STATIONARY PHASE EXPRESSION OF *ARGCBH* IN *ESCHERICHIA COLI*

STATIONARY PHASE EXPRESSION OF THE ARGININE BIOSYNTHETIC OPERON (*ARGCBH*) IN *ESCHERICHIA COLI*

By

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ABSTRACT

In this study, we report that expression of the *argCBH* operon is induced in stationary phase cultures and that this increase is largely dependent on RpoS, the alternative stress sigma factor. Using combinatorial *argR* and *rpoS* mutants, we evaluated the relative contributions of these two regulators to the expression of *argH* using operon *lacZ* fusions. While ArgR was found to be the main factor responsible for de-repression of the *argCBH* operon, RpoS was required for full expression of this biosynthetic operon at concentrations below 10 μg arginine ml^{-1}, a level at which growth of an arginine auxotroph was arginine limited. At high arginine concentrations (>10 μg ml^{-1}), *argCBH* expression was strongly repressed as expected by ArgR. *argCBH* expression was 30 fold higher in *ΔargR* mutants relative to a wild type fully repressed strain and this expression was independent of RpoS. These results indicate that RpoS plays an important role in the regulation of arginine biosynthesis, particularly when the operon is partially de-repressed as would be the case in starvation conditions.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
GDF	Gene Disruption Fragment
J/cm^2	joules per centimetre squared (fluence)
kDa	kilodalton
µCi	microcurie
µF	microfarad
µg	microgram
µl	microlitre
µM	micromolar
mg	milligram
mM	millimolar
ng	nanogram
OD_{600}	optical density at 600 nm
OD_{420}	optical density at 420 nm
PFU	Plaque Forming Unit
UV	ultraviolet

INTRODUCTION

Physiological Roles of Arginine

Under nutrient-limiting conditions, *Escherichia coli* can utilize arginine as both a carbon and a nitrogen source (McFall & Newman, 1996). It is degraded by the *speA* encoded "biosynthetic" arginine decarboxylase to form agmatine. Agmatine is subsequently processed to yield the polyamine putrescine. Putrescine is a precursor for the biosynthesis of another polyamine spermidine, but in its capacity to serve as carbon source it can be further degraded to succinate via several γ-aminobutyrate intermediates. Succinate is readily metabolized in the tricarboxylic acid cycle to contribute to the cells ATP and carbon requirements. Arginine can also be degraded using the arginine succinyltransferase (AST) pathway (Schneider *et al.*, 1998). The ammonia released (as ammonium ions at physiological pH) during arginine catabolism can be utilized for nitrogen metabolism. In addition, L-glutamate a by- product of this process can also be utilized as a source for nitrogen for the cell (Reitzer, 1996).

Polyamines have a vital role in cell growth. These low-molecular weight polycations are present in all living organisms. Spermidine and spermine are two polyamines that are derived from another highly abundant polyamine putrescine. Arginine is the sole precursor for putrescine biosynthesis, hence it is essential for the biosynthesis of all polyamines that are putrescine-derived. Upon severe polyamine deprivation protein and nucleic acid elongation rates diminish, the fidelity of translation is impaired and chromosome may disintegrate in later

stages of polyamine starvation (Davis *et al.*, 1992). Polyamines ensure proper ribosome structure and assist in ribosome binding to mRNA (Amarantos *et al.*, 2002). They also aid in the binding of polymerases and other transcription factors to nucleic acids during replication and transcriptional processes (Amarantos *et al.*, 2002). In many cases polyamines stabilize interactions between macromolecules rather than occupy specific binding sites. Polyamines are also physiologically important by scavenging reactive oxygen species. In particular, spermidine and spermine have been shown to reduce oxidative damage to proteins and DNA by scavenging singlet molecular oxygen ($^1O_2$*) and other highly charged oxygen species (Khan *et al.*, 1992b; Khan *et al.*, 1992a).

It has been suggested that, in phosphate-starved cells, aerobic metabolism of arginine is an important physiological adaptation that is intimately associated with cell survival (Gerard *et al.*, 1999). Arginine is also physiologically important in acid resistance (Castanie-Cornet *et al.*, 1999). There are three main mechanisms through which *E. coli* protects itself from extreme acid challenge in stationary phase. The first is the oxidative or glucose-repressed acid resistance system, the second and third are arginine and glutamate decarboxylase-dependent systems (Cui *et al.*, 2001). In enteropathogenic *E. coli*, arginine is the substrate for the acid-induced "biodegradative" arginine decarboxylase, encoded by *adiA* (Castanie-Cornet *et al.*, 1999). The influx of protons is neutralized by the decarboxylation of arginine and glutamate to agamtine and γ-aminobutyrate,

respectively. These acid resistance systems cooperatively allow the cell to thrive in harsh acidic conditions.

Interestingly, recent studies on uropathogenic *E. coli* have revealed that arginine is required for establishing renal infections in mice (Russo *et al.*, 1996). The authors suggest this observation may be medically important in the treatment of urinary tract infection in humans.

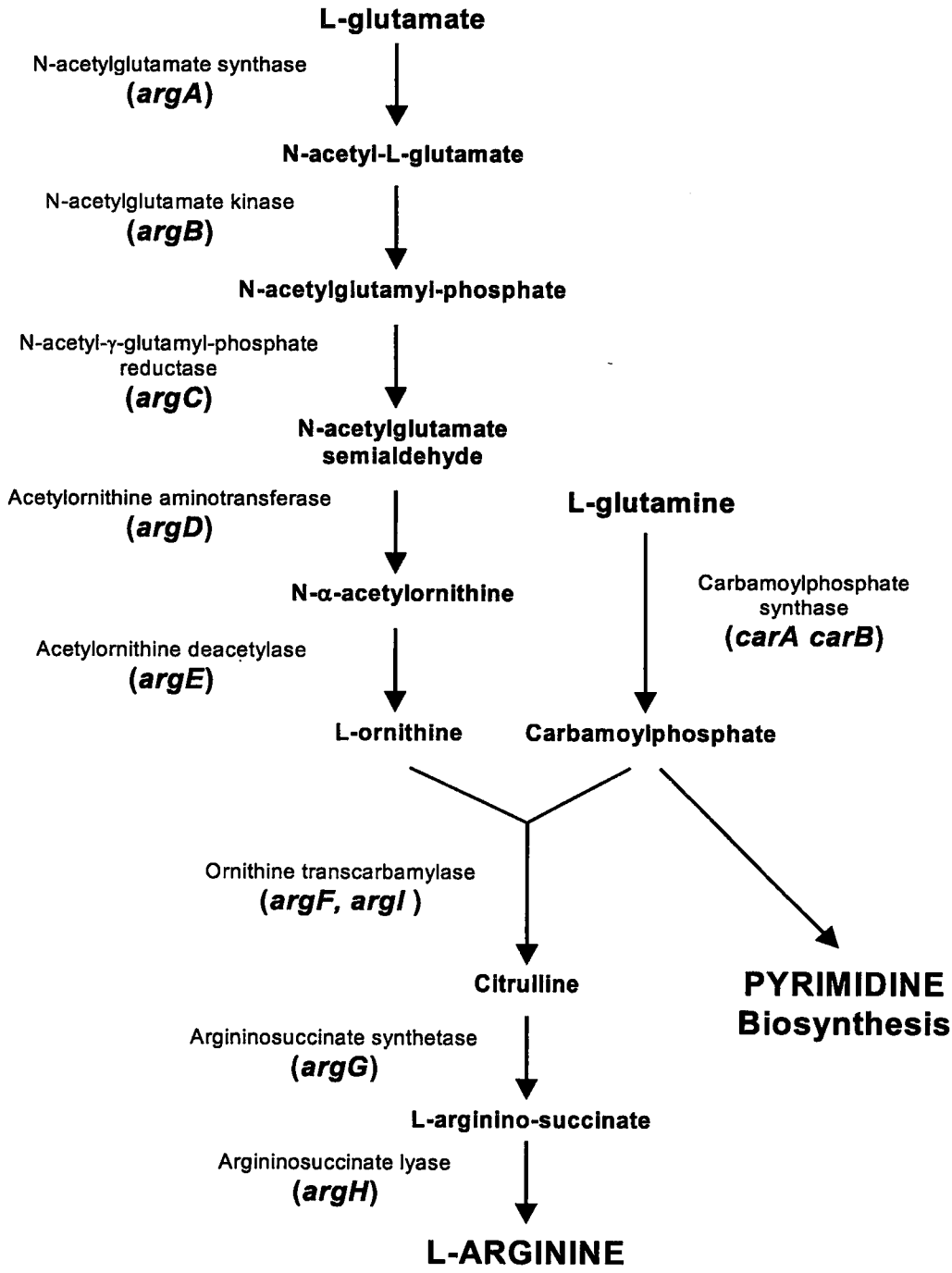
Arginine transport vs. biosynthesis

E. coli can synthesize all 20 essential amino acids. The synthesis of each amino acid is mediated by complex biosynthetic pathways. Each pathway can be regulated and thus the biosynthesis of each amino acid controlled in accordance to the cells' needs. Amino acid biosynthesis is favoured over amino acid transport during periods of starvation. There are two arginine permeases that transport arginine across the periplasm encoded by *argT* and *abpS* (Glansdorff, 1996). A third system encoded by the *artPIQMJ* (Wissenbach *et al.*, 1995) also transports arginine. Regulation of each system is independent of one another and independent of arginine biosynthetic genes. The arginine biosynthetic pathway in *E. coli* K-12 consists of 8 steps, each catalyzed by a unique enzyme (Figure 1). Many of the intermediates in the arginine biosynthetic pathway can be utilized in other tributary metabolic circuits including proline biosynthesis from N-acetlyglutamate-semialdehyde, succinate metabolism from glutamate degradation, pyrimidine biosynthesis from carbamoylphosphate, and polyamine biosynthesis from arginine catabolism.

Figure 1. The arginine biosyhthetic pathway in *E. coli*.

Note that ArgF and ArgI are ornithine transcarbamylase isoenzymes, while CarA and CarB each encode different subunits of carbamoylphosphate synthase.

Adapted from EcoCyc: Encyclopedia of *Escherichia coli* Genes and Metabolism (http://biocyc.org/ecocyc/).


L-glutamate
N-acetylglutamate synthase
(argA)
N-acetyl-L-glutamate
N-acetylglutamate kinase
(argB)
N-acetylglutamyl-phosphate
N-acetyl-γ-glutamyl-phosphate reductase
(argC)
N-acetylglutamate semialdehyde
Acetylornithine aminotransferase
(argD)
L-glutamine
N-α-acetylornithine
Carbamoylphosphate synthase
(carA carB)
Acetylornithine deacetylase
(argE)
L-ornithine
Carbamoylphosphate
Ornithine transcarbamylase
(argF, argI)
Citrulline
PYRIMIDINE Biosynthesis
Argininosuccinate synthetase
(argG)
L-arginino-succinate
Argininosuccinate lyase
(argH)
L-ARGININE

Arginine biosynthesis is regulated by several mechanisms. Accumulation of intracellular arginine results in feedback inhibition of N-acetylglutamate synthase encoded by *argA* (Glansdorff, 1996), the first enzyme in the biosynthetic pathway. The intermediate carbamoylphosphate is common to both arginine and pyrimidine biosynthetic pathways. The carbamoylphosphate synthetase encoded by *carAB* is regulated by elements in both pathways. It is inhibited allosterically by UMP the product of the pyrimidine biosynthetic pathway (Glansdorff, 1996). However, this inhibition is relieved by accumulation of ornithine from the arginine biosynthetic pathway (Glansdorff, 1996). The antagonistic effect of ornithine and UMP ensure balanced distribution of carbamoylphosphate between each metabolic conduit. Aside from regulation by enzyme inhibition, the arginine biosynthesis is tightly regulated at the transcriptional level by the arginine repressor encoded by *argR* (Maas, 1994). The repressor is very sensitive to intracellular arginine concentrations. It represses all its regulon members when arginine is in abundance.

The arginine repressor

The ArgR aporepressor is a hexameric protein with six identical 17 kDa subunits. It is primarily a regulator of arginine biosynthesis, but has a secondary function in site-specific recombination (Summers, 1989). In the presence of exogenous arginine the ArgR repressor interacts with a family of slightly dissimilar operators that constitute a regulon of 9 transcriptional units. For maximum repressor function the aporepressor has to bind to six arginine

molecules which act as corepressors (Kueh *et al.*, 2003; Van Duyne *et al.*, 1996). The magnitude of repressible function is cumulative as each of the 6 binding sites on the aporepessor is occupied by an arginine molecule (Kueh *et al.*, 2003; Van Duyne *et al.*, 1996). Table 1 lists the members of the ArgR regulon with their respective repression coefficients.

The ArgR regulon members have 2 partially conserved 18 bp sequences that display hyphenated symmetry and overlap the promoters to various extents (Tian *et al.*, 1992). These consensus sequences are termed ARG boxes and are separated by 3 bp (Figure 2) (Tian *et al.*, 1992). Stoichiometry of repressor-operator interaction was estimated in *E. coli* by quantitative DNase I footprinting (Charlier *et al.*, 1992) and gel retardation studies with labeled repressor (Tian *et al.*, 1992). It was determined that the repressor binds to 4 consecutive helical turns to only one face of the DNA and that one ArgR repressor molecule binds to both ARG boxes (Tian *et al.*, 1992), suggesting contact with 2 repressor subunits. It has been shown that the activated repressor prevents RNA polymerase binding by steric hindrance, resulting in attenuated expression in some of its members but reduced expression in others. The *argR* gene is autoregulatory (Glansdorff, 1996; Lim *et al.*, 1987). Its expression is under the control of 2 promoters. The first is constitutive, and accounts for a third of the total ArgR protein, while the second, which is slightly downstream carries a pair of ARG boxes separated by 2 bp and is ArgR-regulated (Glansdorff, 1996; Lim *et al.*, 1987). Studies have also estimated the number of repressor molecules per

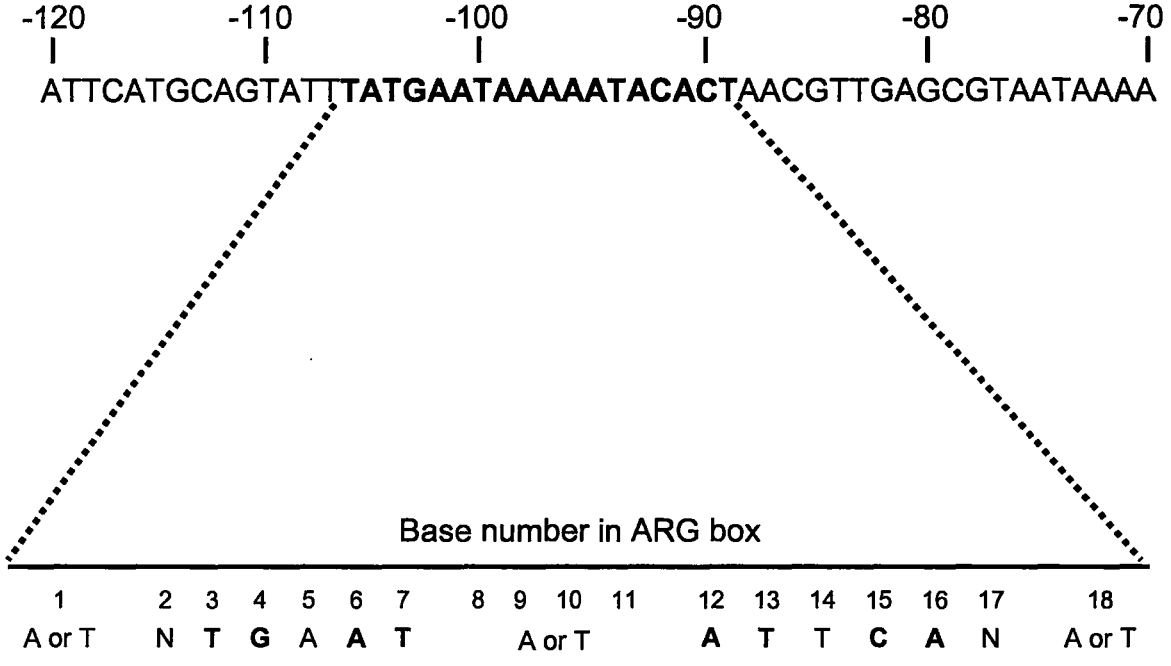
Table 1. Repression response in the arginine regulon of *E. coli* K-12.

*Ratio between specific activities (micromoles per hour per milligram of protein) as assayed in *argR* cells (no arginine added) and in *argR*$^+$ cells (100 µg of arginine added per ml) (Glansdorff, 1996).

Transcriptional unit	Repression coefficient*
argA	50
argCBH	60
argE	60
argD	16
argF	150-200
argI	300-400
argG	ND
argR	15
carAB	50

Figure 2. ARG box consensus sequence derived from an alignment of all the ArgR regulon members.

The residues in bold are highly conserved in all regulon members. An ARG box upstream of the *argCBH* operon is used as an example, base position is relative to the transcriptional start site.


-120
-110
-100
-90
-80
-70
ATTCATGCAGTATTTATGAATAAAAATACACTAACGTTGAGCGTAATAAAA
Base number in ARG box
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A or T N T G A A T A or T A T T C A N A or T

cell is approximately 500-600 in *E. coli* (Tian *et al.*, 1994). Autoregulation brings this number to 300-400 in the presence of excess arginine (Tian *et al.*, 1994).

Surprisingly, apart from regulating arginine biosynthesis, ArgR also functions in *cer*-mediated site-specific recombination to resolve ColE1 plasmid dimers to monomers (Stirling *et al.*, 1988). The naturally occurring plasmid ColE1 which harbours the *cea* confers drug resistance to the bacteriocin, colicin E1. Colicin E1 causes lethal membrane changes in bacteria. ColE1 plasmid dimers that arise from homologous recombination need be resolved to monomers before segregating to daughter cells. Recombinases XerB and XerC catalyse DNA exchange at a family of sites including the *cer* sites (similar to ARG boxes) on ColE1 plasmids (Summers, 1989). ArgR (also referred to as XerA in site specific recombination) acts as a XerB and XerC recombinase recruiter promoting recombination and resolution of ColE1 dimers at *cer* sites (Stirling *et al.*, 1988; Summers, 1989).

