829) at lower temperatures (Table 4).

In supplemented minimal medium, when glycine was omitted (Arg+FEP-Gly), their growth was slower compared to minimal medium Arg+FEP.

The RNase assay was used to verify that new ($\Delta folA$) strains (MH989, MH990, MH991, MH992, and MH993) were correct derivatives of their parents (MH428, MH429, MH430, MH431 and MH432). The RNase phenotype of new strains was checked with control strains, such MH828 and MH829 as *RNase*⁻, and MC4100 as *RNase*⁺ strains. After adding soft agar and incubating, the grown colonies were applied with 1N HCl to precipitate RNA. Only one strain showed bright halo around the colony, which was MC4100 (*RNase*⁺). But, the related strains MH828 and MH829 and all of the new derivatives (MH989, MH990, MH991, MH992, and MH993) did not make halos around their colonies that verified them as *RNase*⁻ derivatives.

The *folA* allele in the new strains and their parents was amplified by “folamnskpn” and “folaplsnar” primers. The sequences of primers are listed in Table 3. The PCR products were analysed on a 1% agarose gel (Figure 4 (Gel B)). As expected, the size of *folA*⁺ allele in strain MH828 was 1575-bp and of the Δ *folA*::*Kan3* allele in the strains MH829 and MH950 was 2565-bp. The PCR products in the new Fol⁻ strains were about 2565 bp compare the PCR product produced for their parents was about 1575 bp. These results confirm that the expected *folA* alleles are present in these strains.

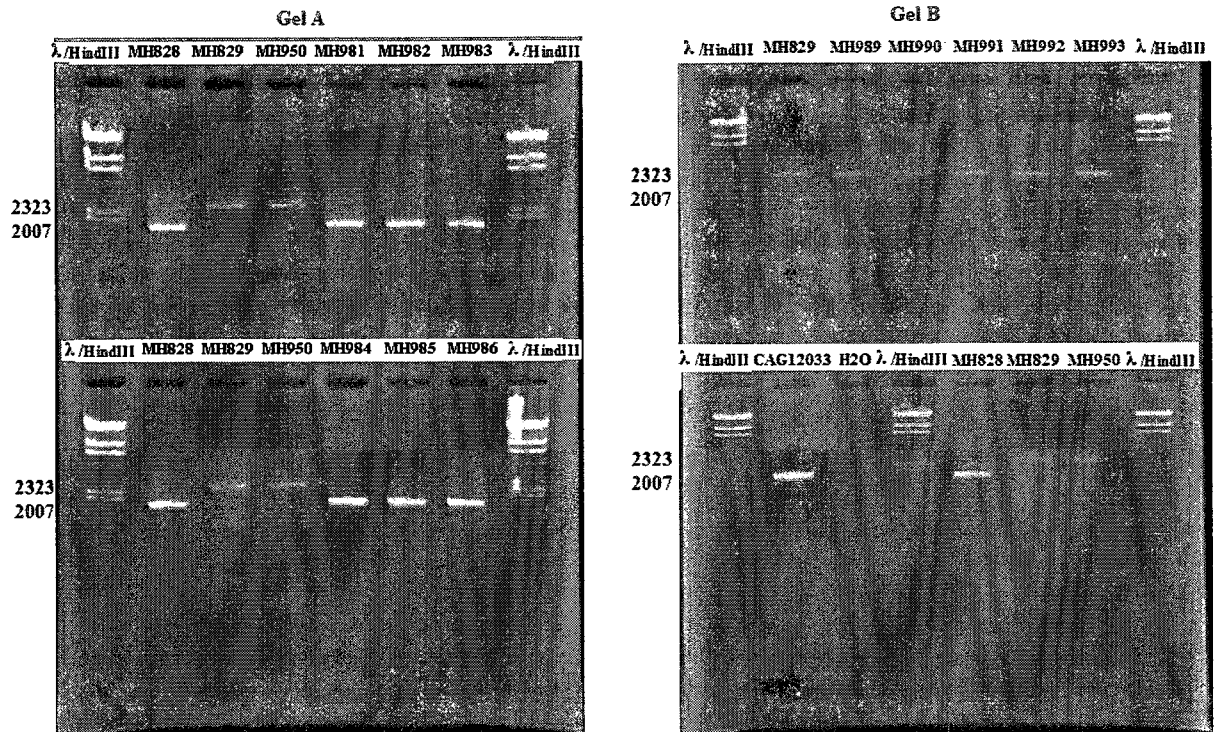


Figure 4. PCR amplification of the *folA* region in different strains; A. The *FolA*⁺ derivatives resulted of gene gorging from the strain MH829 (MH985 and MH986) and from the strain MH950 (MH981, MH982, MH983, and MH986). B. The Δ *folA* derivatives of MH989, MH990, MH991, MH992 and MH993 strains. The parents strains (MH428, 429, 430, 431 and MH432) carry different *thyA* allele. Expected band size for *folA*⁺ is 1575-bp and for Δ *folA*::*Kan3* is 2565-bp (Herrington and Chirwa 1999). The ladder was “Lambda DNA/*Hind* III Marker, 2” from “Fermentas”.

Growth of MH989, MH990, MH991, MH992, and MH993 derivatives and their related strains, MH828 and MH829 (Figure 3) was tested on minimal medium plates. As the parental strains (MH428, MH429, MH430, MH431, and MH432) were methionine requiring, the presence of methionine in medium was necessary for the growth of all derivative strains, as well. To test the growth phenotype of derivatives, the following supplemented minimal medium with folate end products (FEP) was applied: ArgFEP, ArgFEP-Gly, ArgFEP-Gly-Met, and ArgFEP-Met plates incubated at three different used temperatures. Growth was tested also on minimal medium supplemented only with thymidine and arginine (Arg+Thy) at 30°C and 43°C, and on LB+Thy medium at 37°C, as well.

Strain MH828 on ArgFEP-Gly-Met, ArgFEP-Met media, grew after one day of incubation at only 30°C and 37°C. MH829 strain did not grow on media without glycine. On Arg+Thy medium, MH828 showed growth after one day at testing temperatures (30°C and 43°C). The growth of MH829 strain on Arg+Thy medium was observed after 6 days of incubation at 30°C, however growth was abnormal. This strain made colonies but the growth was observed as thin lawn. On minimal medium containing arginine and methionine and supplemented minimal medium without thymidine, MH828 grew only at 30°C and at 37°C it show abnormal growth. In these conditions with no thy added, MH829 did not grew.

As all the derivative strains, MH989-MH993, were methionine requiring, none of them grew on medium without added methionine. Therefore, I did not observe any growth colony on minimal media containing ArgFEP-Gly-Met, ArgFEP-Met, and Arg+Thy.

The growth of derivatives was observed only on ArgFEP at all three testing temperatures. In this medium all strains grew at 30°C the same as on LB+Thy after one day, whereas at the higher temperatures (37°C and 43°C) their growth was very late (6-7 days) compared to LB+Thy plate. The related strain MH828 grew after one day on ArgFEP at all three temperatures and strain MH829 grew only at the two lower temperatures (30 and 37°C) after 3-5 days (Table 4).

These results show that the growth phenotype of these new $\Delta folA::Kan3$ derivatives was influenced by the *met* mutation on minimal medium containing Arg+FEP and Arg+FEP-Gly. The results also show the *thyA* allele did not affect the phenotype of the *folA* mutation in these derivatives (MH989, MH990, MH991, MH992 and MH993), because the folate phenotype of strains with the either *thyA^{ts}* or *thyA⁻* alleles was the same.

3.1.2. Construction of *FolA*⁺ strains

The strain MH829 is the parent strain of strain MH950 (Figure 3). Genotypically, strain, MH829 has a *thyA*(Ts) allele but phenotypically it is *Thy⁻*. The suppressor strain, MH950 was also phenotypically *Thy⁻*. Earlier experiments suggested that this strain remained *Thy⁻* when it was made *FolA⁺*, whereas *FolA⁺* derivatives on MH829 were *Thy^{ts}*. In order to check and determine if the earlier results were correct, I inserted *folA* allele in MH950 and MH829 strains (Table 1) and I constructed *FolA⁺* derivatives of both MH829 and MH950 strains.