The *argCBH* operon

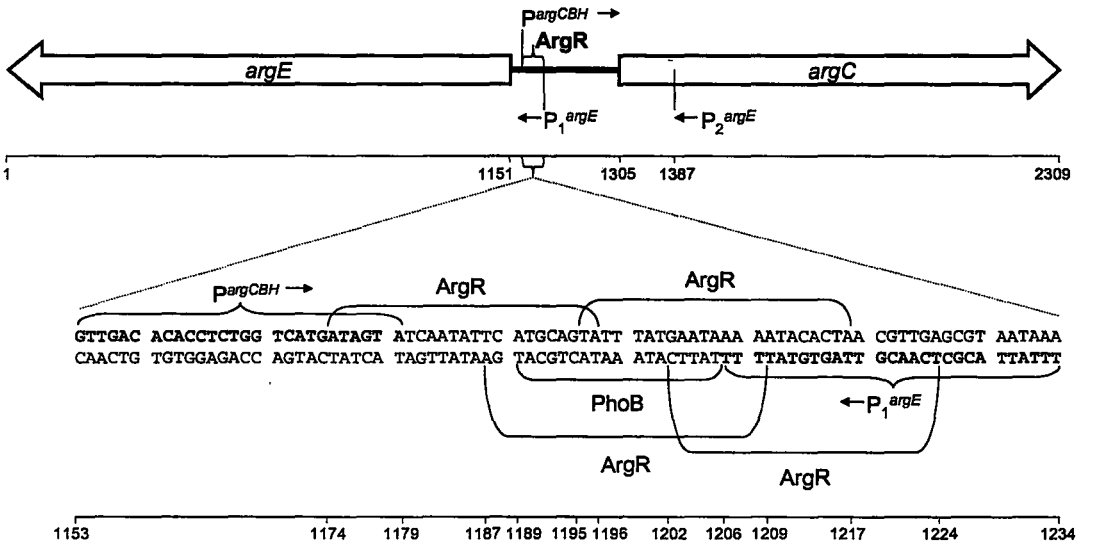
All members of the ArgR regulon are dispersed throughout the *E. coli* genome with the exception of *argC*, *argB*, and *argH*, which are clustered together as a three membered polycistronic operon. A single promoter is thought to drive the expression of this operon (Glansdorff, 1996). It is upstream of *argC* and is under ArgR regulatory control. The organization of this promoter is unique. *argECBH* in *E. coli* is shown to be a divergent operon consisting of 2 arms, *argE* and *argCBH*, with an internal operator region flanked by two convergent

promoters (Figure 3) (Glansdorff, 1996). There are 4 ARG boxes in this internal operator region. One pair is used for *argCBH* ArgR-mediated regulation while the other for *argE*. There also exist a second *argE* promoter insensitive to ArgR repression. It is located upstream of the *argE* transcription start site within the *argC* coding sequence (Cunin *et al.*, 1983; Glansdorff, 1996). A putative PhoB binding site has also been characterized in this region. PhoB is a positive response regulator of phosphate metabolic genes that are part of the *pho* regulon (Rao & Kornberg, 1999). However, a role for PhoB in arginine metabolism has not been shown.

All three members are clearly homologous in other species including *S. typhimurium*, *S. cerevisiae*, *S. pombe*, *B. stearothermophilus*, *C. glutamicum*, and *T. aquaticus* (Glansdorff, 1996). *argB* and *argH* encode proteins N-acetlyglutamokinase and arginosuccinase, respectively. Both proteins have been isolated and their molecular masses have been determined. ArgB is approximately 29 kDa and in its active form shown to be a dimer. The *argH* encoded arginosuccinase, like its mammalian homologue is a collection of 4 subunits, the combined weight of this tetramer is approximately 50 kDa. Interestingly, *argC*, the first member of the operon, is the only member that has been linked to pathogenic *E. coli* virulence. It encodes a 47 kDa N-acetylglutamylphosphate reductase responsible for catalyzing N-acetylglutamylphosphate to N-acetylglutamate semialdehyde. By using *argC-lacZ* fusions it is now known that induction of *argC* is critical for growth in human

Figure 3. An illustration of the *argECBH* operator region.

Curly brackets represent the promoter regions while rounded brackets indicate predicted protein-binding sites. Data was obtained from NCBI (http://www.ncbi.nlm.nih.gov/). Gene lengths are represented in bp.


P argCBH
ArgR
argE
argC
P1 argE
P2 argE
1
1151
1305
1387
2309
P argCBH
ArgR
ArgR
GTTGAC ACACCTCTGG TCATGATAGT ATCAATATTC ATGCAGTATT TATGAATAAA AATACACTAA CGTTGAGCGT AATAAA
CAACTG TGTGGAGACC AGTACTATCA TAGTTATAAG TACGTCATAA ATACTTATTT TTATGTGATT GCAACTCGCA TTATTT
PhoB
P1 argE
ArgR
ArgR
1153
1174
1179
1187 1189 1195 1196
1202
1206 1209
1217
1224
1234

urine (Russo *et al.*, 1996). In addition, previous *in vivo* studies have confirmed significantly diminished urovirulence and reduced capability of uropathogenic *E. coli* to establish urinary tract infections in mouse models (Russo *et al.*, 1996). This operon in conjunction with the other members of the ArgR regulon is co-operatively expressed to biosynthesize arginine. The cell utilizes arginine in many pathways (as indicated earlier) but one in particular is in the biosynthesis of polyamines.

Polyamine biosynthesis and the AST pathway

E. coli can catabolize arginine through two pathways. The first is the arginine decarboxylase (ADC) pathway, and the second is the arginine succinyltransferase (AST) pathway. The primary function of the ADC pathway is to synthesize putrescine when arginine is in abundance (Glansdorff, 1996; Tabor & Tabor, 1985). The initial step requires the *speA* encoded "biosynthetic" arginine decarboxylase to degrade arginine to agmatine. This decarboxylase is a 296 kDa tetramer and is partially located in the periplasm. Agmatine is hydrolyzed by agmatine ureohydrolase encoded by *speB* to putrescine. Putrescine although often considered an intermediate of spermine and spermidine biosynthesis, plays a vital role in bacterial adaptation to growth under hypo-osmotic conditions (Schiller *et al.*, 2000). This diamine is the most abundant polyamine in the cell approximating 34.1 μmol/g of dried cells (Glansdorff, 1996), and when in excess curtails levels of SpeA by negative feedback inhibition (Glansdorff, 1996). Interestingly, both *speA* and *speB* are

organized in a bicistronic operon. Transcription is driven by a promoter upstream of *speA*, but there is a second internal promoter that drives *speB* transcription. Transcription from the second promoter is induced by accumulation of agmatine (Szumanski & Boyle, 1992b). In addition, *speB* unlike *speA* is subject to catabolite repression (Glansdorff, 1996; Shaibe *et al.*, 1985), but this repression can be overridden by the nitrogen-regulated (Ntr) response, an adaptation brought about by conditions of nitrogen limitation (Szumanski & Boyle, 1990). It is also known that mutations in both genes result in poor growth when arginine is supplied as a source of nitrogen (Glansdorff, 1996; Shaibe *et al.*, 1985). All enzymes that degrade agmatine to succinate are Ntr-regulated (Reitzer, 1996). *speAB* is also partially negatively regulated by cyclic AMP mediated by the cyclic AMP receptor protein (Szumanski & Boyle, 1992a). Accumulation of polyamines is deleterious to the cell (Davis *et al.*, 1992). Any excess is excreted via polyamine transporters encoded by two polycistronic operons, *potABCD* and *potFGHIJ*. The *potABCD* transporter binds to both putrescine and spermidine, but preferentially transports the later (Kashiwagi *et al.*, 1996), while the *potFGHIJ* specifically binds putrescine (Pistocchi *et al.*, 1993; Vassylyev *et al.*, 1998). These transporters in conjunction with a third putrescine/ornithine antiporter encoded by *potE* (Kashiwagi *et al.*, 1992; Kashiwagi *et al.*, 2000) largely control the internal concentrations of polyamines.

The AST pathway carries out most arginine degradation in *E. coli*. Its primary role is to produce ammonia to be utilized as a source of nitrogen for the

cell. *E. coli* does not utilize arginine directly as a carbon source (Schneider *et al.*, 1998; Goux *et al.*, 1995). Recently, the ArgR regulator, which has been well characterized as a repressor of arginine biosynthetic genes, has been shown to enhance expression of the *astCADBE* operon (Kiupakis & Reitzer, 2002). This operon encodes arginine degradation enzymes in the AST pathway (Kiupakis & Reitzer, 2002). Transcription is induced under conditions of nitrogen limitation, this response is mediated by the sigma factor RpoN (σ^N) and the nitrogen regulator NtrC. The operon is moderately expressed during general nitrogen limitation and maximally expressed in the presence of arginine (Kiupakis & Reitzer, 2002). There is also a second RpoS (σ^s) dependent promoter, confirmed by primer extension analysis, that upon entry into stationary phase induces *astCADBE* expression (Kiupakis & Reitzer, 2002). ArgR appears to enhance expression from both the RpoN- and RpoS-dependent promoters (Kiupakis & Reitzer, 2002).

The alternative vegetative σ^s factor

Under conditions of nutrient abundance *E. coli* proliferates rapidly, a stage of growth termed exponential phase. Upon exhaustion of a particular nutrient, specific genetic programs are activated to compensate for the nutrient loss. These programs may include induction of a biosynthetic pathway that intracellularlly synthesizes the limiting nutrient or induction of transport systems that scavenges alternative nutrient sources (Hengge-Aronis, 1996). When these systems fail a decrease in cell division is observed, to the point where there is no

net increase in cell density. This condition is termed stationary phase. The physiology and metabolism of cells in each growth phase are very different. For instance, *E. coli* cells under go a change in morphology from a rod shape in exponential phase to a more spherical shape in stationary phase (Lange & Hengge-Aronis, 1991). They also tend to aggregate to one another, a phenomenon not observed in exponentially growing cells (Lange & Hengge-Aronis, 1991). The lack of nutrients and accumulation of excreted fermentative by products like acetate compromise the cells ability to survive in stationary phase (Arnold *et al.*, 2001). Many of the morphological changes and physiological changes are adaptation responses to environmental stresses. Some of these environmental stresses are not exclusive to nutrient starvation but also include extreme temperature, acidity, salinity, and oxidative stress (Hengge-Aronis, 1993). These responses are tightly regulated at the genetic level by various sigma factors. There are 6 sigma factors in *E. coli*, each associates with RNA polymerase to mediate transcription of their respective regulons under specific environmental conditions. The most abundant is RpoD (σ^{70}) which mediates transcription of "house-keeping" genes in exponential phase. The other 5 sigma factors control transcription of a set regulon, each facilitates adaptation to specific stresses. One in particular is the alternative vegetative sigma factor RpoS (σ^{S}).

RpoS is a global regulator facilitating the transcription of more than 100 genes (Ishihama, 2000). Many of the members of this large regulon are

expressed during the transition to stationary phase growth and under various stress-related conditions (Hengge-Aronis, 1996; Loewen & Hengge-Aronis, 1994). Mutations in *rpoS* diminish resistance to oxidative stress (Dukan & Touati, 1996; Loewen & Hengge-Aronis, 1994), acid challenge (Levinthal & Pownder, 1996), starvation survival (McCann *et al.*, 1991), and tolerance to high temperatures (Suh *et al.*, 1999).

The concentration of RpoS in the cell is tightly controlled at the level transcription, post-transcription, and translation. At the level of transcription *rpoS* is regulated by CRP, guanosine 3', 5'-bispyrophosphate (ppGpp), and polyphosphates. CRP in the presence of cAMP is believed to bind to the *rpoS* promotor and repress transcription (Lange & Hengge-Aronis, 1994). In contrast, ppGpp and polyphosphates are activators of *rpoS* transcription. Cells unable to synthesize ppGpp (Hirsch & Elliott, 2002) or polyphosphates (Kornberg *et al.*, 1999; Shiba *et al.*, 1997; Shiba *et al.*, 2000) have been shown to have lower *rpoS* expression and reduced levels of RpoS-dependent proteins, suggesting both are positive regulators of *rpoS* transcription.

There are many proteins at the post-transcriptional level that regulate *rpoS* translation. Two in particluar are H-NS and Hfq. H-NS is a nucleoid histone-like protein encode by the *hns* locus. Mutations in *hns* increased RpoS protein, this suggests H-NS represses translation of RpoS mRNA perhaps by sterically hindering ribosomal binding to the transcript (Yamashino *et al.*, 1995). On the contrary, Hfq has been shown to be a positive regulator of RpoS translation

(Muffler *et al.*, 1996; Muffler *et al.*, 1997). It has been proposed that Hfq, a RNA-binding protein facilitates ribosomal binding to the RpoS transcript, by regulating a small RNA, DsrA (Sledjeski *et al.*, 2001). DsrA has regions of complementarity to the RpoS transcript, and once bound positively regulates RpoS translation (Lease *et al.*, 1998).

In addition, some small untranslated RNA species have also been suggested to regulate RpoS translation by complementary binding to the RpoS mRNA. One of particular interest is DsrA, which has regions of complementarity to RpoS mRNA (Repoila *et al.*, 2003; Sledjeski *et al.*, 1996). Its interaction with RpoS mRNA enhances translation possibly by freeing the translation initiation codon (Majdalani *et al.*, 1998) and facilitating ribosomal binding. Interestingly DsrA also has regions of complementarity to other mRNA species including H-NS and ArgR (Lease *et al.*, 1998). H-NS has been shown to repress RpoS translation, but this repression is alleviated by the DsrA-H-NS mRNA interaction which blocks translation of H-NS mRNA (Lease *et al.*, 1998; Sledjeski *et al.*, 1996). This suggests that DsrA may play a role in *rpoS* translation by both directly interacting with RpoS mRNA and indirectly through *hns*. Though sequences of complementarity of DsrA exist to ArgR (Lease *et al.*, 1998), no experimental evidence has been shown to prove a regulatory role of DsrA on ArgR translation.

Objective of this study

In addition to ArgR, other regulators may be important for control of arginine metabolism. Several enzymes required for arginine catabolism are under RpoS control including those encoded by *adiA* (Castanie-Cornet *et al.*, 1999), *astD* (Baca-DeLancey *et al.*, 1999), and *cstC* (*astC*) (Fraley *et al.*, 1998).

In a previous study (Schellhorn *et al.*, 1998) we screened for RpoS-dependent genes by transduced a *rpoS* null mutation into a library of *rpoS*$^+$ random *lacZ*-fusions to the *E. coli* genome (Schellhorn *et al.*, 1998). From this genetic screen, two fusions that were found to be RpoS-dependent mapped to the *argCBH* operon (Schellhorn *et al.*, 1998). In this study, we will further investigate this preliminary observation using reporter gene assays and Northern blot analyses. We hypothesize that in addition to ArgR, the *argCBH* operon is induced in stationary phase and is partly controlled by the alternative sigma factor, RpoS.

METHODS

Bacterial strains, phage, and plasmids

All strains used are *E. coli* K-12 derivatives. The bacterial strains, phage and plasmids used in this study are listed in Table 2.

Media, chemicals and other reagents

Unless otherwise stated all chemicals were supplied by either Sigma Chemical Co., St. Louis, Mo, USA or Gibco BRL, Burlington, ON, Canada. Cultures were routinely grown in Luria-Bertani (LB) rich broth and in M9 minimal media (Miller, 1992). The concentrations of antibiotics used were as follows: 100 μg ampicillin ml^{-1}, 25 μg chloramphenicol ml^{-1}, 50 μg kanamycin ml^{-1}, 15 μg tetracycline ml^{-1}, and 100 μg streptomycin ml^{-1}. Expression studies were conducted using 50 μg X-Gal ml^{-1} (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and ONPG (o-nitrophenyl-β-D-galactopyranoside) obtained from Diagnostic Chemicals, PE, Canada and Sigma Chemical Co., St. Louis, Mo, USA, respectively.

Growth conditions

All cultures were grown in triplicate from independently isolated colonies. Cell growth was monitored spectrophotometrically (Novaspec® II spectrophotometer; Pharmacia LKB Biochrom Ltd., Cambridge, England) by measuring optical density at 600 nm (OD_{600}). Expression studies in rich media were conducted using cultures that had been maintained in early exponential

Table 2. *E. coli* strains, bacteriophages, and plasmids used in this study.

Strains	Genotype	Source/Reference
A) Strains		
MG1655	Prototrophic *E. coli* K-12, $F^- \lambda^-$ *rph*	CSGC
GC4468	*ΔlacU169 rpsL*	(Schellhorn and Stones, 1992)
GC122	as GC4468 but *rpoS13*::Tn*10*	(Schellhorn and Stones, 1992)
HS1066	as GC4468 but *argH* $^+$-λp*lac*Mu53[Φ(*argH-lacZ*)66](rsd1066)	(Schellhorn *et al.*, 1998)
HS1066p	as HS1066 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS1072	as GC4468 but *argH* $^-$-λp*lac*Mu53[Φ(*argH-lacZ*)72](rsd1072)	(Schellhorn *et al.*, 1998)
HS1072p	as HS1072 but *rpoS13*::Tn*10*	(Schellhorn *et al.*, 1998)
HS2401	as MG1655 but *ΔargR*::cat	This study
HS2402	as GC4468 but *ΔargR*::cat	P1(HS2401)xGC4468→cm^R
HS2403	as GC122 but *ΔargR*::cat	P1(HS2401)xGC122→cm^R
HS2404	as HS1066 but *ΔargR*::cat	P1(HS2401)xHS1066→cm^R
HS2405	as HS1066p but *ΔargR*::cat	P1(HS2401)xHS1066p→cm^R
HS2406	as HS1072 but *ΔargR*::cat	P1(HS2401)xHS1072→cm^R
HS2407	as HS1072p but *ΔargR*::cat	P1(HS2401)xHS1072p→cm^R
B) Phage		
P1*vir*	generalized transducing phage	Laboratory stock
C) Plasmids		
pKD3	Template plasmid for gene disruption, *cat* is flanked by FRT sites. 2804 basepairs	(Datsenko and Wanner, 2000)
pKD46	λ Red recombinase expression plasmid under control of an *araC*-P_{araB} inducible promotor. 6329 basepairs	(Datsenko and Wanner, 2000)

phase (OD_{600} of < 0.3) in antibiotic-free LB media for at least 8 generations, prior to the start of the experiment. Sub-cultures, with a starting OD_{600} of 0.01, were grown in LB in a 1:5 culture to flask volume ratio at 37°C and agitated at 200 rpm. Samples were collected periodically and assayed for β-galactosidase activity.

To determine the RpoS dependence of *argCBH* expression in minimal media as a function of exogenous arginine concentration, overnight liquid minimal media cultures (0.4% glucose) were inoculated with well-isolated colonies from solid minimal media (0.2% glucose) and grown with 40 μg L-arginine ml^{-1} and appropriate antibiotics. Overnight cultures were diluted (1 in 1000) and maintained in early exponential phase (as described above) before the start of the experiment in antibiotic-free minimal media supplemented with the 40 μg L-arginine ml^{-1}. At an OD_{600} of 0.3, cultures were placed on ice for 2 min and then centrifuged for 10 min at 4000 x g at room temperature. The supernatant was decanted and the resulting cell pellets washed twice with arginine-free minimal media to remove all residual exogenous arginine. The arginine-free cell pellets were re-suspended in minimal media to the same optical density as collected. A series of sub-cultures with a starting OD_{600} of 0.05 were made into minimal media supplemented with increasing concentrations of exogenous L-arginine. The sub-cultures were incubated at 37°C and agitated at 200 rpm. For each concentration of L-arginine, samples were taken in mid exponential phase (OD_{600} of 0.3) and assayed for β-galactosidase activity.

Enzyme Assays

β-galactosidase activity was assayed as described by (Miller, 1992). ONPG was used as the substrate, and activity was expressed in Miller units (Miller, 1992). All assays were performed in duplicate with 50 µl sample volumes.

Construction of *ΔargR* mutants

The one-step chromosomal gene inactivation procedure of Datsenko and Wanner (2000) was used to produce a MG1655 *ΔargR* derivative. Using $P1_{vir}$ (Miller, 1992), the mutation was transduced into the other experimental strains (Table 2).

The PCR fragment used for gene inactivation carries the sequence for chloramphenicol resistance (Cm^r) was amplified using the pKD3 plasmid as a template. Primers were designed such that the resulting PCR product includes Cm^r flanked by sequences homologous to the *argR* reading frame in MG1655 chromosome. The PCR primers used were:

forward

5' **CAATAATGTTGTATCAACCACCATATCGGGTGACTT**GTGTAGGCTGAAGCTGCTTC 3'

and

reverse

5'**ACATTTTCCCCGCCGTCAGAAACGACGGGGCAGAGA**CATATGAATATCCTCCTTAG 3' .

The bold text corresponds to *argR* flanking sequences, and normal text represents DNA sequences of the Cm^r cassette.

The PCR amplified Cm^r cassette was transformed into a MG1655 recipient strain carrying pKD46 (which carries the λ Red system for recombination (Datsenko & Wanner, 2000)) by electroporation using a BioRad Gene Pulser II ® (BioRad Laboratories) according to manufacturer's instructions. Electrocompetent MG1655 pKD46 cells were prepared using cultures grown in liquid LB media with appropriate antibiotics and 20 mM L-arabinose at 30°C to induce the λ Red system encoded on pKD46. Following electroporation, transformants that had replaced *argR* with Cm^r were selected on LB agar containing chloramphenicol at 37°C, which cures cells of the temperature sensitive pKD46. Incorporation of Cm^r was confirmed by PCR using genomic DNA as a template, and confirmational primers. The confirmational primers used were the forward primer as above and a reverse primer complementary to a region 100 bp downstream of *argR*: 5' TGTCGCAGTAAAACGCACTA 3'. All primers were synthesized by MOBIX. The *ΔargR* mutation was transduced into the remaining experimental strains by $P1_{vir}$ mediated transduction (Miller, 1992).

RNA Isolation and Northern Analyses

RNA was isolated from cultures grown in LB using the hot phenol method (Kohrer & Domdey, 1991). Primers to the *argH* gene (5'-CGGTTCAAACAATTCAACGA-3' and 5'-GCAGCTTTTTGCCTAACTGG-3') were used to PCR-amplify a DNA probe for hybridization studies to examine stationary phase and RpoS dependence of *argH* expression. RNA samples were prepared, separated by electrophoresis and hybridized as previously described (Schellhorn

et al., 1998). Probes were prepared and radioactively labeled as described in (Sambrook & Russel, 2001). Densitometry analysis of the bands was performed using ImageQuant v 5.2 (Molecular Dynamics).

RESULTS

RpoS dependence of *argCBH* expression

In a previous study we isolated over 100 unique RpoS dependent (rsd) operon-*lacZ* fusions (Schellhorn *et al.*, 1998). Many of these were newly identified members of the RpoS regulon. We identified the sites of *lacZ* insertion by sequencing the DNA (Roy *et al.*, 1995) and found that two of the isolated fusion mutations mapped to the *argCBH* operon (Figure 4). While both fusions were in *argH*, one was intragenic and, as expected, rendered the cell auxotrophic for arginine (rsd1072) and the other fusion (rsd1066) was located between the *argH* coding sequence and a predicted transcriptional terminator (Figure 4). Strains carrying the rsd1066 fusion were not arginine auxotrophs (data not shown). Expression of the rsd1072 and rsd1066 fusions was similar on rich media and was clearly RpoS dependent (Figure 5, only HS1066 is shown). Since ArgR is a known regulator of *argCBH* (Maas, 1994) we constructed combinatorial *argR* and *rpoS* mutants to determine the relative contributions of each regulator to the expression of the operon. Introduction of a *ΔargR* mutation into HS1066 resulted in high constitutive levels of expression in both *ΔargR* and *ΔargR rpoS* mutants (Figure 5). We hypothesized that RpoS-dependent expression of *argCBH* in the *ΔargR* mutant may be masked by strong de-repression as a consequence of loss of ArgR.

Figure 4. Location of operon fusions in strains used in this study.

Arrows indicate the direction of transcription of genes.

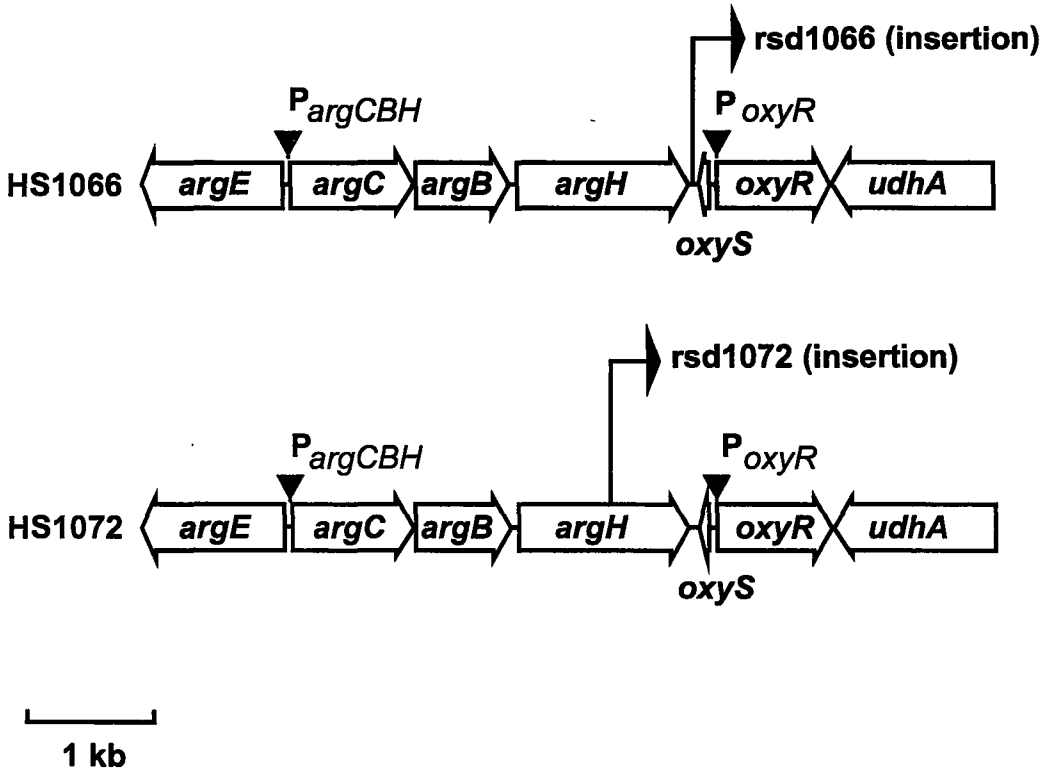
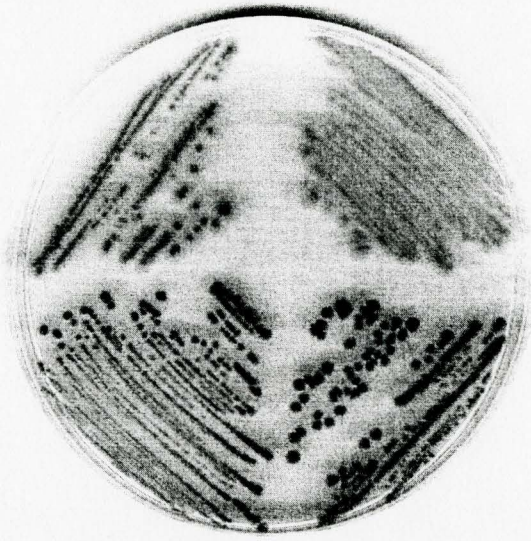

Strain
rsd1066 (insertion)
P argCBH
P oxyR
HS1066
argE
argC
argB
argH
oxyR
udhA
oxyS
rsd1072 (insertion)
P argCBH
P oxyR
HS1072
argE
argC
argB
argH
oxyR
udhA
oxyS
1 kb

Figure 5. Expression of *argCBH-lacZ* in WT, *rpoS*, *ΔargR* and *rpoSΔargR* strains on LB plates containing X-Gal.

WT *rpoS*



ΔargR *rpoS ΔargR*

argCBH expression in rich media

Upon entry into stationary phase, expression increased 5-fold in wild type, but was substantially reduced in the *rpoS* mutant (Figure 6a and 6b). This indicated that *argCBH* expression is not only growth phase-dependent but is also 2-fold RpoS dependent. Regardless of *rpoS* status, *argCBH* expression was much higher in *ΔargR* mutants than in wild type strains (Figure 6a and 7a, 6b and 7b).

Effect of exogenous arginine on *argCBH* expression

RpoS is important for amino acid scavenging in stationary phase (Zinser & Kolter, 1999). Amino acid biosynthesis offers an alternative to the scavenging strategy. It is possible that *argCBH* expression is more dependent on RpoS in stationary phase when arginine is limiting. To test this, expression of the operon in minimal media exogenously supplemented with various concentrations of L-arginine was quantified. In strain HS1066 and its *rpoS* mutant derivative, HS1066p, *argCBH* expression was inversely proportional to arginine concentration (Figure 8a). Normally, in response to arginine limitation, arginine biosynthetic genes including *argCBH* are up regulated (Williams & Rogers, 1987). In exponential phase cultures (Figure 8a), *argCBH* expression was RpoS dependent only at low arginine concentrations. In stationary phase, *argCBH* expression was 2-fold RpoS-dependent at all arginine concentrations (Figure 8b).

Figure 6. Expression of *argCBH* under rich media conditions.

Expression in isogenic wild-type (a) and *rpoS* mutant (b) strains were assessed. Growth (○) and β-galactosidase activity (●) were assayed as described in Methods. O/N, overnight.

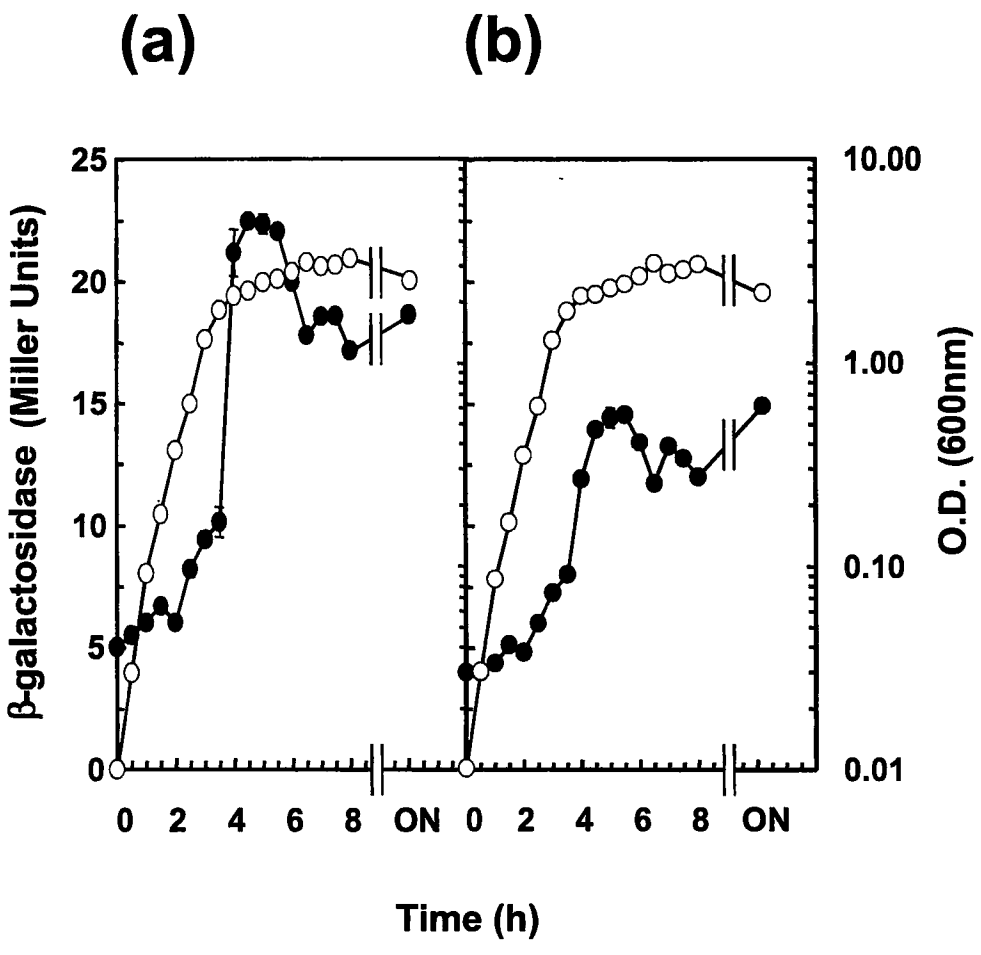

(a)
(b)
β-galactosidase (Miller Units)
O.D. (600nm)
25
20
15
10
5
0
10.00
1.00
0.10
0.01
0 2 4 6 8 ON 0 2 4 6 8 ON
Time (h)

Figure 7. *argCBH* expression in *ΔargR* mutants.

Growth (○) was monitored by measuring optical density at OD_{600}. Expression of isogenic *ΔargR* **(a)** and *ΔargRrpoS* **(b)** mutants were monitored by assaying β-galactosidase activity (●). O/N, overnight.