Strains were constructed by gene gorging, transformation of *FolA⁻* cells to *FolA⁺* using two plasmids. The plasmid pMGSA5 donated *folA⁺* allele to the desired strains,

MH829 and MH950, by company of the plasmid pACBSR that carried I-SceI and λ -Red genes. The competent of MH829 and MH950 strains were combined with pACBSR, pMGSA5 and with a mixture of the two plasmids. 100 μ l of undiluted transformants were plated on LB+Thy plates containing required antibiotics, chloramphenicol (CM) or ampicillin (AMP). Single transformants were selected on CM and on AMP plate and double transformants were selected on AMP+CM plates. On AMP plates, transformed strain MH829 had 40 colonies and transformed strain MH950 had 34 colonies. On CM plates, 41 colonies were observed from MH829 and 30 colonies from MH50 strain. The double transformation was successful, since transformed strains MH829 and MH950 had 43 and 38 colonies, respectively, on AMP+CM plates. So these transformants were used for the gene gorging.

I included both a negative control (no added DNA) and a positive control (pUC18) for each strain. Both strains produced resistant colonies with the positive control on Amp plates. There were no colonies when no DNA was added.

In gene gorging, at $t=0$, 10 μ l of 10-, 100-, and 1000-fold dilutions of transformed cells were spotted on AMP, CM, AMP+CM, and LB+Thy plates and incubated at 37°C. The colonies after one day showed the same growth compare to LB+Thy plate on AMP, CM, AMP+CM plates. These results showed that cells had maintained both plasmids.

After applying L-arabinose and chloramphenicol, and incubation, 100 μ l of 10^{-4} and 10^{-5} aliquots of MH829 and MH950 strains were spotted on LB+Thy, and LB+Thy containing AMP, CM, Kan, AMP+CM, and AMP+CM+Kan plates at 37°C. Also, 100 μ l of 10^{-5} aliquots of MH829 and MH950 strains were plated on LB+Thy and Arg+Thy and incubated at 37°C. As there were roughly the same number of colony (4-5) in different

media compared to LB+Thy, hence transformation and gene gorging worked well. Many of the cells were now AMP sensitive and CM sensitive indicating that the donor plasmid was no longer present (as expected due to it became linearized by I-SceI) and the mutagenesis plasmid was lost in many of the cells. Very low number of colonies (3-5) on CM+AMP plate verified that the donor plasmid had been cut (Herring, Glasner, and Blattner, 2003). These results showed that two plasmids were no longer present in many of the cells. The difference between *FolA*⁺ and *FolA*⁻ colonies was very clear on Arg+Thy plates (Figure 5).

So, from Arg+Thy plates, some of the largest colonies were selected as possible *FolA*⁺ derivatives were chosen (Figure 5). To confirm the recombination and replacement of $\Delta folA::Kan3$ allele with *folA*⁺ allele in strains MH829 and MH950, cells were again screened for kanamycin, ampicillin and chloramphenicol sensitivity (*Kan*^S *AMP*^S *CM*^S) on LB+Thy plates containing these antibiotics. The *Kan*^S *AMP*^S *CM*^S colonies were tested for Thy phenotype on minimal plate containing arginine (Arg) and thymidine (Arg+Thy) at three temperatures (30, 37 and 43°C).

Finally, after screening and successfully purifying, two derivatives of MH829 (MH984, MH985) and four derivatives of MH950 (MH981, MH982, MH983, MH986) were selected for further characterization. The new derivative strains resulted from gene gorging (*Fol*⁺) are listed in Table 1.

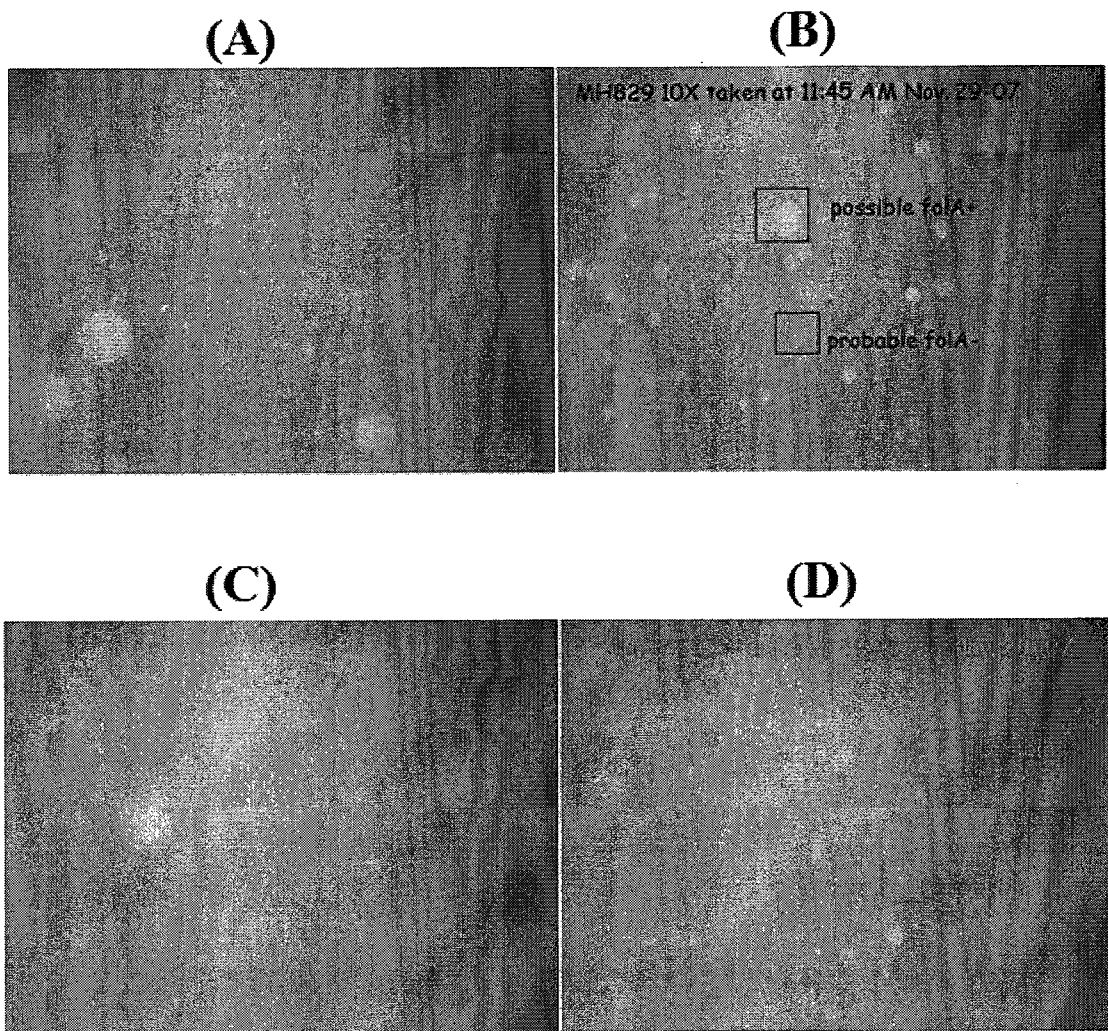


Figure 5. Screening of *FolA*⁺ derivatives by Gene Gorging. Samples from gene gorging were plated on LB+Thy and Arg+Thy media and were incubated at 37°C. (A, B) MH829 on Arg+Thy, (C, D) MH950 on Arg+Thy.

The *folA* region of the *E. coli* chromosome of each putative FolA⁺ strains were PCR amplified and the PCR products were analysed (Figure 4, Gel A). All of the new Fol⁺ strains had the expected size product.

Ten µl of 10⁻⁶ dilutions of the Fol⁺ derivative strains (MH981, MH982, MH983, MH984, MH985, and MH986) and their parents, MH829 and MH950, were spotted on minimal medium containing Arg, Arg+Thy, Arg+FEP-Gly, and on LB+Thy plates. Then plates were incubated at 37°C.

Strain MH829 was able to grow into obvious patches on minimal medium containing arginine and thymidine (minA+Arg+Thy) at 30 °C by 3 days, but at 37 °C and 43°C patches were formed after 5 days. When thymidine was omitted from this medium, strain MH829 grew abnormally at 30 °C and it was not able to grow at 37 °C and 43°C, and strain MH950 did not grow at any of three testing temperatures (30, 37 and 43°C) and it was known as thymine requirement (*thy*⁻).