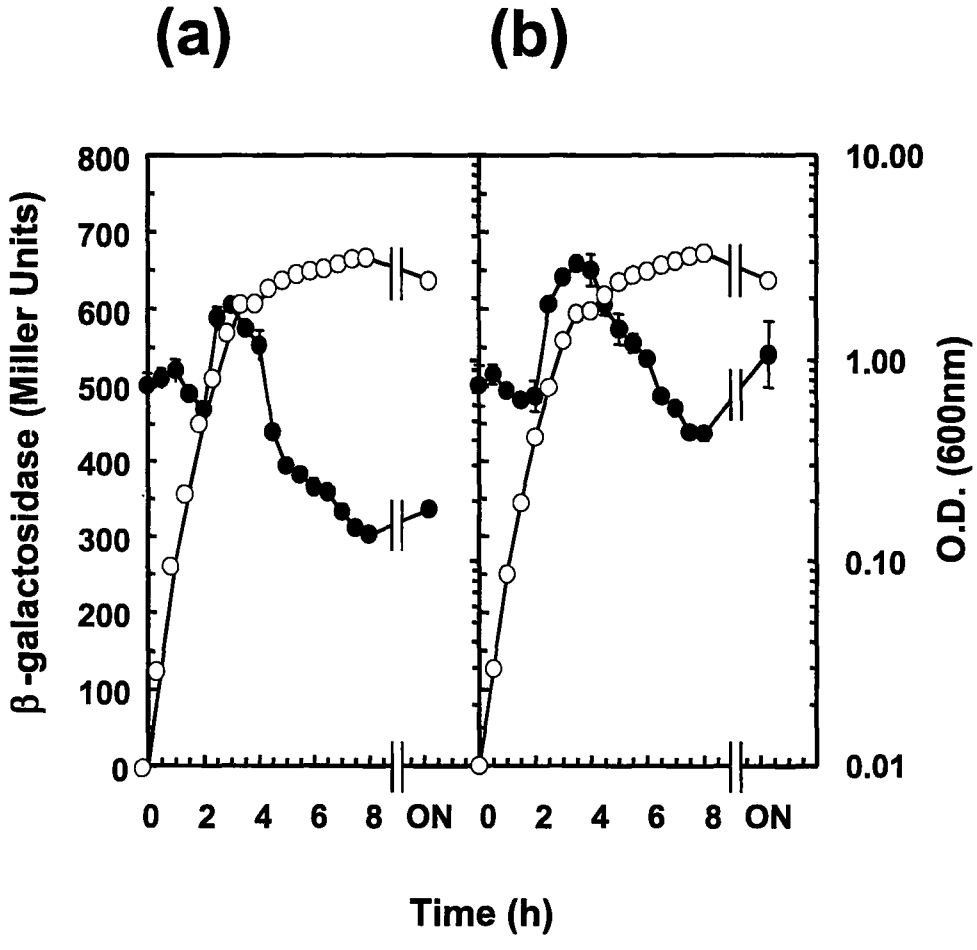
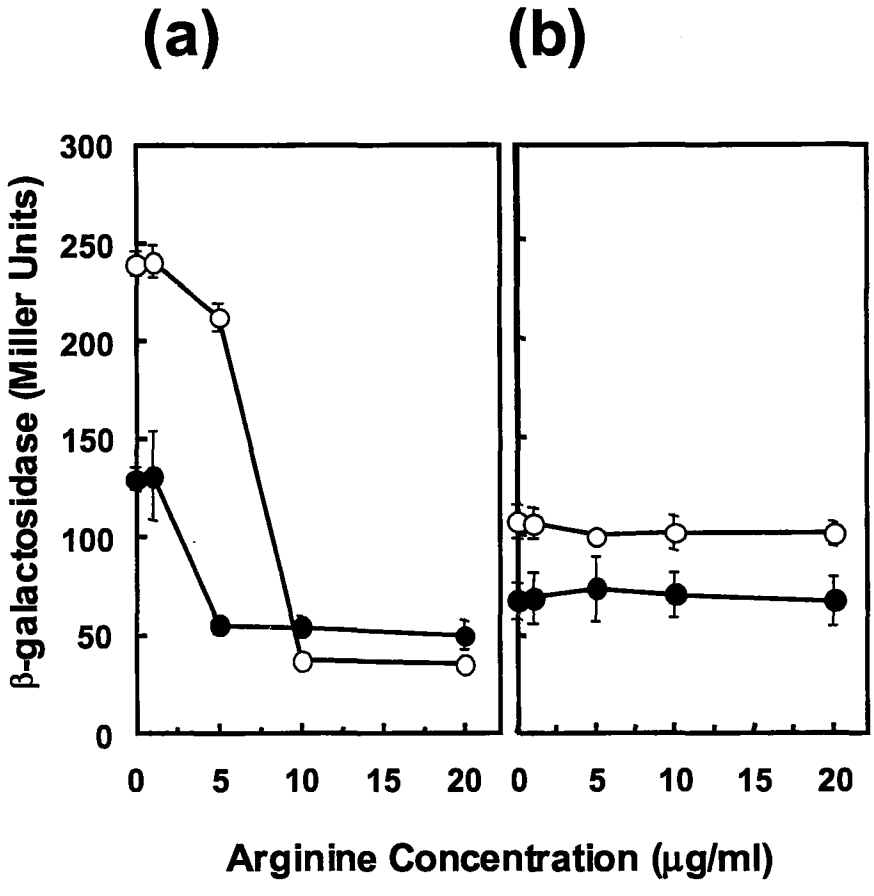

(a)
(b)
β-galactosidase (Miller Units)
800
700
600
500
400
300
200
100
0
10.00
1.00
0.10
0.01
O.D. (600nm)
0 2 4 6 8 ON 0 2 4 6 8 ON
Time (h)

Figure 8. Expression of *argCBH* in isogenic wild type and *rpoS* mutant strains in glucose minimal media supplemented with exogenous arginine.

Expression was monitored in wild type (○) and *rpoS* mutant (●) at **(a)** exponential phase (OD_{600} = 0.3) and **(b)** stationary phase (OD_{600} = 1.5).


(a)
(b)
β-galactosidase (Miller Units)
300
250
200
150
100
50
0
0
5
10
15
20
0
5
10
15
20
Arginine Concentration (μg/ml)

Surprisingly, we found that *ΔargR* mutants were unable to grow on minimal media even when supplemented with arginine indicating that the strains had acquired an uncharacterized nutritional requirement. Many of the intermediates in the arginine biosynthetic pathway are precursors for other pathways. For instance, carbamoylphosphate, the product of carbamoylphosphate synthetase (encoded by the bi-cistronic operon *carAB*) is not only a precursor of arginine but also pyrimidine biosynthesis (Glansdorff, 1996). It is likely that the uncontrolled de-repression of arginine biosynthesis depletes precursors required for synthesis of pyrimidines, rendering *ΔargR* mutants auxotrophic for pyrimidines. If this is the case, addition of pyrimidines should restore growth in minimal media. We examined the growth of *ΔargR* mutants on minimal media supplemented with pyrimidines relative to $argR^+$ strains. As shown in Table 3, the growth deficiency of *ΔargR* mutants could be completely suppressed by the addition of pyrimidines.

The effect of exogenous arginine supplementation on growth

Since strains carrying a mutation in *argH* are auxotrophic for arginine (HS1072 this study), we could use these strains to determine the concentration at which this amino acid becomes growth limiting. In addition, because the mutation in strain HS1066 does not confer auxotrophy but is ArgR dependent, this strain could be used to quantify the ArgR-dependent activation of the operon in response to exogenous arginine limitation. Repression of *argCBH* expression was maximal at concentrations greater than 10 μg arginine ml^{-1} (Figure 9).

Table 3. Effect of exogenous supplemented pyrimidines on *ΔargR* mutants. Overnight cultures were prepared in minimal media with exogenously supplemented 40 μg L-arginine ml^{-1} (to ensure repression of arginine biosynthesis). Cultures were washed in arginine-free minimal media and replica-plated onto minimal plates supplemented with exogenous cytosine (C), thymine (T) and uracil (U). Growth was recorded after overnight incubation at 37°C over a concentration range for each pyrimidine (0 to 20 μg ml^{-1}).

Media	*argR*$^+$		*ΔargR*	
	rpoS$^+$	*rpoS*$^-$	*rpoS*$^+$	*rpoS*$^-$
LB	+	+	+	+
M9 + Glucose	+	+	–	–
M9 + Glucose + C, T, or U	+	+	+	+

Coincidently, this is also the concentration above which growth rate was highest (Figure 9).

Northern analysis of *argH* expression

Although plate expression assays indicate that *argH* expression is clearly RpoS-dependent (Figure 5), the results of reporter gene expression studies in liquid culture showed that RpoS dependence was only 2-fold which is somewhat less than that found for most RpoS regulated genes (Schellhorn *et al.*, 1998). To further examine this (and eliminate the possibility that some artifact associated with the use of *lacZ* fusions may have affected results), we independently measured expression of *argH* by Northern analysis in wild type and *rpoS* mutant backgrounds in exponential and stationary phases (Figure 10). Expression of *argH* was 7-fold higher (determined by densitometry) in stationary phase cultures of the wild type strain than in the GC122 *rpoS* mutant and was much lower in exponential phase samples of both cultures. The hybridizing *argH* transcript co-migrated with the 16S RNA leading to some interference with hybridization (Figure 10). These results in conjunction with the reporter fusion studies support the finding that expression of *argH* is both RpoS and stationary phase dependent in a wild type strain which is typical of many RpoS-dependent genes (Schellhorn *et al.*, 1998).

Figure 9. Effect of exogenous arginine on growth and *argCBH* expression. Growth rate (○) and *argCBH* expression (●) were determined using an arginine auxotrophic strain, HS1072 (○) and an arginine prototrophic strain HS1066 (●), respectively, at the indicated arginine concentrations. Overnight cultures were grown with appropriate antibiotics, sub-cultured into minimal media and maintained in exponential phase for at least 8 generations prior to the start of the experiment.

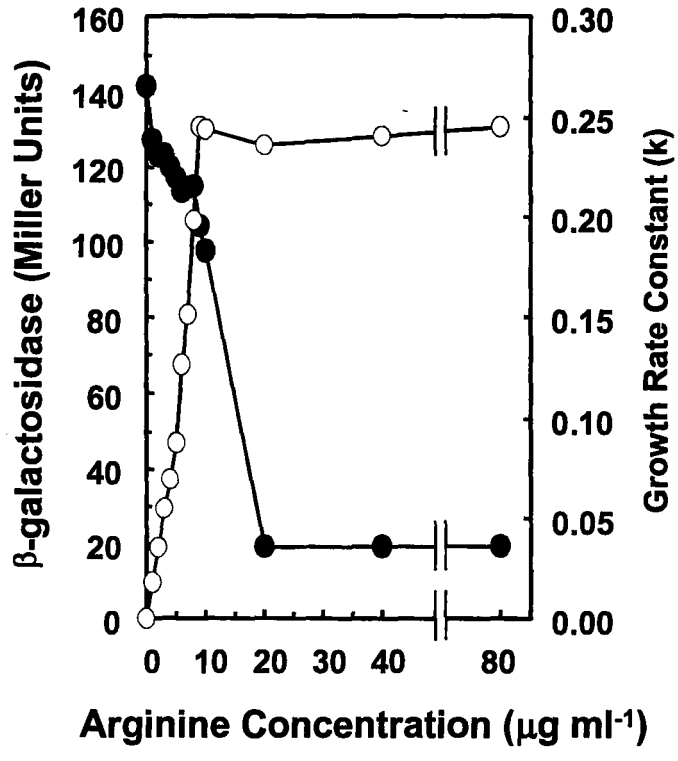
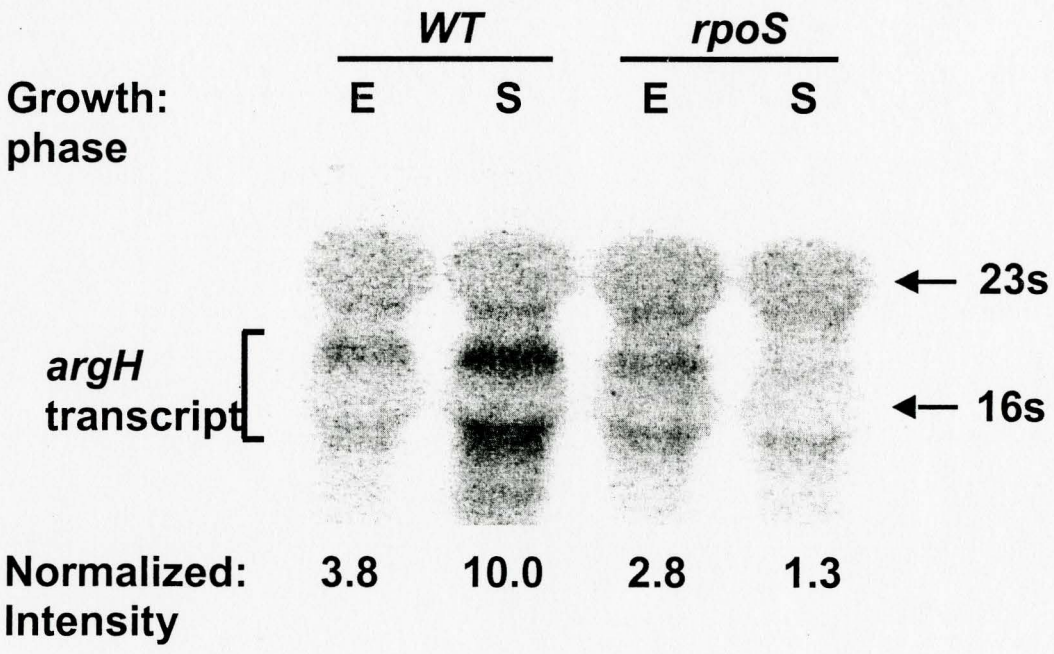

160
140
120
100
80
60
40
20
0
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 10 20 30 40 80
β-galactosidase (Miller Units)
Growth Rate Constant (k)
Arginine Concentration (μg ml-1)

Figure 10. RpoS dependent expression of *argH* in exponential and stationary phase determined by Northern analysis.

RNA was extracted from cultures grown in LB to OD_{600} = 0.3 for exponential phase (E) and OD_{600} = 1.5 for stationary phase (S) using the hot phenol method (as described in Methods). 5 μg of RNA was loaded in each lane. Signal intensity was determined by densitometry and normalized to 10 (arbitrary units).


WT
rpoS
Growth: phase
E
S
E
S
argH transcript
23s
16s
Normalized: Intensity
3.8
10.0
2.8
1.3

DISCUSSION

In this study, we examined the role of RpoS on the expression of *argCBH* in *Escherichia coli*. Using two mutants carrying operon fusions in different positions within the *argCBH* region, we conducted expression studies of this biosynthetic operon by assaying reporter gene fusions and by examining *argH* transcript levels in both a wild type and an *rpoS* mutant by Northern analysis. Since ArgR also regulates this operon (Maas, 1994), we evaluated the relative contributions of both ArgR and RpoS to its expression by constructing appropriate double and single null mutants.

RpoS regulates many genes that play important roles in stress resistance and energy metabolism (Ishihama, 2000), but a subset of these RpoS-dependent genes such as *gabP* (Schellhorn *et al.*, 1998), *proP* (Mellies *et al.*, 1995), *proU* (Manna & Gowrishankar, 1994), *gadAB* (De Biase *et al.*, 1999) and *ldcC* (Kikuchi *et al.*, 1998) aid in amino acid transport and utilization. In a previous genetic screen for RpoS-dependent genes (Schellhorn *et al.*, 1998), we identified one mutant that was auxotrophic for arginine and carried a mutation that mapped to the terminal gene member of the *argCBH* operon. To the best of our knowledge, this is the only RpoS-dependent gene identified to date which, when mutated, renders the cell auxotrophic. As such, examining the regulation of this operon may offer a unique perspective on RpoS-controlled stationary phase metabolism.

Why might arginine biosynthesis be dependent on RpoS in stationary phase? It is known that nutrient scavenging is an important survival strategy of

subpopulations in starved cultures (Ferenci, 1996). Arginine in particular is likely to be an important metabolite in stationary phase cultures for several reasons. As arginine represents 11 percent of the cells total nitrogen (Kiupakis & Reitzer, 2002), this amino acid is probably an important nitrogen reservoir for starving populations. Arginine is also a precursor for the biosynthesis of polyamines which stabilize and condense DNA during senescence (Davis *et al.*, 1992) and protect it against oxidative damage (Ha *et al.*, 1998; Khan *et al.*, 1992b; Khan *et al.*, 1992a). Finally, *de novo* protein synthesis in non-growing stationary phase cells is required for the expression of stationary phase adaptive proteins. This may impose a significant biosynthetic demand upon the cell both because there are many such proteins produced (see Ishihama, 2000 for review) and because some of these are expressed in extraordinarily high levels. For example, Dps, a highly RpoS-dependent DNA binding protein (Altuvia *et al.*, 1994), is almost undetectable in exponential phase, but accumulates to 200,000 molecules per cell in stationary phase (approx. 5 % of total cellular protein) (Martinez & Kolter, 1997). Many genes are induced upon entry into stationary phase and it is likely that this creates a high demand for amino acids for *de novo* protein synthesis. The up-regulation of amino acid biosynthetic operons such as *argCBH* may provide a means to satisfy this demand.

Biosynthetic regulons, particularly those required for amino acid biosynthesis, are often controlled by a transcriptional repressor (e.g TrpR and ArgR). In this study, we found that the *argCBH* operon is controlled by RpoS in

stationary phase cultures but the dynamic range of this control was small in relation to that exerted by ArgR. Interestingly, another member of the ArgR regulon are RpoS-dependent to a similar degree including *cstC*, a member of the *astCADBE* operon (Baca-DeLancey *et al.*, 1999). These observations in conjunction with this study, suggest that RpoS plays an important role in coordinately regulating arginine metabolism in stationary phase.

ArgA-mediated synthesis of N-acetylglutamate is the first committed step in the arginine biosynthetic pathway and is controlled by 1) cumulative feedback inhibition by arginine and 2) ArgR at the level of transcription (Maas, 1994). Since ArgC and ArgB catalyze early steps in the pathway and ArgH catalyzes the final biosynthetic reaction (Figure 1), it is likely that control of these key steps by RpoS regulates the arginine biosynthesis pathway.

In an *argR* wild type background, the levels of *argCBH* expression in arginine-deficient media was only one third those of the *ΔargR* mutant indicating that ArgR can effectively repress expression even in the absence of exogenous arginine. This suggests that, even under starved conditions, limited amounts of synthesized arginine can function as a co-repressor. Growth phase dependent expression of *argCBH* is independent of the ArgR repressor in rich media since expression increased during entry into stationary phase even in the absence of ArgR.

Fully de-repressed cells (*ΔargR* mutants), were found to be unable to grow on minimal media even when supplied with excess arginine (this study). Since

this requirement could be satisfied by the addition of pyrimidines (Table 3) it is likely that excess arginine biosynthesis depletes carbamoylphosphate, a precursor that is common to the arginine and pyrimidine pathways. The need for *de novo* arginine synthesis, therefore, appears to be balanced against other biosynthetic requirements of the cell.

ArgR-dependent de-repression of *argCBH* occurs when arginine becomes growth limiting at concentrations below 10 μg ml^{-1} (this study). In LB, the concentration is approximately 10 μg arginine ml^{-1} (Cui *et al.*, 2001); however, the arginine concentration is likely to fall below this level in exponential phase where most growth occurs. The need to synthesize arginine *de novo* in stationary phase is necessary to alleviate this arginine deficiency (this study), hence the observed increase in *argCBH* expression in stationary phase in LB. In summary, *argCBH* expression is clearly controlled by a finely balanced mechanism mediated by two signals: 1) a general nutrient stress signal exerted through RpoS and, 2) specific control through arginine dependent modulation of the ArgR repressor.

APPENDIX A – RAW DATA

***argCBH* expression data from HS1066 (WT) grown under rich media conditions.** Growth and β-galactosidase activity were assayed as described in Methods. O/N, overnight.