The growth phenotype of Fol⁺ derivatives (MH981, MH982, MH983, MH984, MH985, and MH986) and parents (MH829 and MH950) showed clear differences on the Arg+Thy medium. On this medium the Fol⁺ derivatives grew quickly, as after one day at 37°C they had fairly large size colonies. In contrast the parent strains, MH829 and MH950, grew slowly on both Arg+Thy and LB+Thy at 30°C after 3 days.

On minimal medium containing arginine (minA+Arg) at 30°C, 37°C and 43°C only derivatives of MH829 (MH984 and MH985) had growth after 6 days only at 30°C. There was no growth of MH950 derivatives (MH981, MH982, MH983, and MH986) at any three testing temperatures. The results suggested that MH984 and MH985 strains were ThyA(Ts) and were not able to grow at higher temperature (43°C) without thymidine

added. Also, it confirmed that other FolA⁺ derivatives (MH981, MH982, MH983 and MH986) were Thy⁻.

The new Fol⁺ derivatives showed faster growth on Arg+FEP-Gly than their parents, MH829 and MH950. During 1-2 days Fol⁺ derivatives were observed at 30 and 37°C. Strain MH829 showed no growth on Arg+FEP-Gly and strain MH950 had growth after 3-4 days. These results showed that Fol⁺ derivatives had the same growth as related Fol⁺ strain MH828.

These results showed that *thyA* gene did not have influence on the growth phenotype of Fol⁺ derivatives. This conclusion is based on the fact that the FolA⁺ derivatives of MH950 (MH981, MH982, MH983, and MH986) behaved as Thy⁻ and Fol⁺ derivatives of wheares the MH829 derivatives (MH984 and MH985) were FolA⁺ and Thy^{ts}.

3.2. The sequence of thyA and lgt genes

To determine what mutations, if any were present in the *lgt-thyA* operon of strain MH829 and MH950, the region was sequenced. For each strain, five PCR products spanning the *lgt-thyA* region were sequenced using the primers listed in Table 2. Figure 6 shows the region and the approximate sizes of the sequences obtained.

The BLAST analysis of all sequences showed high identities to sub-strain of MG1655 of strain *E. coli* K-12 (Table 5).

After combination of sequenced regions shown in Figure 7, the 5' to 3' strand of these two genes and their operons were confirmed by BLAST analysis. The BLAST analysis showed 1898/1899 identities to sub-strain of MG1655 of strain *E. coli* K-12.

Primers	Primer's sequence	Strains	BLAST Hit	Sequence's direction & Mismatch position
thyA_Pm_R_X	GTGCTCGAGCAATGGGAGCTGTC TCAGG	MH829	2963341...2962644	NM
		MH950	2963342...2962721	5' to 3', (T → A) 2963047
thyA_reg_L	AAAAAAGACGAGGCCAGTTT	MH829	2962298...2962939	5' to 3', (T → A) 2963047
		MH950	2962298...2962902	NM
thyA_reg_R	CTGATTCGCGGAGGGTTAT	MH829	2964172...2963545	NM
		MH950	2964172...2963552	NM
RT_thyA_L	GCCGTCTGCCACATAGAACT	MH829	2962793...2963456	NM
		MH950	2962783...2963479	3' to 5', (A → T) 2963047
RT_thyA_R	ATGTCACCATCTGGGACGAA	MH829	2962870...2962248	3' to 5', (A → T) 2963047
		MH950	2962888...2962275	NM

pts_r_R	TGCAATGTTACGAGCTCTGG	MH829	2965570...2963686	NM
		MH950	2964470...2963641	NM
		MH841	2964375...2963646	NM
		MH956	2964444...2963498	NM
		MH958	2964460...2963652	NM
		MH829	2963613...2962847	NM
RT_lgt_R_test	CCACTCAITCCGTTTGGTCT	MH950	2963604...2962838	5' to 3', (T→A) 2963047
		MH841	2963614...2962799	5' to 3', (T→A) 2963047
		MH956	2963614...2962799	5' to 3', (T→A) 2963047
		MH958	2963605...2963014	5' to 3', (T→A) 2963047
		MH829	2962197...2962962	5' to 3', (T→A) 2963047
		MH950	2962185...2962961	NM
ppdA_r_L	AGTCCACTTGGCCTTAGCAT	MH841	2962173...2963015	NM
		MH956	2962184...2962962	NM
		MH958	2962199...2963008	NM

thyA_R_t	TAGTCGAAAGATGGATTCCGGG	MH829	2962421...2962122	NM
		MH950	2962413...2962122	NM
		MH841	2962413...2962122	NM
		MH956	2962420...2962122	NM
		MH958	2962413...2962122	NM
		MH829	2963828...2964510	NM
t_lgt_2	GTCCCAGACACGGGAACAGAT	MH950	2963833...2964510	NM
		MH841	2963828...2964510	NM
		MH956	2963828...2964510	NM
		MH958	2963830...2964510	NM

Table 5. Summary of BLAST analyses of sequences. Ten primers were used to sequence *thyA-Igt* region and its operon in five strains. Two strains MH829 and MH950 were sequenced with all ten primers, but three strains MH841, MH956, and MH958 were sequenced with five primers that covered the desired region and its operon. The BLAST hit positions given is the range with either 100% identity or a single mismatch. The Location of mismatch in all five sequences is nucleotide 2963047 with the change of T→A on 5' to 3' direction of sequence. NM notifies when there was no mismatch in the sequence and the identity was 100%.

Thus, both strains contain a single base pair change in the *thyA* gene located at position 2963047.

The BLAST analysis of translated sequence (protein) showed complete (291/291) identity to wild type Lgt (phosphatidylglycerol-prolipoprotein diacylglyceryl transferase ref[NP_417305.1]) and a single amino acid change (263/264) when compared to wild type ThyA (thymidylate synthetase ref[NP_417304.1]).

The desired region (*thyA* and *lgt*) in strains MH829, MH841, MH950, MH956, and MH958 was sequenced and the used primers and BLAST results shown in Table 5. When strain MH829 was also sequenced, the assembled sequence spanned nucleotides 2964510-2962421 with a single mismatch (A to T) at position 2963047.

When strain MH950 was sequenced, the assembled sequence spanned 2964510-2962122 nucleotides with the same mismatch (A to T) at 2963047. The assembled sequence of spanned nucleotides 2964510-2962122 of strains MH841, MH956, and MH958 (Table 6) was contained the same mismatch (A to T) at nucleotide 2963047. This mismatch was seen on only one spanned nucleotide sequenced by RT_lgt_R_t primer.

These results indicate that both group of strains with different Thy phenotypes (Thy^{ts} and Thy⁻) showed the same nucleotide change. These results demonstrated a single AT→TA mutation in *thyA* suggesting that a second site mutation results in the different Thy phenotype; Thy⁻ in one strain and Thy^{ts} in another. These result demonstrated a single AT→TA mutation in *thyA* located within the region where the *thyA^{ts}* allele was mapped by Belfort, Maley and Pedersen-Lane (1983). Since all the strains have the same mutation, the Thy⁻ phenotype must result from a mutation outside of the *thyA-lgt* region.

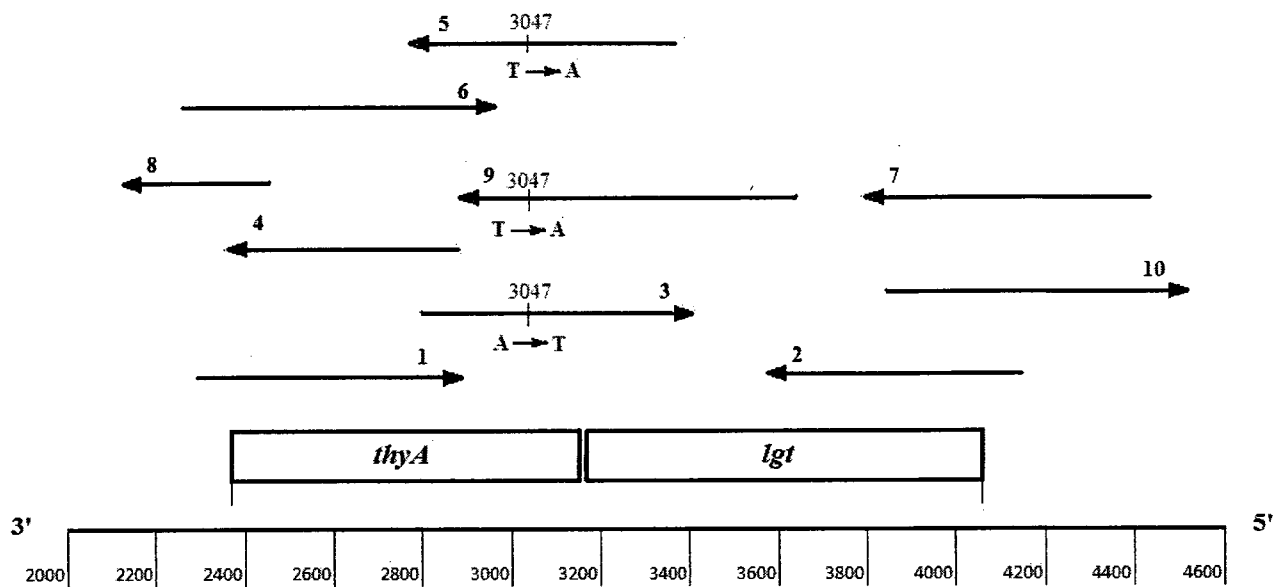


Figure 6. Assembled sequences for the *thyA* and *lgt* region. This region shown corresponds to nucleotides 2962122-2964510 of *E. coli* K-12 strain MG1655. In the diagram only the last four numbers are shown. The arrows show the approximate length of sequence obtained with each primer, with the arrowhead indicating the 5' end. The numbers correspond to the primers used: 1. *thyA*_reg_L, 2. *thyA*_reg_R, 3. RT_*thyA*_L, 4. RT_*thyA*_R, 5. *thyA*_Pm_R_X, 6. *ppdA*_r_L, 7. *Pts*_r_R, 8. *thyA*_R_t, 9. RT_*lgt*_R_t, and 10. *t_lgt*_2. The position of the only mutation found in the region is also shown.

2964240 CATGGGCGGG CTGATTGCGG GAGGGTATA GCGGGGATCA TATACATATC TTTTAAAGGT
-10 Igt ↗

2964180 ATCCGGCAAC CAGCCAGGTC CCCTTGTGC ATTATTCGCA CCTTTGGAGC GCCTGAAACC
RBS

2964120 TCGGGCGCGC ATTTCAATCG CTGTTCTCTT TCAGCGAAT AACAGAACT TGTGGTGACA

2964060 GATGACCAGT AGCTATCTGC ATTTCCGGA GTTTGATCCG GTCATTTTCT CAATAGGACC
M

2964000 CGTGGCGCCT CACTGGTACG GCCTGATGTA TCTGGTGGGT TTCATTTTTC CAATGTGGCT

2963940 GGCALCACGA CGGGCGAATC GTCCGGGCGG CGGCTGGACC AAAAATGAAG TTGAAAACCT
2963880 ACTCTATGCG GGCTTCTCG GCGTCTTCT CGGGGGACGT ATTGGTTATG TTCGTCTCTA
2963820 CAATTTCCCG CAGTTTATGG CCGATCCGCT GTATCTGTTC CGTGTCTGGG ACGGCGGCAT
2963760 GTCTTTCCAC GCGGGCCTGA TTGGCCTTAT CGTGGTGTAT ATTATCTTCG CCCGCCGTAC
2963700 TAAACGTTC TTCTTCCAGG TCTCTGATTT TATCGCACCA CTCATTCGGT TTGGTCTTGG
2963640 TGCCGGGCGT CTGGGCAACT TTATTAACGG TGAATGTGTG GGCCGCGTTC ACCCGAACTT
2963580 CCCGTTTGGC ATGCTGTTC CTGGCTCCCG TACAGAAGAT ATTTTGTCTG TCGAAACCAA
2963520 CCCGCAGTGG CAATCCATT TCGACACTTA CGGTGTGCTG CCGCGCCACC CATCACAGCT
2963460 TTACGAGCTG CTGCTGGAAG GTGTGGTGT GTTATTTATC CTCAACCTGT ATATTCGTAA
2963400 ACCACGCCCA ATGGGAGCTG TCTCAGTTF GTTCTGTATT GGTTACGCGC CGTTTCCCAT

(-35) (-10)

2963340 CATTGTTGAG TTTTCCGCC AGCCCGACGC GCAGTTTACC GGTGCCCTGG TGCAGTACAT
thyA ↗

2963280 CAGCATGGGG CAAATTCCTT CCATCCCGAT GATTGTGCGG GGTGTGATCA TGATGTTCTG

(End of Igt) S.D.

2963220 GGCATATCGT CGCAGCCAC AGCAACAGT TTCC GGG ACCATGAAAC AGTATTTAGA

2963160 ACTGATGCAA AAAGTGCTCG ACGAAGGCAC ACAGAAAAC GACCGTACCG GAACCGGAAC
MspAII

2963100 GCTTTCCATT TTTGGTCAFC AGATGCGTTF TAACCTGCAA GATGGATTCC CGCAGGGTGAC

2963040 AACTAAACGT TGCCAOCCTGC GTTCATCAT CCATGAACTG CTGTGGTTTC TGCAGGGCGA

2962980 CACTAACATT GCTTATCTAC ACGAAAACHA TGTCAOCHC TGGGACGAAT GGGCCGATGA

2962920 AAACGGCCGAC CTCGGGOCAG TGTATGGTAA ACATGGGCGC GCCTGGCCAA CGCCAGATGG
MspAII

2962860 TCGTCATATT GACCAGATCA CTACGGTACT GAACCACTG AAAAAGGACC CGGATTCGCG
2962800 CCGCAITATT GTTTCAGCGT GGAACGTAGG CGAAGTGGAT AAAATGGCGC TGGCACCGTG
2962740 CCAAGCATT TCCAGTTC ATGTGGCAGA CGGCAAACCTC TCTTGCACAGC TTTATCAGCG
2962680 CTCTGTGAC GTCTTCCCTG GCCTGCCGTT CACATTGOC AGCTACGGGT TATTGGTGC
2962620 TATGATGSCG CAGCAGTGGC ATCTGGAAGT GGGTGTATTT GTCTGSAOCC GTGGCGACAC
2962580 GCATCTGTAC AGCAAACCAI TGGATCAAAC TCATCTGCAA TTAAGCCGCG AACCCGCTCC
2962520 GCTGCCGAAG TTGATTATCA AACGTAAACC CGAATCCATC TTCGACTACC GTTTCGAAGA
(End of thyA)

2962460 CTTTGAATTT GAAGGCTACG ATCCGCATCC GGGCATTAAA GCGCCGCTGG CTATCTA GGG

2962420 ACGAAACATC CTGCCAGAGC CGACGCCAGT GTGCGTGGT TTTTATACC TCCGTAAAT

Figure 7. The sequence of *thyA* and *lgt* genes in MH829 and MH950 strains of *E. coli* K-12. The double boxed A is the only nucleotide difference between the wild type sequence and *thy^{ts}* allele. This is located in the region where this allele was mapped by Belfort Maley and Pedersen-Lane (1983).

The start codons of the two genes are shown in dark gray boxes, and the translation-stop site of *thyA* and *lgt* genes are shown in very dark gray. The positions of Shine-Dalgarno sequence (S.D.) of the ribosome binding site shown in dashed boxes placed 3 nucleotides upstream of the start codon. The *MspAII* restriction enzyme sites are in dark gray in *thyA* ORF.

The underlined is the part that Belfort and Pedersen-Lane (1984) reported for the ThyA mutation they found. The double-boxed nucleotide shows the inverted nucleotide (A to T) in *thyA* gene of the strains MH829, MH841, MH950, MH956 and MH958. The mutated nucleotide in *thyA* gene is mentioned as nucleotide “A”, which is on the 5’ to 3’ strand. The transcription start sites for *lgt* and *thyA* are indicated with arrows, the promoters and ribosome binding sites of these two genes, also the translation start codon of *lgt* and *thyA*, are specified (Gan, Sankaran, and Williams, 1995). The poly-T region, which is shown in gray, represents the transcriptional termination of the *thyA* gene after a G-C-rich region (Gan, Sankaran, and Williams, 1995).