Isolate 1

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.075	4408	0.05	0.044	0.045	0.045	0.021	0.024	1.4
0.5	0.085	4408	0.05	0.071	0.070	0.071	0.021	0.050	2.6
1	0.094	4408	0.05	0.137	0.140	0.139	0.021	0.118	5.7
1.5	0.191	4408	0.05	0.280	0.290	0.285	0.021	0.264	6.3
2	0.385	4408	0.05	0.509	0.517	0.513	0.021	0.492	5.8
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.655	1106	0.05	0.322	0.333	0.328	0.018	0.310	8.5
3	1.320	1106	0.05	0.759	0.750	0.755	0.018	0.737	10.1
3.5	1.850	1106	0.05	0.969	0.919	0.944	0.018	0.926	9.1
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.170	360	0.05	0.815	0.881	0.848	0.032	0.817	20.9
4.5	2.220	360	0.05	1.054	1.010	1.032	0.032	1.001	25.0
5	2.445	360	0.05	0.971	1.010	0.991	0.032	0.959	21.8
5.5	2.570	360	0.05	1.030	1.041	1.036	0.032	1.004	21.7
6	2.740	360	0.05	1.005	1.000	1.003	0.032	0.971	19.7
6.5	3.030	360	0.05	1.010	0.994	1.002	0.032	0.971	17.8
7	2.920	360	0.05	0.992	0.987	0.990	0.032	0.958	18.2
7.5	2.890	360	0.05	0.993	0.993	0.993	0.032	0.962	18.5
8	3.130	360	0.05	0.969	1.025	0.997	0.032	0.966	17.1
ON	2.480	360	0.05	0.847	0.852	0.850	0.032	0.818	18.3

Isolate 2

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.078	4408	0.05	0.041	0.045	0.043	0.021	0.022	1.3
0.5	0.079	4408	0.05	0.074	0.084	0.079	0.021	0.058	3.3
1	0.092	4408	0.05	0.134	0.134	0.134	0.021	0.113	5.6
1.5	0.168	4408	0.05	0.276	0.283	0.280	0.021	0.259	7.0
2	0.356	4408	0.05	0.492	0.476	0.484	0.021	0.463	5.9
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.627	1106	0.05	0.280	0.281	0.281	0.018	0.263	7.6
3	1.265	1106	0.05	0.669	0.651	0.660	0.018	0.642	9.2
3.5	1.770	1106	0.05	1.029	1.004	1.017	0.018	0.999	10.2
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.060	360	0.05	0.784	0.739	0.762	0.032	0.730	19.7
4.5	2.260	360	0.05	1.030	1.016	1.023	0.032	0.992	24.4
5	2.485	360	0.05	1.017	1.034	1.026	0.032	0.994	22.2
5.5	2.590	360	0.05	1.053	1.047	1.050	0.032	1.019	21.8
6	2.790	360	0.05	1.045	1.037	1.041	0.032	1.010	20.1
6.5	3.150	360	0.05	1.050	1.043	1.047	0.032	1.015	17.9
7	3.020	360	0.05	1.037	1.044	1.041	0.032	1.009	18.6
7.5	3.060	360	0.05	1.032	1.094	1.063	0.032	1.032	18.7
8	3.270	360	0.05	1.062	1.038	1.050	0.032	1.019	17.3
ON	2.510	360	0.05	0.880	0.897	0.889	0.032	0.857	19.0

Isolate 3

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.084	4408	0.05	0.045	0.048	0.047	0.021	0.026	1.4
0.5	0.089	4408	0.05	0.000	0.000	0.000	0.021	-0.021	0.0
1	0.090	4408	0.05	0.141	0.147	0.144	0.021	0.123	6.2
1.5	0.181	4408	0.05	0.294	0.291	0.293	0.021	0.272	6.8
2	0.366	4408	0.05	0.513	0.507	0.510	0.021	0.489	6.1
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.602	1106	0.05	0.298	0.306	0.302	0.018	0.284	8.5
3	1.335	1106	0.05	0.677	0.698	0.688	0.018	0.670	9.1
3.5	1.830	1106	0.05	1.130	1.169	1.150	0.018	1.132	11.2
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.165	360	0.05	0.926	0.931	0.929	0.032	0.897	23.0
4.5	2.290	360	0.05	1.049	1.065	1.057	0.032	1.026	24.9
5	2.545	360	0.05	1.083	1.099	1.091	0.032	1.060	23.1
5.5	2.615	360	0.05	1.076	1.119	1.098	0.032	1.066	22.6
6	2.875	360	0.05	1.029	1.115	1.072	0.032	1.041	20.1
6.5	3.240	360	0.05	1.073	1.060	1.067	0.032	1.035	17.7
7	3.050	360	0.05	1.058	1.084	1.071	0.032	1.040	18.9
7.5	3.180	360	0.05	1.099	1.086	1.093	0.032	1.061	18.5
8	3.380	360	0.05	1.075	1.065	1.070	0.032	1.039	17.1
ON	2.600	360	0.05	0.902	0.903	0.903	0.032	0.871	18.6

Growth (OD 600)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	0.075	0.078	0.084	0.00	0.00	0.08	0.01
0.5	0.085	0.079	0.089	0.01	0.00	0.08	0.03
1	0.094	0.092	0.090	0.00	0.00	0.09	0.09
1.5	0.191	0.168	0.181	0.01	0.01	0.18	0.18
2	0.385	0.356	0.366	0.01	0.01	0.37	0.37
2.5	0.655	0.627	0.602	0.03	0.02	0.63	0.63
3	1.320	1.265	1.335	0.04	0.02	1.31	1.31
3.5	1.850	1.770	1.830	0.04	0.02	1.82	1.82
4	2.170	2.060	2.165	0.06	0.04	2.13	2.13
4.5	2.220	2.260	2.290	0.04	0.02	2.26	2.26
5	2.445	2.485	2.545	0.05	0.03	2.49	2.49
5.5	2.570	2.590	2.615	0.02	0.01	2.59	2.59
6	2.740	2.790	2.875	0.07	0.04	2.80	2.80
6.5	3.030	3.150	3.240	0.11	0.06	3.14	3.14
7	2.920	3.020	3.050	0.07	0.04	3.00	3.00
7.5	2.890	3.060	3.180	0.15	0.08	3.04	3.04
8	3.130	3.270	3.380	0.13	0.07	3.26	3.26
9	2.480	2.510	2.600	0.06	0.04	2.53	2.53

Specific Activity (Miller Units)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	1.4	1.3	1.4	0.07	0.04	1.4	5.0
0.5	2.6	3.3		0.49	0.34	3.0	5.5
1	5.7	5.6	6.2	0.34	0.19	5.8	6.0
1.5	6.3	7.0	6.8	0.37	0.21	6.7	6.7
2	5.8	5.9	6.1	0.13	0.08	5.9	6.0
2.5	8.5	7.6	8.5	0.56	0.32	8.2	8.2
3	10.1	9.2	9.1	0.56	0.32	9.4	9.4
3.5	9.1	10.2	11.2	1.07	0.62	10.1	10.1
4	20.9	19.7	23.0	1.69	0.97	21.2	21.2
4.5	25.0	24.4	24.9	0.35	0.20	24.8	22.5
5	21.8	22.2	23.1	0.68	0.39	22.4	22.4
5.5	21.7	21.8	22.6	0.51	0.29	22.1	22.1
6	19.7	20.1	20.1	0.24	0.14	20.0	20.0
6.5	17.8	17.9	17.7	0.08	0.05	17.8	17.8
7	18.2	18.6	18.9	0.35	0.20	18.6	18.6
7.5	18.5	18.7	18.5	0.13	0.07	18.6	18.6
8	17.1	17.3	17.1	0.12	0.07	17.2	17.2
9	18.3	19.0	18.6	0.32	0.19	18.6	18.6

***argCBH* expression data from HS1066p (*rpoS* mutant) grown under rich media conditions.** Growth and β-galactosidase activity were assayed as described in Methods. O/N, overnight.

Isolate 1

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.085	4408	0.05	0.029	0.027	0.028	0.021	0.007	0.4
0.5	0.081	4408	0.05	0.050	0.047	0.049	0.021	0.028	1.5
1	0.083	4408	0.05	0.093	0.090	0.092	0.021	0.071	3.9
1.5	0.148	4408	0.05	0.192	0.189	0.191	0.021	0.170	5.2
2	0.316	4408	0.05	0.342	0.356	0.349	0.021	0.328	4.7
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.552	1106	0.05	0.265	0.250	0.258	0.018	0.240	7.8
3	1.220	1106	0.05	0.519	0.488	0.504	0.018	0.486	7.2
3.5	1.700	1106	0.05	0.758	0.780	0.769	0.018	0.751	8.0
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.035	362	0.05	0.485	0.462	0.474	0.032	0.442	12.0
4.5	2.075	362	0.05	0.553	0.549	0.551	0.032	0.520	13.8
5	2.215	362	0.05	0.587	0.578	0.583	0.032	0.551	13.7
5.5	2.355	362	0.05	0.630	0.634	0.632	0.032	0.601	14.1
6	2.585	362	0.05	0.651	0.653	0.652	0.032	0.621	13.3
6.5	2.920	362	0.05	0.659	0.668	0.664	0.032	0.632	12.0
7	2.630	362	0.05	0.658	0.656	0.657	0.032	0.626	13.1
7.5	2.750	362	0.05	0.644	0.673	0.659	0.032	0.627	12.6
8	2.860	362	0.05	0.663	0.666	0.665	0.032	0.633	12.2
ON	2.130	362	0.05	0.595	0.597	0.596	0.032	0.565	14.6

Isolate 2

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.085	4408	0.05	0.033	0.038	0.036	0.021	0.015	0.8
0.5	0.085	4408	0.05	0.054	0.052	0.053	0.021	0.032	1.7
1	0.087	4408	0.05	0.109	0.106	0.108	0.021	0.087	4.5
1.5	0.168	4408	0.05	0.210	0.210	0.210	0.021	0.189	5.1
2	0.363	4408	0.05	0.399	0.392	0.396	0.021	0.375	4.7
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.629	1106	0.05	0.285	0.297	0.291	0.018	0.273	7.8
3	1.285	1106	0.05	0.521	0.539	0.530	0.018	0.512	7.2
3.5	1.775	1106	0.05	0.789	0.806	0.798	0.018	0.780	7.9
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.100	362	0.05	0.484	0.492	0.488	0.032	0.457	12.0
4.5	2.140	362	0.05	0.576	0.575	0.576	0.032	0.544	14.0
5	2.350	362	0.05	0.651	0.640	0.646	0.032	0.614	14.4
5.5	2.485	362	0.05	0.683	0.679	0.681	0.032	0.650	14.4
6	2.655	362	0.05	0.678	0.674	0.676	0.032	0.645	13.4
6.5	3.050	362	0.05	0.675	0.707	0.691	0.032	0.660	11.9
7	2.740	362	0.05	0.696	0.701	0.699	0.032	0.667	13.4
7.5	2.830	362	0.05	0.706	0.698	0.702	0.032	0.671	13.1
8	3.060	362	0.05	0.689	0.697	0.693	0.032	0.662	11.9
ON	2.260	362	0.05	0.629	0.630	0.630	0.032	0.598	14.6

Isolate 3

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank				0.021	0.021	0.021	0.021	0.000	
0	0.087	4408	0.05	0.038	0.037	0.038	0.021	0.017	0.9
0.5	0.088	4408	0.05	0.057	0.052	0.055	0.021	0.034	1.7
1	0.086	4408	0.05	0.111	0.109	0.110	0.021	0.089	4.7
1.5	0.171	4408	0.05	0.210	0.213	0.212	0.021	0.191	5.1
2	0.353	4408	0.05	0.419	0.417	0.418	0.021	0.397	5.1
Blank				0.018	0.018	0.018	0.018	0.000	
2.5	0.631	1106	0.05	0.297	0.311	0.304	0.018	0.286	8.2
3	1.330	1106	0.05	0.548	0.578	0.563	0.018	0.545	7.4
3.5	1.795	1106	0.05	0.836	0.803	0.820	0.018	0.802	8.1
Blank				0.029	0.034	0.032	0.032	0.000	
4	2.145	362	0.05	0.478	0.491	0.485	0.032	0.453	11.7
4.5	2.185	362	0.05	0.579	0.584	0.582	0.032	0.550	13.9
5	2.300	362	0.05	0.640	0.680	0.660	0.032	0.629	15.1
5.5	2.395	362	0.05	0.682	0.690	0.686	0.032	0.655	15.1
6	2.655	362	0.05	0.683	0.683	0.683	0.032	0.652	13.6
6.5	3.210	362	0.05	0.680	0.695	0.688	0.032	0.656	11.3
7	2.770	362	0.05	0.688	0.696	0.692	0.032	0.661	13.2
7.5	2.910	362	0.05	0.691	0.691	0.691	0.032	0.660	12.5
8	3.100	362	0.05	0.694	0.692	0.693	0.032	0.662	11.8
ON	2.150	362	0.05	0.636	0.633	0.635	0.032	0.603	15.5

Growth (OD 600)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	0.085	0.085	0.087	0.00	0.00	0.09	0.01
0.5	0.081	0.085	0.088	0.00	0.00	0.08	0.03
1	0.083	0.087	0.086	0.00	0.00	0.09	0.09
1.5	0.148	0.168	0.171	0.01	0.01	0.16	0.16
2	0.316	0.363	0.353	0.02	0.01	0.34	0.34
2.5	0.552	0.629	0.631	0.05	0.03	0.60	0.60
3	1.220	1.285	1.330	0.06	0.03	1.28	1.28
3.5	1.700	1.775	1.795	0.05	0.03	1.76	1.76
4	2.035	2.100	2.145	0.06	0.03	2.09	2.09
4.5	2.075	2.140	2.185	0.06	0.03	2.13	2.13
5	2.215	2.350	2.300	0.07	0.04	2.29	2.29
5.5	2.355	2.485	2.395	0.07	0.04	2.41	2.41
6	2.585	2.655	2.655	0.04	0.02	2.63	2.63
6.5	2.920	3.050	3.210	0.15	0.08	3.06	3.06
7	2.630	2.740	2.770	0.07	0.04	2.71	2.71
7.5	2.750	2.830	2.910	0.08	0.05	2.83	2.83
8	2.860	3.060	3.100	0.13	0.07	3.01	3.01
9	2.130	2.260	2.150	0.07	0.04	2.18	2.18

Specific Activity (Miller Units)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	0.4	0.8	0.9	0.26	0.15	0.7	4.0
0.5	1.5	1.7	1.7	0.10	0.06	1.7	4.0
1	3.9	4.5	4.7	0.44	0.26	4.4	4.4
1.5	5.2	5.1	5.1	0.07	0.04	5.1	5.1
2	4.7	4.7	5.1	0.24	0.14	4.8	4.8
2.5	7.8	7.8	8.2	0.20	0.12	8.0	6.0
3	7.2	7.2	7.4	0.12	0.07	7.3	7.3
3.5	8.0	7.9	8.1	0.07	0.04	8.0	8.0
4	12.0	12.0	11.7	0.19	0.11	11.9	11.9
4.5	13.8	14.0	13.9	0.11	0.06	13.9	13.9
5	13.7	14.4	15.1	0.68	0.39	14.4	14.4
5.5	14.1	14.4	15.1	0.51	0.30	14.5	14.5
6	13.3	13.4	13.6	0.15	0.09	13.4	13.4
6.5	12.0	11.9	11.3	0.38	0.22	11.7	11.7
7	13.1	13.4	13.2	0.17	0.10	13.3	13.3
7.5	12.6	13.1	12.5	0.31	0.18	12.7	12.7
8	12.2	11.9	11.8	0.22	0.13	12.0	12.0
9	14.6	14.6	15.5	0.50	0.29	14.9	14.9

***argCBH* expression data from HS2404 (*ΔargR*) grown under rich media conditions.** Growth and β-galactosidase activity were assayed as described in Methods. O/N, overnight.

Isolate 1

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.034	40	0.05	0.067	0.067	0.067	0.013	0.054	794.1
0.5	0.058	40	0.05	0.094	0.092	0.093	0.013	0.080	689.7
1	0.100	40	0.05	0.147	0.145	0.146	0.013	0.133	665.0
1.5	0.222	40	0.05	0.260	0.261	0.261	0.013	0.248	557.4
2	0.500	40	0.05	0.521	0.525	0.523	0.013	0.510	510.0
2.5	0.845	15	0.05	0.436	0.425	0.431	0.013	0.418	658.8
3	1.405	15	0.05	0.668	0.686	0.677	0.013	0.664	630.1
3.5	1.900	15	0.05	0.811	0.830	0.821	0.013	0.808	566.7
4	1.920	15	0.05	0.810	0.795	0.803	0.013	0.790	548.3
4.5	2.180	15	0.05	0.764	0.748	0.756	0.013	0.743	454.4
5	2.420	15	0.05	0.768	0.693	0.731	0.013	0.718	395.3
5.5	2.660	15	0.05	0.812	0.838	0.825	0.013	0.812	407.0
6	2.730	15	0.05	0.777	0.767	0.772	0.013	0.759	370.7
6.5	2.770	15	0.05	0.832	0.761	0.797	0.013	0.784	377.1
7	2.900	15	0.05	0.800	0.766	0.783	0.013	0.770	354.0
7.5	3.080	15	0.05	0.800	0.765	0.783	0.013	0.770	333.1
8	3.130	15	0.05	0.771	0.796	0.784	0.013	0.771	328.2
ON	2.410	15	0.05	0.664	0.651	0.658	0.013	0.645	356.6

Isolate 2

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.035	40	0.05	0.071	0.069	0.070	0.013	0.057	814.3
0.5	0.058	40	0.05	0.094	0.094	0.094	0.013	0.081	698.3
1	0.096	40	0.05	0.146	0.146	0.146	0.013	0.133	692.7
1.5	0.227	40	0.05	0.261	0.260	0.261	0.013	0.248	545.2
2	0.500	40	0.05	0.523	0.516	0.520	0.013	0.507	506.5
2.5	0.840	15	0.05	0.422	0.401	0.412	0.013	0.399	632.5
3	1.410	15	0.05	0.690	0.652	0.671	0.013	0.658	622.2
3.5	1.890	15	0.05	0.857	0.829	0.843	0.013	0.830	585.5
4	1.950	15	0.05	0.825	0.837	0.831	0.013	0.818	559.3
4.5	2.260	15	0.05	0.778	0.787	0.783	0.013	0.770	454.0
5	2.420	15	0.05	0.782	0.760	0.771	0.013	0.758	417.6
5.5	2.560	15	0.05	0.744	0.787	0.766	0.013	0.753	391.9
6	2.610	15	0.05	0.829	0.767	0.798	0.013	0.785	401.0
6.5	2.690	15	0.05	0.749	0.785	0.767	0.013	0.754	373.7
7	2.840	15	0.05	0.758	0.786	0.772	0.013	0.759	356.3
7.5	3.000	15	0.05	0.770	0.715	0.743	0.013	0.730	324.2
8	3.070	15	0.05	0.749	0.742	0.746	0.013	0.733	318.1
ON	2.340	15	0.05	0.625	0.645	0.635	0.013	0.622	354.4

Isolate 3

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.037	40	0.05	0.069	0.069	0.069	0.013	0.056	756.8
0.5	0.054	40	0.05	0.092	0.092	0.092	0.013	0.079	731.5
1	0.096	40	0.05	0.138	0.135	0.137	0.013	0.124	643.2
1.5	0.220	40	0.05	0.261	0.257	0.259	0.013	0.246	559.1
2	0.505	40	0.05	0.492	0.496	0.494	0.013	0.481	476.2
2.5	0.840	15	0.05	0.396	0.397	0.397	0.013	0.384	608.7
3	1.410	15	0.05	0.682	0.701	0.692	0.013	0.679	641.6
3.5	2.000	15	0.05	0.869	0.886	0.878	0.013	0.865	576.3
4	1.950	15	0.05	0.927	0.882	0.905	0.013	0.892	609.6
4.5	2.460	15	0.05	0.859	0.856	0.858	0.013	0.845	457.7
5	2.730	15	0.05	0.855	0.862	0.859	0.013	0.846	412.9
5.5	2.940	15	0.05	0.862	0.876	0.869	0.013	0.856	388.2
6	3.040	15	0.05	0.860	0.825	0.843	0.013	0.830	363.8
6.5	3.190	15	0.05	0.855	0.911	0.883	0.013	0.870	363.6
7	3.350	15	0.05	0.810	0.852	0.831	0.013	0.818	325.6
7.5	3.560	15	0.05	0.800	0.890	0.845	0.013	0.832	311.6
8	3.580	15	0.05	0.801	0.815	0.808	0.013	0.795	296.1
ON	2.780	15	0.05	0.729	0.725	0.727	0.013	0.714	342.4

Growth (OD 600)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	0.034	0.035	0.037	0.00	0.00	0.04	0.01
0.5	0.058	0.058	0.054	0.00	0.00	0.06	0.03
1	0.100	0.096	0.096	0.00	0.00	0.10	0.10
1.5	0.222	0.227	0.220	0.00	0.00	0.22	0.22
2	0.500	0.500	0.505	0.00	0.00	0.50	0.50
2.5	0.845	0.840	0.840	0.00	0.00	0.84	0.84
3	1.405	1.410	1.410	0.00	0.00	1.41	1.41
3.5	1.900	1.890	2.000	0.06	0.04	1.93	1.93
4	1.920	1.950	1.950	0.02	0.01	1.94	1.94
4.5	2.180	2.260	2.460	0.14	0.08	2.30	2.30
5	2.420	2.420	2.730	0.18	0.10	2.52	2.52
5.5	2.660	2.560	2.940	0.20	0.11	2.72	2.72
6	2.730	2.610	3.040	0.22	0.13	2.79	2.79
6.5	2.770	2.690	3.190	0.27	0.16	2.88	2.88
7	2.900	2.840	3.350	0.28	0.16	3.03	3.03
7.5	3.080	3.000	3.560	0.30	0.17	3.21	3.21
8	3.130	3.070	3.580	0.28	0.16	3.26	3.26
9	2.410	2.340	2.780	0.24	0.14	2.51	2.51

Specific Activity (Miller Units)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	794.1	814.3	756.8	29.19	16.85	788.4	500.0
0.5	689.7	698.3	731.5	22.08	12.75	706.5	510.0
1	665.0	692.7	643.2	24.80	14.32	667.0	520.5
1.5	557.4	545.2	559.1	7.61	4.40	553.9	490.0
2	510.0	506.5	476.2	18.57	10.72	497.6	469.2
2.5	658.8	632.5	608.7	25.03	14.45	633.3	588.2
3	630.1	622.2	641.6	9.75	5.63	631.3	604.3
3.5	566.7	585.5	576.3	9.44	5.45	576.2	575.0
4	548.3	559.3	609.6	32.68	18.87	572.4	552.8
4.5	454.4	454.0	457.7	2.04	1.18	455.4	438.8
5	395.3	417.6	412.9	11.77	6.79	408.6	393.5
5.5	407.0	391.9	388.2	9.96	5.75	395.7	381.7
6	370.7	401.0	363.8	19.80	11.43	378.5	364.9
6.5	377.1	373.7	363.6	7.02	4.05	371.5	358.2
7	354.0	356.3	325.6	17.13	9.89	345.3	332.7
7.5	333.1	324.2	311.6	10.81	6.24	323.0	311.1
8	328.2	318.1	296.1	16.43	9.49	314.1	302.4
9	356.6	354.4	342.4	7.61	4.39	351.1	335.9

***argCBH* expression data from HS2405 (*ΔargR rpoS* mutant) grown under rich media conditions.** Growth and β-galactosidase activity were assayed as described in Methods. O/N, overnight.

Isolate 1

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.033	40	0.05	0.067	0.067	0.067	0.013	0.054	818.2
0.5	0.054	40	0.05	0.091	0.090	0.091	0.013	0.078	717.6
1	0.091	40	0.05	0.130	0.131	0.131	0.013	0.118	645.6
1.5	0.204	40	0.05	0.243	0.243	0.243	0.013	0.230	563.7
2	0.465	40	0.05	0.465	0.466	0.466	0.013	0.453	486.6
2.5	0.755	15	0.05	0.400	0.368	0.384	0.013	0.371	655.2
3	1.280	15	0.05	0.654	0.638	0.646	0.013	0.633	659.4
3.5	1.670	15	0.05	0.877	0.855	0.866	0.013	0.853	681.0
4	1.760	15	0.05	0.947	0.941	0.944	0.013	0.931	705.3
4.5	2.000	15	0.05	0.986	0.982	0.984	0.013	0.971	647.3
5	2.370	15	0.05	1.032	1.011	1.022	0.013	1.009	567.4
5.5	2.580	15	0.05	1.099	1.041	1.070	0.013	1.057	546.3
6	2.660	15	0.05	1.108	1.129	1.119	0.013	1.106	554.1
6.5	2.840	15	0.05	1.050	1.070	1.060	0.013	1.047	491.5
7	2.950	15	0.05	1.144	1.056	1.100	0.013	1.087	491.3
7.5	3.160	15	0.05	1.027	1.067	1.047	0.013	1.034	436.3
8	3.190	15	0.05	1.115	1.070	1.093	0.013	1.080	451.2
ON	2.270	15	0.05	0.996	0.996	0.996	0.013	0.983	577.4

Isolate 2

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.031	40	0.05	0.070	0.069	0.070	0.013	0.057	911.3
0.5	0.052	40	0.05	0.090	0.089	0.090	0.013	0.077	735.6
1	0.083	40	0.05	0.125	0.124	0.125	0.013	0.112	671.7
1.5	0.187	40	0.05	0.222	0.224	0.223	0.013	0.210	561.5
2	0.400	40	0.05	0.426	0.430	0.428	0.013	0.415	518.8
2.5	0.730	15	0.05	0.368	0.378	0.373	0.013	0.360	657.5
3	1.250	15	0.05	0.661	0.634	0.648	0.013	0.635	676.8
3.5	1.690	15	0.05	0.876	0.874	0.875	0.013	0.862	680.1
4	1.740	15	0.05	0.949	0.960	0.955	0.013	0.942	721.5
4.5	2.070	15	0.05	0.935	0.958	0.947	0.013	0.934	601.3
5	2.350	15	0.05	1.013	1.035	1.024	0.013	1.011	573.6
5.5	2.530	15	0.05	1.117	1.122	1.120	0.013	1.107	583.1
6	2.600	15	0.05	1.119	1.041	1.080	0.013	1.067	547.2
6.5	2.760	15	0.05	1.077	1.090	1.084	0.013	1.071	517.1
7	2.890	15	0.05	1.073	1.064	1.069	0.013	1.056	487.0
7.5	3.030	15	0.05	1.088	1.042	1.065	0.013	1.052	462.9
8	3.200	15	0.05	1.134	1.108	1.121	0.013	1.108	461.7
ON	2.120	15	0.05	1.014	0.977	0.996	0.013	0.983	617.9

Isolate 3

Time Pt. (h)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		15		0.013	0.013	0.013	0.013	0.000	
0	0.032	40	0.05	0.068	0.069	0.069	0.013	0.056	867.2
0.5	0.052	40	0.05	0.085	0.085	0.085	0.013	0.072	692.3
1	0.086	40	0.05	0.125	0.127	0.126	0.013	0.113	657.0
1.5	0.197	40	0.05	0.225	0.225	0.225	0.013	0.212	538.1
2	0.390	40	0.05	0.448	0.446	0.447	0.013	0.434	556.4
2.5	0.730	15	0.05	0.374	0.373	0.374	0.013	0.361	658.4
3	1.215	15	0.05	0.623	0.636	0.630	0.013	0.617	676.5
3.5	1.690	15	0.05	0.882	0.870	0.876	0.013	0.863	680.9
4	1.720	15	0.05	1.018	1.004	1.011	0.013	0.998	773.6
4.5	2.150	15	0.05	1.026	1.003	1.015	0.013	1.002	621.1
5	2.450	15	0.05	1.164	1.170	1.167	0.013	1.154	628.0
5.5	2.670	15	0.05	1.196	1.162	1.179	0.013	1.166	582.3
6	2.810	15	0.05	1.165	1.170	1.168	0.013	1.155	547.8
6.5	3.080	15	0.05	1.165	1.122	1.144	0.013	1.131	489.4
7	3.240	15	0.05	1.162	1.152	1.157	0.013	1.144	470.8
7.5	3.410	15	0.05	1.129	1.198	1.164	0.013	1.151	449.9
8	3.540	15	0.05	1.114	1.206	1.160	0.013	1.147	432.0
ON	2.910	15	0.05	1.037	1.060	1.049	0.013	1.036	474.5

Growth (OD 600)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	0.033	0.031	0.032	0.00	0.00	0.03	0.01
0.5	0.054	0.052	0.052	0.00	0.00	0.05	0.03
1	0.091	0.083	0.086	0.00	0.00	0.09	0.09
1.5	0.204	0.187	0.197	0.01	0.00	0.20	0.20
2	0.465	0.400	0.390	0.04	0.02	0.42	0.42
2.5	0.755	0.730	0.730	0.01	0.01	0.74	0.74
3	1.280	1.250	1.215	0.03	0.02	1.25	1.25
3.5	1.670	1.690	1.690	0.01	0.01	1.68	1.68
4	1.760	1.740	1.720	0.02	0.01	1.74	1.74
4.5	2.000	2.070	2.150	0.08	0.04	2.07	2.07
5	2.370	2.350	2.450	0.05	0.03	2.39	2.39
5.5	2.580	2.530	2.670	0.07	0.04	2.59	2.59
6	2.660	2.600	2.810	0.11	0.06	2.69	2.69
6.5	2.840	2.760	3.080	0.17	0.10	2.89	2.89
7	2.950	2.890	3.240	0.19	0.11	3.03	3.03
7.5	3.160	3.030	3.410	0.19	0.11	3.20	3.20
8	3.190	3.200	3.540	0.20	0.12	3.31	3.31
9	2.270	2.120	2.910	0.42	0.24	2.43	2.43

Specific Activity (Miller Units)

Time Pt. (h)	Isolate 1	Isolate 2	Isolate 3	SD	SE	Average	Intrapolation
0	818.2	911.3	867.2	46.58	26.89	865.6	500.0
0.5	717.6	735.6	692.3	21.74	12.55	715.2	515.0
1	645.6	671.7	657.0	13.08	7.55	658.1	493.4
1.5	563.7	561.5	538.1	14.21	8.21	554.4	481.6
2	486.6	518.8	556.4	34.96	20.18	520.6	486.3
2.5	655.2	657.5	658.4	1.68	0.97	657.1	605.6
3	659.4	676.8	676.5	9.99	5.77	670.9	640.5
3.5	681.0	680.1	680.9	0.51	0.30	680.7	658.1
4	705.3	721.5	773.6	35.72	20.62	733.5	650.0
4.5	647.3	601.3	621.1	23.10	13.34	623.2	604.9
5	567.4	573.6	628.0	33.36	19.26	589.7	573.8
5.5	546.3	583.1	582.3	21.05	12.15	570.6	555.9
6	554.1	547.2	547.8	3.85	2.22	549.7	535.6
6.5	491.5	517.1	489.4	15.44	8.91	499.4	486.2
7	491.3	487.0	470.8	10.81	6.24	483.0	470.4
7.5	436.3	462.9	449.9	13.32	7.69	449.7	437.8
8	451.2	461.7	432.0	15.04	8.68	448.3	436.8
9	577.4	617.9	474.5	73.96	42.70	556.6	540.7

***argCBH* expression in HS1066 (WT) when grown in minimal media supplemented with exogenous arginine.** Expression was monitored only in exponential phase (OD_{600} = 0.3).

Isolate 1

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	0.248	162	0.05	0.537	0.513	0.525	0.045	0.480	238.9
1	0.251	162	0.05	0.527	0.510	0.519	0.045	0.474	233.4
5	0.276	162	0.05	0.519	0.491	0.505	0.045	0.460	205.8
10	0.272	1237	0.05	0.661	0.674	0.668	0.045	0.623	37.1
20	0.255	1237	0.05	0.571	0.537	0.554	0.045	0.509	32.3
LB	0.315	1237	0.05	0.309	0.292	0.301	0.045	0.256	13.1
LB+100	0.223	1237	0.05	0.278	0.276	0.277	0.045	0.232	16.8

Isolate 2

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	0.221	162	0.05	0.462	0.464	0.463	0.045	0.418	234.04
1	0.226	162	0.05	0.480	0.484	0.482	0.045	0.437	239.25
5	0.223	162	0.05	0.432	0.423	0.428	0.045	0.383	211.76
10	0.236	1237	0.05	0.548	0.582	0.565	0.045	0.520	35.70
20	0.234	1237	0.05	0.559	0.565	0.562	0.045	0.517	35.80
LB	0.293	1237	0.05	0.271	0.260	0.266	0.045	0.221	12.17
LB+100	0.272	1237	0.05	0.288	0.280	0.284	0.045	0.239	14.21

Isolate 3

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	0.237	162	0.05	0.540	0.494	0.517	0.045	0.472	246.4
1	0.231	162	0.05	0.489	0.533	0.511	0.045	0.466	249.6
5	0.247	162	0.05	0.480	0.487	0.484	0.045	0.439	219.6
10	0.277	1237	0.05	0.723	0.731	0.727	0.045	0.682	39.9
20	0.248	1237	0.05	0.631	0.606	0.619	0.045	0.574	37.5
LB	0.277	1237	0.05	0.315	0.321	0.318	0.045	0.273	16.0
LB+100	0.228	1237	0.05	0.281	0.271	0.276	0.045	0.231	16.4

***argCBH* expression in HS1066 (WT) when grown in minimal media supplemented with exogenous arginine.** Expression was monitored only in stationary phase (OD_{600} = 1.5).

Isolate 1

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	1.400	81	0.05	0.659	0.636	0.648	0.045	0.603	106.3
1	1.285	81	0.05	0.657	0.628	0.643	0.045	0.598	114.8
5	1.345	81	0.05	0.594	0.604	0.599	0.045	0.554	101.7
10	1.245	81	0.05	0.520	0.511	0.516	0.045	0.471	93.3
20	1.430	81	0.05	0.656	0.628	0.642	0.045	0.597	103.1
LB	2.105	81	0.05	0.481	0.471	0.476	0.045	0.431	50.6
LB+100	2.120	81	0.05	0.516	0.479	0.498	0.045	0.453	52.7

Isolate 2

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	1.480	81	0.05	0.634	0.636	0.635	0.045	0.590	98.4
1	1.400	81	0.05	0.639	0.625	0.632	0.045	0.587	103.5
5	1.495	81	0.05	0.656	0.644	0.650	0.045	0.605	99.9
10	1.345	81	0.05	0.641	0.654	0.648	0.045	0.603	110.6
20	1.445	81	0.05	0.665	0.656	0.661	0.045	0.616	105.2
LB	1.965	81	0.05	0.469	0.453	0.461	0.045	0.416	52.3
LB+100	1.930	81	0.05	0.482	0.452	0.467	0.045	0.422	54.0

Isolate 3

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		1237		0.039	0.051	0.045	0.045	0.000	
0	1.330	81	0.05	0.673	0.666	0.670	0.045	0.625	115.9
1	1.515	81	0.05	0.652	0.657	0.655	0.045	0.610	99.3
5	1.520	81	0.05	0.645	0.631	0.638	0.045	0.593	96.3
10	1.565	81	0.05	0.676	0.672	0.674	0.045	0.629	99.2
20	1.440	81	0.05	0.601	0.580	0.591	0.045	0.546	93.5
LB	2.175	81	0.05	0.474	0.475	0.475	0.045	0.430	48.8
LB+100	2.290	81	0.05	0.457	0.496	0.477	0.045	0.432	46.5

***argCBH* expression in HS1066p (*rpoS* mutant) when grown in minimal media supplemented with exogenous arginine.** Expression was monitored only in exponential phase (OD_{600} = 0.3).