3.3. Detection of Curli protein

I wanted to study the curli proficiency of strains, because some Fol^- derivatives (MH950) (Figure 3) surprisingly made red colonies on Congo-red plates. As Congo-red plates are variable I developed a new rapid method for detecting curli.

Crude curli preparations were made from the wild-type strain MC4100 and MHR204 as well as Fol^+ derivatives of MH829 (MH984 and MH985) and of MH950 (MH981, MH982, MH983, and MH986).

3.3.1. Detecting curli by SDS-Agarose gels

SDS-Agarose gels have been used to analyze partially dissociated amyloid proteins (Bagriantsev et al., 2006). Since curli are amyloid like, I examined their behavior during SDS-agarose gel electrophoresis (Figure 8).

When the gel was stained with EZ-blue buffer and washed briefly, the only protein visible was in the wells of the gel (Figure 9.A). Since this protein was present in the samples from MC4100 and not from MHR204 it could be predominantly curli fibres.

Protein was also visible in SDS-agarose gel of samples from MH981, MH982, MH983, and MH986. These strains are red on Congo-red plates suggesting they make curli.

Protein was not visible in samples of MH984 and MH985. These strains are less red on Congo-red suggesting they make few if any curli.

Similar results were observed when proteins were extracted from cells after three days of incubation (Figure 9.A) and after one day (Figure 9.B).

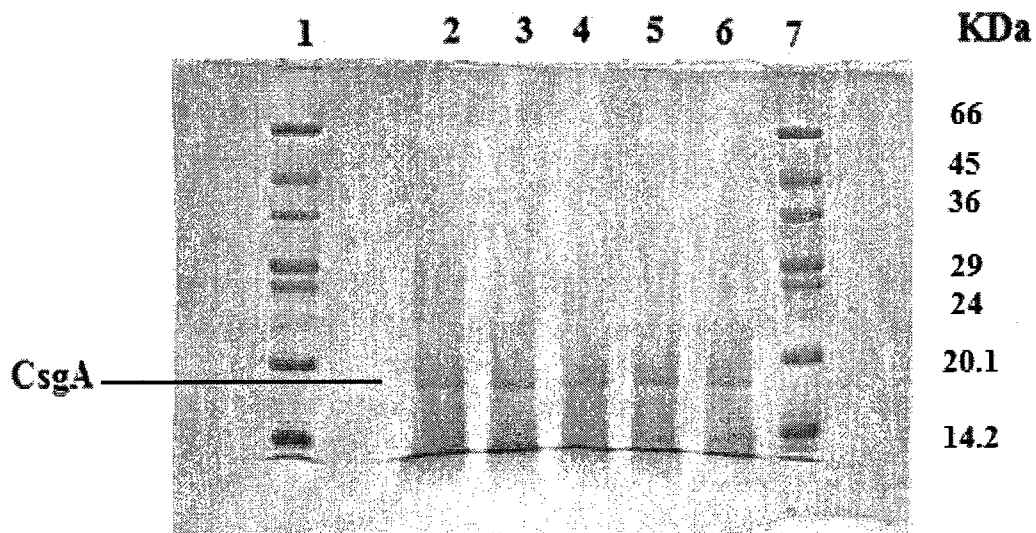


Figure 8. Protein analysis by SDS-PAGE. CsgA and CsgB were purified (Chapman et al., 2002; Collinson, Emody, and Muller, 1991). Lanes 2- 6 contained 0.83 μ g of purified CsgA (18 KDa (Chapman et al. 2002, Olsen, Arnqvist, and Hammar, 1993; Collinson, Emody, and Trust, 1992)) and CsgB (15.3 KDa (Bian and Normark, 1997)) from MC4100. Lanes 1 & 7 contains 10 μ g of protein markers.

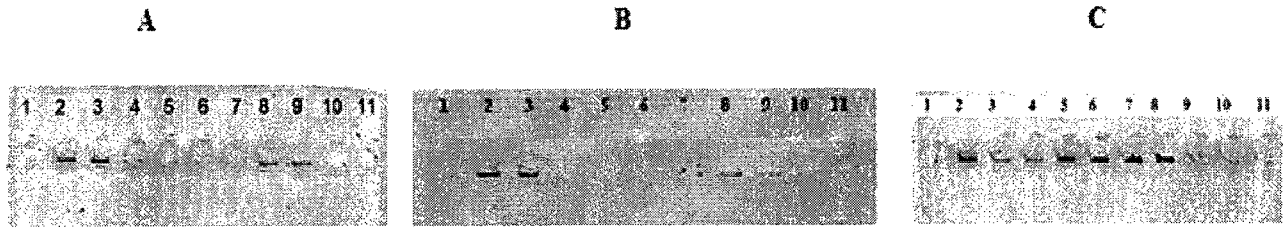


Figure 9. The SDS-Agarose gel and analysis of crude extracts. The known amount, 20-30 μg , protein from washed samples were loaded on a 1.2% SDS-agarose gel. The voltage of 72 was applied for 45 minutes. After staining the gel with the EZ Blue Reagent and washing twice with water, the gel was observed with the Odyssey Infrared imager. (A) Proteins extracted from cells after 3 days incubation of YESCA plates at 30°C. Lanes represent, 1: Ladder, 2 & 3: MC4100, 4 & 5: MHR204, 6 & 7: MH829, 8 & 9: MH950, 10: empty, 11: Ladder. (B) Proteins extracted from cells after 1 day incubation of YESCA plates at 30°C. Loaded samples on the gel contained 20 μg proteins. Lanes represent, 1: Ladder, 2 & 3: MC4100, 4 & 5: MHR204, 6 & 7: MH829, 8 & 9: MH950, 10: empty, 11: Ladder. (C) The protein samples including curli protein from new Fol⁺ derivatives and their parents (MH829 and MH950). 30 μg of calculated protein by BCA assay (Sorensen and Brodbeck, 1986) from washed sample were loaded in each well. Lanes 1 & 10 indicate protein marker (MWM), and 9 and 11 are empty; and the rest of the lanes are: 2:MC4100; 3:MH984; 4:MH985; 5:MH981; 6:MH982; 7:MH983; 8:MH986.

As Figure 9.A shows, the wells, containing 20 μ g of quantified protein, of gel belong to strains MHR204 and MH829 do not have protein in the wells in contrast to strains MC4100 and MH950. As well, the first well from left that belongs to the protein marker (Dalton Marker VII-L, molecular weight marker, from Sigma), which does not have curli protein, showed the same results as MHR204. As it is seen in Figure 9.C, 30 μ g of quantified protein from Fol⁺ derivatives of MH950 had clearly observed protein in the wells compared to Fol⁺ derivatives of MH829. The image B of this figure showed that if the strains would be incubated for one day on YESCA plate at 30°C, they could give similar results as three days incubation on same medium at 30°C.

As shown in Figure 10, there were clear difference of existing curli protein in the known curli-proficient and non-curli-proficient strains corresponding to MC4100 and MHR204. The amount of 30 μ g of calculated protein from washed samples of the strains MC4100 and MHR204 were loaded in wells of the SDS-Agarose gel. It was discovered that overnight staining along with shaking produced a result that clearly distinguished between curli-proficient and non-curli proficient strains.

In order to determine the curli proficiency of Fol⁺ derivatives, the protein of strains MH981, MH982, MH983, MH984, MH985, and MH986 were purified (Collinson, Emody, and Trust, 1992) and quantified by BCA assay (Sorensen and Brodbeck, 1986). Then 30 μ g of purified protein was loaded in the wells on SDS-agarose gel. After 45 minutes running and 15 minutes staining, the gel was twice washed with distilled water. As shown in Figure 9.C, FolA⁺ derivatives of MH950 had material in the related wells compared to the MH829 FolA⁺ derivatives. These results verified that Fol⁺ derivatives of curli- proficient strain, MH950, were also curli-proficient.