Isolate 1

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	0.224	339	0.05	0.508	0.549	0.529	0.024	0.505	132.9
1	0.219	339	0.05	0.561	0.552	0.557	0.024	0.533	143.5
5	0.239	339	0.05	0.225	0.234	0.230	0.024	0.206	50.7
10	0.230	339	0.05	0.220	0.205	0.213	0.024	0.189	48.5
20	0.232	339	0.05	0.178	0.196	0.187	0.024	0.163	41.5
LB	0.279	339	0.05	0.081	0.089	0.085	0.024	0.061	12.9
LB+100	0.285	339	0.05	0.093	0.074	0.084	0.024	0.060	12.3

Isolate 2

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	0.226	339	0.05	0.553	0.520	0.537	0.024	0.513	133.79
1	0.226	339	0.05	0.577	0.585	0.581	0.024	0.557	145.40
5	0.232	339	0.05	0.265	0.256	0.261	0.024	0.237	60.27
10	0.225	339	0.05	0.251	0.233	0.242	0.024	0.218	57.16
20	0.231	339	0.05	0.235	0.236	0.236	0.024	0.212	54.13
LB	0.258	339	0.05	0.106	0.108	0.107	0.024	0.083	19.02
LB+100	0.263	339	0.05	0.103	0.100	0.102	0.024	0.078	17.39

Isolate 3

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	0.218	339	0.05	0.493	0.463	0.478	0.024	0.454	123.1
1	0.223	339	0.05	0.431	0.410	0.421	0.024	0.397	105.1
5	0.221	339	0.05	0.231	0.230	0.231	0.024	0.207	55.3
10	0.216	339	0.05	0.235	0.235	0.235	0.024	0.211	57.8
20	0.214	339	0.05	0.229	0.217	0.223	0.024	0.199	55.0
LB	0.252	339	0.05	0.104	0.098	0.101	0.024	0.077	18.1
LB+100	0.253	339	0.05	0.087	0.093	0.090	0.024	0.066	15.4

***argCBH* expression in HS1066p (*rpoS* mutant) when grown in minimal media supplemented with exogenous arginine.** Expression was monitored only in stationary phase (OD_{600} = 1.5).

Isolate 1

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	1.325	246	0.05	1.055	1.062	1.059	0.024	1.035	63.5
1	1.460	246	0.05	1.159	1.093	1.126	0.024	1.102	61.4
5	1.515	246	0.05	1.104	1.057	1.081	0.024	1.057	56.7
10	1.555	246	0.05	1.147	1.134	1.141	0.024	1.117	58.4
20	1.485	246	0.05	0.978	0.996	0.987	0.024	0.963	52.7
LB	2.175	246	0.05	0.541	0.553	0.547	0.024	0.523	19.5
LB+100	2.140	246	0.05	0.528	0.530	0.529	0.024	0.505	19.2

Isolate 2

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	1.505	247	0.05	1.141	1.121	1.131	0.024	1.107	59.6
1	1.695	247	0.05	1.274	1.328	1.301	0.024	1.277	61.0
5	1.385	247	0.05	1.250	1.304	1.277	0.024	1.253	73.3
10	1.380	247	0.05	1.259	1.220	1.240	0.024	1.216	71.3
20	1.445	247	0.05	1.338	1.352	1.345	0.024	1.321	74.0
LB	1.825	247	0.05	0.548	0.550	0.549	0.024	0.525	23.3
LB+100	1.870	247	0.05	0.524	0.553	0.539	0.024	0.515	22.3

Isolate 3

Arginine Conc. (ug/ml)	OD600	Rxn. Time (min)	Volume Assayed (ml)	OD420					Specific Activity (Miller Units)
				Sample 1	Sample 2	Avg	Blank	Avg-Blk	
Blank		339		0.021	0.027	0.024	0.024	0	
0	1.220	247	0.05	1.185	1.182	1.184	0.024	1.160	77.0
1	1.180	247	0.05	1.241	1.233	1.237	0.024	1.213	83.2
5	1.075	247	0.05	1.188	1.219	1.204	0.024	1.180	88.8
10	1.080	247	0.05	1.103	1.080	1.092	0.024	1.068	80.0
20	1.120	247	0.05	1.075	1.010	1.043	0.024	1.019	73.6
LB	1.615	247	0.05	0.525	0.495	0.510	0.024	0.486	24.4
LB+100	1.560	247	0.05	0.525	0.518	0.522	0.024	0.498	25.8

***argCBH* expression in HS1066 (WT) when grown in minimal media supplemented with exogenous arginine.** Data was compiled from both exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5).

Specific activity in exponential phase

Arginine Conc. (ug/ml)	Isolate 1	Isolate 2	Isolate 3	SD	Average
0	238.9	234.0	246.4	6.2	239.8
1	233.4	239.2	249.6	8.2	240.7
5	205.8	211.8	219.6	6.9	212.4
10	37.1	35.7	39.9	2.1	37.6
20	32.3	35.8	37.5	2.6	35.2
LB	13.1	12.2	16.0	2.0	13.8
LB+100	16.8	14.2	16.4	1.4	15.8

Specific activity in stationary phase

Arginine Conc. (ug/ml)	Isolate 1	Isolate 2	Isolate 3	SD	Average
0	106.3	98.4	115.9	8.8	106.9
1	114.8	103.5	99.3	8.0	105.9
5	101.7	99.9	96.3	2.7	99.3
10	93.3	110.6	99.2	8.8	101.1
20	103.1	105.2	93.5	6.2	100.6
LB	50.6	52.3	48.8	1.8	50.5
LB+100	52.7	54.0	46.5	4.0	51.1

***argCBH* expression in HS1066p (*rpoS* mutant) when grown in minimal media supplemented with exogenous arginine.** Data was compiled from both exponential phase (OD_{600} = 0.3) and stationary phase (OD_{600} = 1.5).

Specific activity in exponential phase

Arginine Conc. (ug/ml)	Isolate 1	Isolate 2	Isolate 3	SD	Average
0	132.9	133.8	123.1	5.9	129.9
1	143.5	145.4	105.1	22.7	131.3
5	50.7	60.3	55.3	4.8	55.4
10	48.5	57.2	57.8	5.2	54.5
20	41.5	54.1	55.0	7.5	50.2
LB	12.9	19.0	18.1	3.3	16.7
LB+100	12.3	17.4	15.4	2.5	15.0

Specific activity in stationary phase

Arginine Conc. (ug/ml)	Isolate 1	Isolate 2	Isolate 3	SD	Average
0	63.5	59.6	77.0	9.1	66.7
1	61.4	61.0	83.2	12.7	68.5
5	56.7	73.3	88.8	16.1	72.9
10	58.4	71.3	80.0	10.9	69.9
20	52.7	74.0	73.6	12.2	66.8
LB	19.5	23.3	24.4	2.5	22.4
LB+100	19.2	22.3	25.8	3.3	22.4

Effect of exogenous arginine on growth and *argCBH* expression. Growth rate and *argCBH* expression were determined using an arginine auxotrophic strain, HS1072 and an arginine prototrophic strain HS1066, respectively, at the indicated arginine concentrations. Overnight cultures were grown with appropriate antibiotics, sub-cultured into minimal media and maintained in exponential phase for at least 8 generations prior to the start of the experiment.

Strain: HS1072

Time Pt. (h)	OD600													
	Arginine Concentration (ug/ml)													
	0	1	2	3	4	5	6	7	8	9	10	20	40	80
0	0.001	0.001	0.001	0.001	0.001	0.002								
1	0.001	0.006	0.005	0.004	0.004	0.008	0.004	0.004						
2	0.001	0.024	0.026	0.027	0.023	0.032	0.025	0.028	0.029	0.043	0.033	0.031	0.037	0.034
3	0.001	0.027	0.049	0.075	0.079	0.100	0.085	0.088	0.091	0.127	0.100	0.095	0.100	0.096
4	0.001	0.030	0.056	0.085	0.115	0.147	0.167	0.203	0.201	0.256	0.245	0.236	0.246	0.247
Slope of best fit line	0.000	0.008	0.015	0.024	0.030	0.038	0.055	0.066	0.086	0.107	0.106	0.103	0.105	0.107
g Generation time (0.301/slope)	0.000	38.101	19.545	12.594	9.934	7.880	5.483	4.581	3.500	2.826	2.840	2.937	2.880	2.826
K Growth Rate Constant (ln2/g)	0.000	0.018	0.035	0.055	0.070	0.088	0.126	0.151	0.198	0.245	0.244	0.236	0.241	0.245

Strain: HS1066

		Blank	Arginine Concentration (ug/ml)													
			0	1	2	3	4	5	6	7	8	9	10	20	40	80
OD600 (4h)			0.282	0.300	0.318	0.307	0.381	0.337	0.289	0.317	0.324	0.390	0.363	0.294	0.292	0.303
OD420	Sample 1	0.028	0.525	0.456	0.487	0.468	0.647	0.423	0.408	0.463	0.466	0.503	0.432	1.063	1.022	1.078
	Sample 2	0.027	0.505	0.531	0.523	0.511	0.675	0.467	0.446	0.477	0.497	0.544	0.487	1.015	1.050	1.075
	Average	0.028	0.515	0.494	0.505	0.490	0.661	0.445	0.427	0.470	0.482	0.524	0.460	1.039	1.036	1.077
	Blank	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028	0.028
	Average-Blank	0	0.488	0.466	0.478	0.462	0.634	0.418	0.400	0.443	0.454	0.496	0.432	1.012	1.009	1.049
Time (min)			61	61	61	61	61	61	61	61	61	61	61	898	898	898
Vol of Cells (ml)			0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Miller Units (4h)			149.7	134.8	130.2	130.7	142.2	108.2	121.1	121.5	121.8	110.0	103.8	19.7	19.8	19.8

APPENDIX B - PROTOCOLS

All strains should be grown on Luria Bertani (LB) solid with appropriate antibiotics to establish a pure culture. Liquid cultures are grown in a 1:5, volume: flask ratio to ensure adequate aeration of the culture. Cultures for each strain are grown as follows:

Culturing conditions for assessing *argCBH* expression in rich media

Cultures are grown in triplicate. Inoculate each of three culture tubes with a single colony. The tubes are filled with LB media with the appropriate antibiotics.

1. Grow each culture overnight at 37 °C and agitate at 200 rpm.
2. The following day, subculture 1/1000 into a culture flask with antibiotic-free LB media.
3. Incubate at 37 °C, agitate at 200 rpm, and monitor growth spectrophotometrically. For each flask, transfer 1ml of culture into a plastic cuvette. The optical density of the culture is determined by measuring its absorbance at 600 nm (OD600). Continue incubating until an OD600 of 0.3 is reached.
4. Subculture into another set of culture flasks containing antibiotic-free LB media so that the initial OD600 is 0.01.
5. From each replicate, collect 1 ml cell samples in borosilicate glass tubes every 30 min over a period of 8 hrs and an overnight sample the following day.
6. To each sample (time point) immediately add chloramphenicol (final concentration of 150 μg/ml) to stop cell proliferation, vortex and record OD600.
7. Store samples on ice or at 4°C until ready to assay expression.
8. Evaluate expression by assaying β-galactosidase activity from each time point (see protocol for Assaying β-galactosidase activity from whole cells).

Culturing conditions for assessing *argCBH* expression in minimal media

1. Follow steps 1 through 5 of the protocol for "culturing conditions for assessing *argCBH* expression in rich media" (see above). Except, instead of LB media use minimal media supplemented with glucose (final concentration 0.4%) as a carbon source (MM).
2. From each replicate, collect 1 ml cell samples in borosilicate glass tubes every 30 min for the first 5 hrs, and every 1 hr for a total period of 15 hrs.
3. Follow steps 7 and 9 of the protocol for "culturing conditions for assessing *argCBH* expression in rich media" (see above).

Culturing conditions for assessing *argCBH* expression (de-repression) as a function of arginine concentration

1. Follow steps 1 through 4 of the protocol for growing cells in rich media. Except, instead of LB media use MM supplemented with L-arginine (final concentration 40 μg/ml) to repress *argCBH* expression.
2. Transfer the cultures into 50 ml polypropylene conical tubes and centrifuge at 5,000 x g for 15 min. Decant the media and re-suspend the resulting cell pellet in the remaining volume.
3. Remove all traces of arginine by washing the cell suspension with arginine-free MM. Add an equal volume of arginine-free MM to the cell suspension, vortex, and centrifuge at 5,000 x g for 15 min to pellet the cells.
4. Decant supernatant and repeat the washing step twice more.
5. Prepare a series of culture flasks with MM supplemented with an increasing concentration of L-arginine (final concentrations of L-arginine in the series should be 0 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, and 20 μg/ml).
6. Re-suspend the resulting cell pellet to OD600 of 0.3 and subculture 1/10 into the prepared series of flasks containing MM with the various arginine concentrations.
7. Incubate each flask at 37 °C, agitate at 200 rpm, and monitor growth spectrophotometrically.
8. Collect exponential phase cell samples when at OD600 of 0.3 is reached and stationary phase cell samples when an OD600 of 1.5 is attained. Collect 1 ml of culture from each flask for both growth phases in borosilicate glass tubes.
9. Follow steps 7 and 9 of the protocol for "culturing conditions for assessing *argCBH* expression in rich media" (see above).

Culturing conditions for assessing *argCBH* expression (de-repression) and growth rate as a function of arginine supplementation

To monitor expression use HS1066 (contains a *lacZ* fusion downstream of *argCBH*), and to monitor growth rate use HS1072 (an *argH* insertion mutant – an arginine auxotroph).

Note: For HS1072 overnight cultures supplement MM with L-arginine (final concentration 40 μg/ml).

1. For each strain, follow steps 1 through 4 of the protocol for "culturing conditions for assessing *argCBH* expression (de-repression) as a function of arginine concentration" (see above).
2. Prepare a series of culture flasks with MM supplemented with an increasing concentration of L-arginine (final concentrations of L-arginine in the series

should be 0 μg/ml to 10 μg/ml in 1 μg/ml increments, 20 μg/ml, 40 μg/ml, and 80 μg/ml).

3. Re-suspend the resulting cell pellet to OD600 of 0.3. Subculture to a final OD600 of 0.01 into the prepared series of flasks containing MM with the various arginine concentrations.
4. Incubate each flask at 37 °C, agitate at 200 rpm, and monitor growth spectrophotometrically.
5. To measure growth rate of the HS1072 as a function of arginine supplementation, calculate the growth rate constant over the first 4 h of incubation at each arginine concentration.

   The growth rate constant is calculated as follows:

$$K = \frac{\ln 2}{G}$$

   Where K is the growth constant and G is the generation time. Generation time is calculated as follows:

$$G = \frac{0.301}{S}$$

   Where S is the rate of change in OD600 over the first 4 h of incubation (slope of the line of best fit on a OD600 vs. time plot).

6. To measure *argCBH* expression in HS1066 in response to increasing arginine supplementation, incubate the series of flasks overnight. Collect a 1 ml cell sample in borosilicate glass tubes and add chloramphenicol (final concentration of 150 μg/ml) to stop cell growth.
7. Assay for β-galactosidase activity from each overnight sample (see protocol for Assaying β-galactosidase activity from whole cells) to assess expression in response to arginine supplementation.

Assaying β-galactosidase activity from whole cells

Gene expression can be monitored by using transcriptional reporter gene fusions. Reporter genes that are commonly used encode an easily assayable enzyme like β-galactosidase, luciferase, or alkaline phosphatase. To evaluate the expression of a gene of interest e.g. argC, a reporter gene (e.g. lacZ, which encodes β-galactosidase) is fused downstream to the argC promotor. For every argC transcript produced a lacZ transcript is co-transcribed. The resulting β-galactosidase enzyme activity is proportional to the expression of the gene of interest. The following is a protocol for assaying β-galactosidase activity from

whole cell samples. Each assay reaction should be performed in duplicate to ensure reproducibility.

Prepare the assaying buffer (Z-buffer) in distilled deionized water (ddH_2O). Final concentration of the salts in Z-buffer are as follows: 0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, and 0.01 M Mg_2SO_4. Add a final concentration of 0.03M β-mercaptoethanol just before use.

For each cell sample (time point) assay 50 µl of cells. Add 50 µl of cells and 950 µl of Z-buffer to a borosilicate glass tube. Include a control of 50 µl water instead of cells.

1. Add 50 µl 100% chloroform and 25 µl 0.1% SDS to the "cells + Z-buffer" mixture (sample tube) and the control tube.
2. Vortex for 5 sec. and equilibrate the mixture for 5 min at room temperature.
3. Prepare an o-nitrophenyl β-D-galactopyranoside stock (ONPG) as follows: Dissolve in ddH_2O for a final concentration of 4 mg/ml. Filter sterilize, store at 4°C, and protect from exposure to light. ONPG is a substrate analogue of lactose.
4. Add 200 µl of ONPG to each sample tube and the control tube. Vortex for 5 sec. and record the time of addition.
5. Protect the tubes from exposure to light by storing them in the dark. Allow the tubes to incubate at room temperature until a yellow colour develops. β-galactosidase hydrolyzes ONPG releasing o-nitrophenol which results in a yellow colour. The intensity of the colour can be measured by absorbance at a wavelength of 420nm (OD420).
6. Add 0.5 ml of 1 M $NaCO_3$ to the sample tubes that have turned yellow. Vortex for 5 sec. and allow for the chloroform to settle. Record the time of addition. The addition of Na_2CO_3 raises the pH to 11. At this pH β-galactosidase is no longer active.
7. Transfer 1 ml of the mixture into a 1.5 ml microfuge tube, taking care no to transfer any of the chloroform. Centrifuge the mixture at 13,000 x g for 15 min to pellet any cell debris.
8. Transfer the supernatant into a plastic cuvette and record the absorbance at OD420.
9. Calculate the specific activity in Miller Units using the following formula:

$$\text{Specific Activity (Miller Units)} = \frac{\text{OD420 x 1000}}{\text{OD600 x vol.of cells assayed (ml) x time of reaction (min)}}.$$

PCR amplifying DNA

The Polymerase Chain Reaction uses the DNA polymerase isolated from *Thermus aquaticus* (Taq) to amplify trace amounts of template DNA. The double stranded template DNA is denatured to single stranded template DNA by

heating. Taq replicates complementary strands by extending appropriate primers that anneal to the single stranded template DNA. Therefore, a DNA template of interest can be amplified to yield several copies. Only the number of free nucleotides and free primers limit the amplification yield. The following protocol outlines a typical 50 µl PCR reaction:

For each PCR reaction add the following reagents to a PCR thermo-tube:
Note: Include a negative control e.g. same reaction mix, but substitute the template with ddH_2O.