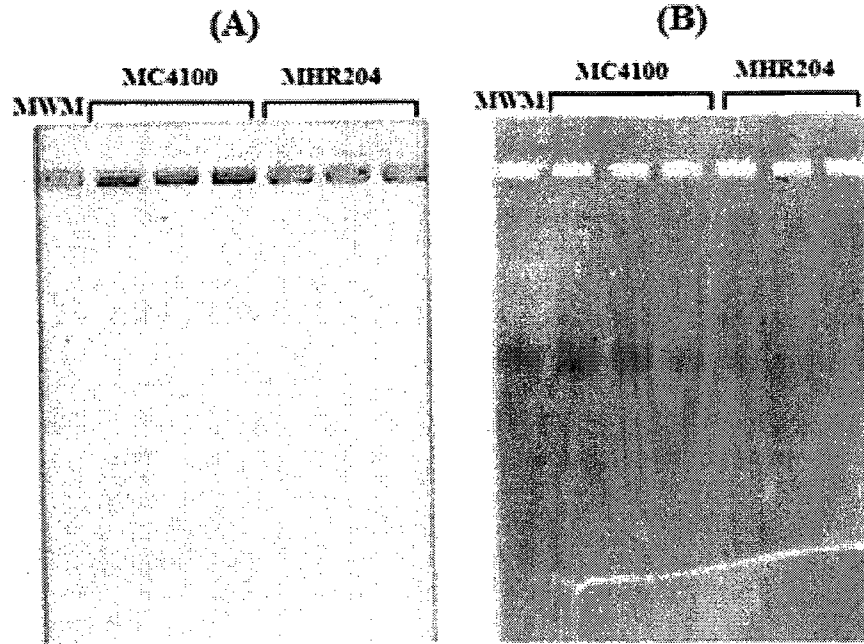


Figure 10. A longer staining time Allows Detection of Protein in the Gel. (A) After protein purification, the different amounts of washed samples of the strains, MC4100 and MHR204,

were loaded in SDS-Agarose gel. After 45 minutes running at 72 volt and staining, the gel was scanned in Odyssey. From left, first well contains 20 μg of molecular weight marker; the 2nd, 3rd and 4th wells contain 30 μg of washed sample of the curli-proficient

strain MC4100. The 5th, 6th and 7th wells contain 30 µg of washed sample of the non-curli-proficient strain MHR204. (B) The SDS-Agarose gel of image (A) was left overnight on EZ Blue Reagent and was washed twice.

3.4. The material in the well is predominantly CsgA

Highly purified curli were prepared (Collinson, Emody, and Muller, 1991), treated with formic acid and run on an SDS-PAGE gel (Figure 8). The predominant protein migrated at 18 KD as reported for CsgA (Chapman et al. 2002, Olsen, Arnqvist, and Hammar, 1993; Collinson, Emody, and Trust, 1992).

To find out if the protein in the well of an SDS-agarose gel was curli; I recovered these materials from wells of those gels after 15 minutes staining. The recovered material was washed extensively with water to remove the stain. After treating with 90% formic acid and analyzing in a SDS-PAGE, results showed that wild-type strain MC4100 had a protein with the same migration of CgsA (Figure 8). As shown in Figure 9.C, only Fola+ strains MH981, MH982, MH983 and MH986 (derivatives of MH950) had materials in the wells of SDS-agarose gel. In order to check these materials, SDS-PAGE analyses of the materials in the well from these Fola+ strains showed the presence of curli subunit CgsA (Figure 11).

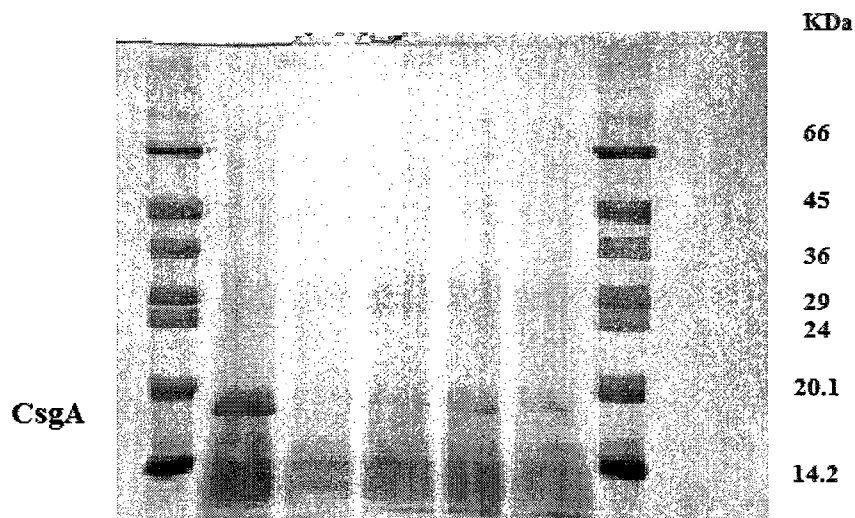


Figure 11. Protein analysis by SDS-PAGE. The stocked materials in SDS-agarose of F_{olA}⁺ strains derived from MH950 wells were recovered and 2 μ g of these materials were loaded in 12% SDS-PAGE. Wells number 1 and 7 are Dalton Mark VII-L (Sigma), and wells number 2 to 6 are MC4100, MH981, MH982, MH983, and MH986. The bands of CsgA (18 KDa (Chapman (2002); Bian and Normark, 1997)) is shown.

4. Discussion:

This study looks at the relationship between two folate pathway genes *FolA* and *ThyA*. Strains deleted for *folA* gene grow poorly and their growth is improved slightly by mutation in *thyA* gene that reduces or eliminates TS activity (Herrington and Chirwa, 1999). Our laboratory has isolated faster growing derivatives (MH840, MH841, MH842 and MH950, MH951, MH952, MH953) of a strain with the genotype of $\Delta folA:Kan^3 thyA(Ts)$ (Figure 3). All of these strains require thymidine both at low and high temperature (Herrington and Chirwa, 1999; This study). Interestingly, when some of these strains are made *folA*⁺ (MH956, MH958, MH991, MH992, MH993, MH994, MH995 and MH996) (Figure 3), the strains became *Thy*⁻ phenotypically whereas the parent strain MH829 is *Thy*^{ts} (Metallic, T. and Herrington, M.B, unpublished; This study).

The hypothesis that came from *FolA*⁺ derivatives that showed *Thy*⁻ phenotype rather than *Thy*^{ts}, was the presence of a second mutation in *thyA-lgt* operon that results in auxotrophy for *Thy* even at the permission temperature 30°C. Hence, it was thought that cells are not able to grow related to the second site mutation carrying *Thy*⁻ phenotype. So, the *lgt-thyA* region was sequenced in the both *thy*^{ts} and *thy*⁻ strains; MH829, MH841, MH950, MH956 and MH958. This concluded that there was no additional mutation in the *thy*⁻ derivatives.

Looking at the sequence of *thyA* and *lgt* genes in both the MH829 and MH950 strains verified the presence of only one single bp change (AT→TA mutation) at position

of 2963047 in *thyA* gene of *E. coli* chromosome in both the Thy^{ts} and Thy⁻ strains (Figure 6). It was expected to observe the change only in Thy^{ts} strains, while Belfort and Pedersen-Lane (1984) mapped *thyA*^{ts} allele in strain N4316 to a 79 nucleotide segment of *thyA* (Figure 7). The changed nucleotide we observe in strains (MH829, MH841, MH950, MH956 and MH958) is located in this segment. This change results in a single amino acid change (L→Q) in thymidylate synthase, which is related to converting of CUG (Leu) to CAG (Glu) codon. This change in TS is presumably responsible for the reduced activity of TS in strains with the *thyA*^{ts} allele, while its activity is 19% of wild-type in cells grown at a lower temperature (Herrington and Chirwa, 1999).

There was no additional mutation either in *thyA* gene to influence the activity or production of TS enzyme, or in *lgt* gene to affect the expression of TS enzyme. This suggested the presence of a second mutation elsewhere in the chromosome responsible for the Thy⁻ phenotype of the F_{olA}⁺ strains. This second mutation can affect the activity of thymidylate synthase (TS) protein by influencing either the expression of *thyA* gene or blocking the enzyme activity. This second mutation might affect the *lgt* gene so that it alters the expression of *thyA* gene. So, this effect on *lgt* gene might block the expression of *thyA* gene and then the strains acquire Thy⁻ phenotype. Another possibility is that the *thyA* gene is expressed and a second mutation affects its activity by either inhibiting or eliminating the folding of thymidylate synthase (TS) protein, or blocking the activity of assembled-folded subunits of TS protein. Another possibility is that the activity of this second mutation might affect the posttranslational modification of TS amino acids. A posttranslational modification that changes the range of protein functions by attaching

other biochemical groups (acetate, phosphate, lipids and carbohydrates), therefore changing the chemical nature of amino acid or protein structural changes.

Since both the Thy^{ts} FolA^+ and Thy^- FolA^+ strains had the same mutation in the *lgt-thyA* operon and it was expected that Thy^- strains might have a mutation outside of the region that affect their phenotype, I attempted to map this change by the high frequency of recombination (Hfr) mapping technique (Miller, 1992), but the results were not clear.

The FolA^+ derivatives of MH950 and MH841 were made first by Herrington and Metallic (unpublished data), and then I made FolA^+ strains in this study by the gene gorging method (Herring, Glasner, and Blattner, 2003) in the parent strain MH829 and its faster growing derivative MH950. As expected the FolA^+ derivatives of MH829 were Thy^{ts} and the FolA^+ derivatives of MH950 were Thy^- . When the activity of DHFR (*folA*) and TS (*thyA*) are absent ($\Delta\text{folA}::\text{Kan3 } \text{thyA}^-$), the pool of methylene-THF is high. It, however, cannot be converted to DHF, but it can be produced from THF by SHMT (*glyA*) (Figure 1). When the pool of methylene-THF is high and TS is inactivated, its conversion can be applied to THF through other ways (Figure 1). Also, the limit pool of DHF converts to THF for a short time and the amount of THF can be enough to produce methylene-THF. Therefore, FolA^+ Thy^{ts} derivatives compared to their parent MH829 ($\text{FolA}^- \text{Thy}^{\text{ts}}$) strains can manage their growth, because they have the *folA* gene.

When the derivative MH950 ($\Delta\text{folA}::\text{Kan3 } \text{Thy}^-$) shows improved growth compared to parent strain MH829 ($\Delta\text{folA}::\text{Kan3 } \text{thyA}^{\text{ts}}$), it can be suggested that the growth of $\text{FolA}^- \text{Thy}^-$ strains could be better when there is little or no TS activity present rather than the low activity pressed from the strain with *thy^{ts}* allele. Thymidylate synthase

(TS) is the only enzyme that uses a folate cofactor both for a one carbon unit and for reducing power (Neuhard and Kelln, 1996), and thus is a source of DHF. It is possible that in a $\Delta folA::Kan3$ background the presence of a partially active TS results in lower amounts of THF than a completely inactive enzyme. So, as a result the growth would be more limited.

It was at first surprising that strains lacking DHFR protein were viable (This study; Herrington and Chirwa, 1999; Howell, Foster, and Foster, 1988; Giladi, Altman-Price, and Levin, 2003), because *folA* deleted strains can make detectable amount of folate cofactors (Hamm-Alvarez, SancarY1, and Rajagopalan, 1990). Hamm- Alvarez et al (1990) suggested that in the absence of DHFR, dihydropteridine reductase (DHPR) could be responsible for synthesis of THF and the five different one-carbon compound of reduced folate pool found in *E. coli* (Figure 1). Also, the mixture of THF and five different one-carbon compounds (Figure 1) can affect the rate of FEP production and initiation of protein synthesis, since protein synthesis can only be initiated with *met*-tRNA^{*met*} and cannot be supplied exogenously (Baumstark et al. 1977). Hence, $\Delta folA::kan3$ strains must generate enough CHO-THF to formylate *met*-tRNA^{*met*}. But the expression regulation of gene and enzyme activity is complex and not completely known (Matthews 1996).

Since *folA*⁻ *thyA*⁻ strains derived from the *folA*⁻ *thyA*^{*ts*} strain MH829 grew better than their parent MH829, we tested whether strains with different *thyA* alleles grew differently. In this study when the *folA*⁺ strains carrying different *thyA* alleles (*thyA*^{*ts*} and *thyA*⁻) were made *FolA*⁻ (*thyA*⁻ $\Delta folA$ and *thyA*(*Ts*) $\Delta folA$), they either were not able to grow or grew very slowly on supplemented minimal medium with folate end products

(FEP). There were no differences among the strains indicating that the type of *thyA* allele did not affect the growth. In these strains carrying different *thyA* alleles, the FolA activity is absent and the conversion of DHF to THF is decreased or blocked. In these conditions the pools of methylene-THF, DHF and THF are limited and the cells grow very slowly or the growth is inhibited. Methylene-THF cannot be converted to DHF and the existence pool of DHF is not able to be converted to THF, as thymidylate synthase is not active (Thy^-) or at lower temperature can be active (Thy^{ts}). The limited amount of methylene-THF and THF can produce folate cofactors required for growth of cell for a while and when the pool is very low, the cells do not grow. Also conversion of methylene-THF and THF through the other processes by FolD, MetF, MetH and MetE (Figure 1) rather than TS and DHFR cause the production of cofactors in longer time and the cells grow slower. Therefore, methylene-THF pool is sharing for both DHF production, also for the production of methenyl-THF and methyl-THF. The conversion of DHF to THF is blocked, as well, because of the absence of DHFR enzyme (ΔfolA) and it is required for *de novo* synthesis of THF and for recycling DHF (Herrington and Chirwa, 1999). Accumulation of DHF could inhibit the DHFS activity of DHFS-FPGS (bifunctional enzyme folylpolyglutamate synthetase-dihydrofolate synthetase (FPGS-DHFS) by *folC* gene) (Herrington and Chirwa, 1999).

In addition, Gilali et al (2003) found the *thyA* ΔfolA and *thyA*⁻ ΔfolA mutants are viable and they grew in minimal medium supplemented only with thymidine. They reported *folM* gene that is responsible for the reduction of DHF in *E. coli*. They reported this enzyme in *E. coli* that carries out the *de novo* synthesis of THF from DHF. Although the *E. coli* *thyA*⁻ ΔfolA mutant is viable and grows in minimal medium supplemented

with thymidine alone, and its growth rate is significantly reduced unless *folM* is overexpressed (Gilali et al., 2003). Hence, the low growth of our strains might also result by *folM* activity that could reduce DHF pool in strains carrying different *thyA* alleles (*thy*⁻, *thy*^{ts}).

Detection of Curli

E. coli and *Salmonella* spp. produce extracellular, adhesive fibers that are thin and highly aggregated, called curli. Curli encourages clumping of bacterial cells in culture and binding to surfaces such as glass and polystyrene, making them important for biofilm formation (Chapman et al., 2002; Collinson, Emody, and Trust, 1992; Collinson, Emody, and Muller, 1991).

The goal is to have a rapid assay that will allow us to detect and quantities the amount of curli made by strain. Some of the F_{olA}⁻ derivatives that grew better made curli compared to their parent MH829. Curli proficiency was detected by Congo red binding (Hammar, Arnqvist, and Bian, 1995). The ability to bind the dye Congo-red is a fast and easy way to determine expression of curli fiber (Hammar, Arnqvist, and Bian, 1995; Gophna, Barlev, and Seijffers, 2001). The simplest way of detection the presence or absence of curli proficiency is by the color of the colony, while curli-proficient strains make red colonies and non curli proficient colonies are white. Also, binding of curli to Congo-red can be assayed in solution (Gophna, Barlev, and Seijffers, 2001; Chirwa and Herrington, 2003). But in this study and in other work in our laboratory the Congo-red assays did not work consistently.

Curli is also able to bind to fibronectin with high affinity (Gophna, Oeschlaeger, and Hacker, 2002; Collinson, Emody, and Muller, 1991). This assay depends on multiple binding sites of fibronectin to extracellular matrix receptors and it was demonstrated that binding to soluble fibronectin needs high expression levels of curli fibers to such an amount that will promote their uptake (Gophna, Oeschlaeger, and Hacker, 2002). The importance of curli expression levels for fibronectin binding limits the usefulness of assay. The more sensitive adherence assay is another fibronectin binding assay, which was reported earlier (Flock, Heimdahl, and Schennings, 1996; Sorensen and Brodbek, 1986). This method does not need the high expression of curli (Flock, Heimdahl, and Schennings, 1996), but the results of this assay did not show difference in adherence rate between curli-proficient and non curli-proficient strains in this study.

The use of SDS-PAGE for detection of curli is not straightforward. To detect a clear band of CsgA in a SDS-PAGE gel, the protein needs to be purified and prior to electrophoresis, the sample should be treated in formic acid. The purification is very long and time consuming (Collinson, Emody, and Muller, 1991; Chapman et al., 2002) and using the unpurified sample needs Western-blotting after analyzing the sample in SDS-PAGE gel. Also, the formic acid treatment of unpurified sample is not proper and the depolymerization and denaturation of protein in unpurified sample cannot occur correctly and so detection of the major subunit (CsgA) of curli protein is not expected. Hence, I used partially purified (washed) (Collinson, Emody, and Trust, 1992) samples and SDS-agarose gel (Horsten, 2003) to detect the presence of curli protein in the samples. In this study, I only treated the washed samples with formic acid and the CsgA band (18 KD) of

curli fiber protein (Figure 8) identified by protein gel electrophoresis, SDS-PAGE. This is highly accurate method, but time consuming. It also required highly purified curli.

It was of interest to find an easier, fast and sensitive way to detect and quantify the presence of curli protein. We got idea from Horsten (2003) and Luo, Wehr, and Levine (2006) and Bagriantsev, Kushnirov and Liebman (2006) studies and we used SDS-agarose gel, which was stained with EZBlue™ buffer, for identifying and quantitating the curli protein. Luo, Wehr, and Levine (2006) discovered that protein in gel or on blot could be quantities with Coomassie Blue G-250. The Coomassie Brilliant Blue G-250 based protein stain of EZBlue reagent is used for ultrasensitive detection on polyacrylamide gels as low abundance proteins can be detected. Also, the background staining is reduced and protein bands can be viewed directly during the staining process and when the proteins are sufficiently stained the staining process can be stopped, so this visualization of the bands saves time. Also, this staining does not need destaining process as other Coomassie-based stains, while rinsing the gel with water after the staining step will enhance the sensitivity (Laemmli, 1970).

It was shown by Horsten (2003) and Bagriantsev, Kushnirov and Liebman (2006) that purified protein can be analyzed in a horizontal SDS-agarose gel. Horsten (2003) reported a method that could quantify the proteins from SDS-agarose gel. Then the suitability of SDS-agarose technique for the analysis of large proteins and SDS-stable high molecular weight complexes was suggested (Bagriantsev, Kushnirov and Liebman, 2006). The SDS-agarose gel, however, is made easier and faster compared to SDS-PAGE, this gel can be used in the horizontal apparatus for running, as well, and it is a fast way to determine the existence of curli in strains. Therefore, I used combination of a fast

protein purification (washed) method (Collinson, Emody, and Trust, 1992) and SDS-agarose gel as a rapid assay for curli protein identification and quantitating. The positive control (MC4100) was able to make curli and negative control (MHR204) did not have this ability. With MC4100 extracts a significant amount of protein remained in the wells of the SDS-agarose gel. In contrast, little or no protein was present in the wells containing samples from MHR204.

The recovered materials of MC4100 from the well of a SDS-agarose gel was formic acid treated and analyzed on a SDS-PAGE gel and the major subunit of curli, CsgA, was observed. Since F_{olA}⁻ strain (MH950) have proficiency of making curli protein, it was interesting to check this ability in F_{olA}⁺ derivatives and their parent strains in this study. As it was expected, MH950 and its F_{olA}⁺ derivatives had materials in the wells of SDS-agarose gel, whereas MH829 and its F_{olA}⁺ derivatives did not have. The curli protein of materials in the wells for MH950 and its F_{olA}⁺ derivatives was analyzed after formic acid treatment in a SDS-PAGE (Figure 11). The materials in SDS-agarose wells contain CsgA subunit (Bian and Normark, 1997) of curli and some other proteins. These proteins other than CsgA might be either smaller proteins that were maintained with curli in the wells of SDS-agarose gel, or the formic acid treatment digests curli protein as it has some proteins with lower molecular weight compared to CsgA. It was thought that smaller band might be CsgB (15.3 KDa) subunit, but CsgB might normally present in curli at low amount, which was not detected and was covered under CsgA band after depolymerization with formic acid (Bian and Normark, 1997).

In conclusion, this assay is suitable to detect the presence of fimbriae (curli) of cell, but needs some further work, such as quantitating the amount of loaded protein that give

detectable amount of putative curli. First, it is needed to identify the adequate amount of purified preparation to be detected in SDS-agarose gel. Then sufficient amount of recovered materials from SDS-agarose well that can identify the presence of curli in SDS-PAGE. It would be very interesting to determine the least amount of purified curli protein and recovered material that can be detected, respectively, in SDS-agarose gel and SDS-PAGE.

The problem in this method is that during staining, especially overnight, and washing the gel, most of the materials in the wells is lost. It makes a problem in quantification of material in the well, since it is needed to be recovered for identifying the CsgA band in SDS-PAGE. I tried to solve this problem by filling the wells of SDS-agarose gel with samples and melted agarose, before putting gel in running buffer (Figure 12). But this system needs more work to have enough concentrated protein in the almost 20 μ l of melted agarose to fill the wells with required detectable amount of protein.

Also the materials inside the gel of SDS-agarose after running needs further study while differences between MC4100 (putative curli) and MHR204 (non-putative curli) are clearly visible (Figure 10). Purified samples of MC4100 have staining material both in the well and inside the gel of SDS-agarose whereas samples of MHR204 only have material in the gel. It was expected that material inside the gel for MC4100 would be less than of strain MHR204, since the same amount of sample was loaded. However,

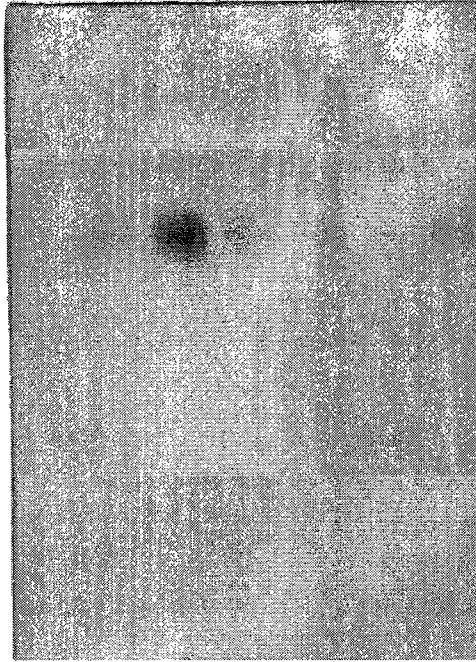


Figure 12. SDS-agarose gel of curli-proficient strain MC4100 loaded with melted agar in the wells. The second and last wells contain 10 μ g of molecular weight marker, and 3rd has 50 μ g of crude sample and 4th, 5th and 6th wells have dilution of 1/5 (10 μ g), 1/10 (5 μ g) and 1/100 (0.5 μ g) of crude sample. These samples were loaded with melted agar in the wells of SDS-agarose gel.

MHR204 had less staining material in the gel. One explanation is that might be the interference of BCA assays with purified protein, in which there is something in the purified materials that BCA ingredients interfere with and affect the calculation of protein. So, that interference can affect the measurement of protein concentration in samples of each curli-making and non-curli-making strain. It is possible that curli protein or other proteins interfere with BCA assay reagents and show an error between the real amount of protein and calculated protein. Also, there might be interfering between purified protein of non-curli proficient strains and the BCA assay ingredients. But, BCA assay has most stable curve for protein assay. Therefore, this assay was found the most accurate and standard for protein assay in this study.

5. Conclusion

- 1- The *folA* gene does not affect the *thyA* gene.
- 2- The type of *thyA* allele (*thyA*(*Ts*), *thyA*⁻) does not affect the growth of *folA*⁻ strains on supplemented minimal medium.
- 3- SDS-agarose gel is a rapid method to detect the presence of curli in the strains.
- 4- The materials in the well of SDS-agarose gel can be recovered and the band corresponding to the major subunit of curli protein (CsgA) identified in SDS-PAGE gel.

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