Reagents to add for a 50 µl PCR reaction (Only stock concentrations are indicated)	**Volume (µl)**
1. Template DNA (20 µg/µl) to be amplified e.g. *cat*	1
2. Forward primer (25 µM)	2
3. Reverse primer (25 µM)	2
4. A mixture of dATP, dTTP, dGTP, and dCTP (10 mM for each deoxyribonucleotide)	1
5. 10 x PCR buffer stock (concentration of components in the 10 x PCR buffer stock obtained from Fermentas are as follows: Tris-Cl (pH 8.8), 750mM; $(NH_4)_2SO_4$, 200mM; Tween 20, 0.1%).	5
6. $MgCl_2$ (25 mM)	3
7. Taq DNA polymerase (1 unit/µl)	2
8. ddH_2O	35

9. Place all reaction tubes into the PCR themocycler. Program the thermocycler with the settings indicated in table B1:

PCR Step	**Temperature**	**Duration**	**Number of Cycles**
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	30
Annealing	Melting temperature of primers - 5°C	30 sec	
Extension	72°C	2 min at least (1 min for every 1 kb to amplified)	
Terminal extension	72°C	7 min	

Table B1: Typical PCR thermocycler settings for amplifying template DNA.

10. To confirm that amplification was successful, run a sample of the PCR mixture on an agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis).
11. After amplification is confirmed, store the remaining PCR mixture at -20°C.

Visualizing DNA by agarose gel electrophoresis

This technique allows one to resolve visualize DNA isolated from a given procedure e.g. a PCR amplification or a plasmid extraction. This is accomplished as follows:

1. Prepare a 1.2% agarose gel, with the appropriate well sizes. Add 0.0025 μg/ml of ethidium bromide to the gel mixture before it solidifies. Dissolve the agarose in TAE buffer. The final concentrations of the components in TAE buffer are as follows: 40 mM Tris-acetate; 1 mM EDTA)
2. Mix 5 μl of the DNA (e.g. PCR product or plasmid extraction) with 1 μl of a 6 x loading dye. Composition of the 6 x loading dye is as follows: 0.25%, bromophenol blue; 30%, glycerol. The loading dye facilitates visualizing the sample migration across the gel. The glycerol component of the dye binds to the DNA and forces it to sink to the bottom of the well.
3. Use TAE as the running buffer. Run the gel at 100 V for 50 min.
4. Visualize the DNA bands under a ultraviolet trans-illuminator.

Creating an *ΔargR* mutant

The one-step gene inactivation procedure developed by Datsenko and Wanner (2000) can be used to obtain a *ΔargR* mutant. In this procedure, a PCR amplified fragment that carries a selectable marker (e.g. a chloramphenicol resistance cassette (Cm^r)) is used to replace the chromosomal copy of the gene of interest (e.g. *argR*). The PCR amplified fragment contains a selectable marker flanked by homologous regions that are upstream and downstream to the gene of interest (e.g. *argR*). The fragment is then introduced by electroporation into a host strain, where it replaces the gene of interest by homologous recombination. Recombinants can be selected for, by plating on selective media (e.g. chloramphenicol). Homologous recombination is facilitated by the λ Red recombinase system (RED). The RED system is encoded on a plasmid and is under the control of a L-arabinose inducible promotor. The plasmid is electroporated into the host, and its expression induced, prior to introducing the linear PCR fragment (this PCR product will be abbreviated to GDF – Gene Disruption Fragment, from here on in this section). Described below is a more detailed protocol for obtaining a *ΔargR* mutant.

Designing the GDF to replace the chromosomal copy of *argR*:

The GDF will carry Cm^r to select for *ΔargR* mutants. The pKD3 plasmid that encodes *cat* (chloramphenicol acetyltransferase which confers chloramphenicol resistance (Cm^r)) will be used as a template for PCR amplification. All primers are synthesized by MOBIX. Use the primers listed in Table B2 to amplify the fragment:

Primer	Sequence (oriented 5' to 3')	Melting Temperature
Forward	**CAATAATGTTGTATCAACCACCATATCGGGTGA** **CTT**GTGTAGGCTGAAGCTGCTTC	63.9°C
Reverse	**ACATTTTCCCCGCCGTCAGAAACGACGGGGCA** **GAGA**CATATGAATATCCTCCTTAG	50.9°C

Table B2: Sequence of primers used to amplify the GDF that will replace the chromosomal copy of *argR*. The regions in bold text correspond to *argR* flanking sequences. Normal text represents DNA sequences complementary to the Cm^r cassette encoded on pKD3. The melting temperatures of each primer are also indicated.

1. To amplify the GDF add the necessary components to a PCR thermo-tube as indicated in the protocol "PCR amplifying DNA" (see above). Use pKD3 as the template for the amplification and the primers indicated in Table B2.
2. Program the PCR thermocycler with the PCR conditions indicated in Table B3:

PCR Step	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	30
Annealing	50°C	30 sec	
Extension	72°C	2 min	
Terminal extension	72°C	7 min	

Table B3: PCR Thermocycler settings for amplifying the GDF that will replace the chromosomal copy of *argR*.

3. To confirm that amplification was successful, run a sample of the PCR mixture on an agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis).
4. After amplification is confirmed, store the remaining PCR mixture at -20°C.

Preparing competent cells for electroporation:

Plasmids and fragments of foreign DNA can be introduced into a bacterial cell by electroporation. This process uses an electrical current to stimulate uptake of large molecules like DNA. For this process to be successful cells have to be made competent for DNA uptake. The following instructions describe how this can be accomplished:

1. Prepare an overnight liquid culture by inoculating a well-isolated colony in LB media, with appropriate antibiotics if necessary. Incubate overnight at 37°C with agitation, 200 rpm.
2. The following day, subculture 1/250 in 50 ml antibiotic-free LB media. Incubate at 37°C with agitation, 200 rpm, until an OD600 of 0.4 is reached.
3. Transfer the culture into a 50 ml polypropylene conical tube and incubate the cells on ice for 30 min.
4. Centrifuge the culture to pellet the cells. Centrifuge at 4°C.
5. Decant the media and re-suspend the cell pellet in 25 ml chilled ddH_2O.
6. Centrifuge the cell suspension at 4°C to pellet the cells.
7. Decant the ddH_2O, and re-suspend the cell pellet in 50 ml of chilled 10% glycerol.
8. Centrifuge one final time at 4°C, decant the glycerol supernatant and re-suspend the pellet in the remaining volume (cells are concentrated to approximately 10^{10} cells/ml).
9. Aliquot 100 µl volumes into sterile chilled microfuge tubes and use immediately for electroporation or store at -80°C for later use.

Transforming the RED encoding plasmid, pKD46 into the host strain:

The temperature sensitive plasmid pKD46, encodes the RED system which is under the control of an L-arabinose inducible promoter (araBp). The RED system consists of the λ Red recombinase genes *gam*, *bet* and *exo*. For the purposes of one-step gene inactivation, the functions of these protein products are as follows: Gam inhibits the host *recBC* encoding nuclease (exonuclease V) thus preventing hydrolysis of dsDNA e.g. an introduced PCR amplified GDF; Bet and Exo facilitate homologous recombination e.g. between homologous regions in the introduced PCR amplified GDF and the host chromosome. The plasmid also encodes an ampicillin resistance marker. The protocol outlined below describes how pKD46 is transformed into a host strain (e.g. MG1655) by electroporation:

1. Keep all cells and reagents on ice. Prepare the competent cells of recipient strain (e.g. MG1655), as described in the protocol "Preparing competent cells for electroporation."

2. In an electroporation cuvette mix 50 µl of competent cells (from a 10^{10} cells/ml stock) with 2 µl of pKD46 plasmid DNA (from a 50 ng/µl stock)
3. Insert the cuvette into the electroporator and follow the manufacturer's operating instructions. If you are using a Bio-Rad Gene Pulser II, set the voltage to 2.25 volts and capacitance to 25 µF and electroporate.
4. Add 1 ml of LB media to the electroporated cell+DNA mixture, resuspend the mixture in the media and transfer to a 15 ml polypropylene tube.
5. Incubate the mixture for 1 h at 30°C with agitation, 200 rpm.
6. Plate 100 µl of the cell mixture on solid LB agar media supplemented with 100 µg/ml ampicillin.
7. Incubate overnight at 30°C and select for pKD46 transformants (MG1655-pKD46).

Confirming the presence of the pKD46 plasmid in transformants:

To confirm that the electroporation is successful and the ampicillin resistant isolates that arise harbour the pKD46 plasmid, a plasmid preparation should be performed. The procedure for a small scale preparation of plasmid DNA (miniprep) by alkaline lysis with SDS (sodium dodecyl sulphate) is described below:

1. Inoculate a single colony of the plasmid harbouring strain (e.g. MG1655 pKD46 transformant) in 10 ml LB media supplemented with the appropriate antibiotics.
2. Incubate the culture overnight at 30°C with agitation, 200 rpm.
3. Collect 1.5 ml of the culture in a 1.5 ml microfuge tube.
4. Centrifuge the sample at 13,000 x g for 5 min to pellet the cells.
5. Decant the supernatant media and re-suspend the cell pellet in 100 µl of Alkaline Lysis Solution I. The final concentrations of the components in solution I is as follows: 50 mM glucose; 25 mM Tris-Cl, pH 8.0; 10 mM ethylene-diaminetetra-acetic acid (EDTA), pH 8.0.
6. To the re-suspended cell pellet add 200 µl of Alkaline Lysis Solution II. The final concentrations of the components in solution II is as follows: 0.2 N NaOH, 1% SDS. Solution II must be prepared fresh.
7. Mix the cell suspension by inverting the tube several times, and then incubate it on ice for 10 min.
8. To the cell suspension add 150 µl of Alkaline Lysis Solution III. The final concentrations of the components in solution III is as follows: 60 ml 5 M Potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml ddH_2O.
9. Mix the cell suspension by inverting the tube several times, and then incubate it on ice for 10 min or until a white precipitate is observed.

10. The Alkaline Lysis Solutions serve to lyse the cells and release the plasmid and chromosomal DNA. Once a white precipitate is observed centrifuge the cell mixture at 13,000 x g for 15 min.
11. Transfer the DNA rich supernatant to a fresh microfuge tube and add 1 ml of a phenol+chloroform+isoamyl alcohol solution. The composition of the phenol+chloroform+ isoamyl alcohol solution is in a ratio of 25:24:1, respectively. The solution serves to precipitate chromosomal DNA and heavy proteins.
12. Vortex the mixture for 30 sec and then centrifuge at 13,000 x g for 10 min.
13. Transfer the upper aqueous phase to a fresh microfuge tube taking care not to transfer any of the lower organic phase.
14. To the aqueous phase add 1 ml of chloroform, and vortex for 30 sec and then centrifuge at 13,000 x g for 10 min.
15. Transfer the upper aqueous phase to a fresh microfuge tube, again taking care not to transfer any of the lower organic phase.
16. To the aqueous phase add 1 ml of 100% ethanol and 50 µl of 3M sodium acetate (pH 5.2). Vortex for 30 sec and place in ice for 30 min to 1 hr. The ethanol helps precipitate DNA and the sodium acetate provides a counter ion for facilitating DNA precipitation.
17. The suspension is then centrifuged at 13,000 x g for 10 min, to pellet the plasmid DNA.
18. The supernatant is decanted and the resulting pellet is washed once with 1 ml of 70% ethanol.
19. Again, the suspension is then centrifuged at 13,000 x g for 10 min, to pellet the plasmid DNA.
20. The supernatant is removed and the resulting DNA pellet is re-suspended in 50 µl TE buffer containing 20 µg/ml of DNase-free RNase A. The final concentrations of the components in TE buffer are as follows: 10 mM Tris-Cl, pH 8.0; 1 mM EDTA, pH 8.0.

The DNA pellet should contain the pKD46 plasmid DNA. This can be confirmed by running a sample of the plasmid extraction on an agarose gel (see Visualizing DNA by agarose gel electrophoresis). The pKD46 plasmid is very large and should migrate to a band size approximating 10 Kb.

Transforming the PCR amplified GDF into a host strain harbouring the pKD46 plasmid:

Expression of the RED system is necessary for 1. inhibiting the host exonuclease V and 2. facilitating homologous recombination between the PCR amplified GDF (e.g. GDF specific for disrupting *argR*) and the chromosomal copy of the gene of interest (e.g. *argR*). Since the RED system is under the control of *araBp*, exogenous L-arabinose has to be supplemented to induce RED expression. Only when host cells are expressing the RED system can the PCR amplified GDF be transformed. If the host cells are not expressing the RED

system, the PCR amplified GDF will be degraded by the host exonuclease V, and successful gene-disruption-mutants will not arise. For successful incorporation of the GDF into the host chromosome the host strain harbouring pKD46 has to 1. express the RED system and must 2. be made electrocompetent to allow transformation with the PCR amplified GDF. This is accomplished as follows:

1. Prepare an overnight culture of the pKD46 transformant (e.g. MG1655-pKD46). Inoculate 10 ml LB media supplemented with 100 μg/ml ampicillin and incubated overnight at 30°C, with agitation 200 rpm.
2. The following day, subculture 1/250 in 50 ml antibiotic-free LB media supplemented with 20 mM L-arabinose. Incubate at 30°C with agitation, 200 rpm, until an OD600 of 0.4 is reached. The L-arabinose induces expression of the RED system encoded on pKD46.
3. Prepare competent cells by following steps 3 through 9 of the protocol "Preparing competent cells for electroporation".
4. Keeping all cells and reagents on ice, mix 50 μl of competent cells (from a 10^{10} cells/ml stock) with 2 μl of the PCR amplified GDF (from a 50 ng/μl stock) (e.g. GDF specific for disrupting *argR*) in an electroporation cuvette.
5. Insert the cuvette into the electroporator and follow the manufacturer's operating instructions. If you are using a Bio-Rad Gene Pulser II, set the voltage to 2.25 volts and capacitance to 25 μF and electroporate.
6. Add 1 ml of LB media to the electroprated cell+DNA mixture, re-suspend the mixture in the media and transfer to a 15 ml polypropylene tube.
7. Incubate the mixture for 1 h at 37°C with agitation, 200 rpm.
8. Plate 100 μl of the cell mixture on solid LB agar media supplemented with 25 μg/ml chloramphenicol.
9. Incubate overnight at 37°C and select for chloramphenicol resistant transformants. The transformants that arise will be gene-disruption-mutants e.g. MG1655-*ΔargR*. Successful incorporation of the GDF into the host chromosome can be confirmed by PCR amplification using confirmational primers (see Confirming successful incorporation of the GDF into the host chromosome by PCR amplification using confirmational primers).

Confirming successful incorporation of the GDF into the host chromosome by PCR amplification using confirmational primers:

To confirm that the GDF disrupts exclusively the gene of interest and is not inserted anywhere else in the host chromosome; a PCR amplification must be performed using a set of confirmational primers. As an example, to authenticate the deletion of the chromosomal copy of *argR* in the chloramphenicol transformants, the confirmational primers indicated in Table B4 can be used:

Primer	Sequence (oriented 5' to 3')	Melting Temperature
Forward	CAATAATGTTGTATCAACCACCATATCGGGT GACTTGTGTAGGCTGAAGCTGCTTC	63.9°C
Reverse	TGTCGCAGTAAAACGCACTA	62.9°C

Table B4: Sequence of primers used to confirm incorporation of the GDF into *argR*. The forward primer used to amplify the GDF will also serve as the forward confirmational primer. The reverse confirmational primer is complementary to a region 100 bp downstream of *argR*. The melting temperatures of each primer are also indicated.

1. To confirm incorporation of the GDF is exclusively in *argR* a PCR amplification using the above primers (see Table B4) should yield a product when using genomic DNA extracted from MG1655-*ΔargR* as DNA template. If no amplified product results, then incorporation of the GDF was not successful. Add the necessary components to a PCR thermo-tube as indicated in the protocol "PCR amplifying DNA" (see above). Use genomic DNA extracted from MG1655-*ΔargR* as the template for the amplification.
2. Program the PCR thermocycler with the PCR conditions indicated in Table B5:

PCR Step	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	30
Annealing	57°C	30 sec	
Extension	72°C	2 min	
Terminal extension	72°C	7 min	

Table B5: PCR Thermocycler settings for amplifying the incorporated GDF that has replaced the chromosomal copy of *argR*.

3. To confirm that amplification was successful, run a sample of the PCR mixture on an agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis).
4. After amplification is confirmed, store the remaining PCR mixture at -20°C.

Transducing mutations into different strain backgrounds using P1 phage

Transduction is the transfer of bacterial genes via a bacterial virus vector (a phage vector). Using generalized $P1_{vir}$ phage transduction it is possible to transfer mutations from one strain (a donor) to another (a recipient). This is accomplished in two steps. First, a high titer lysate from the donor strain is prepared (approximately 10^{10} plaque forming units (PFU)/ml). A small population of the $P1_{vir}$ phage will have packaged donor DNA (e.g. a gene of interest fused to a selectable marker as well as other host DNA) and will not be able to complete the lytic lifecycle. These phage are called transducing particles. The transducing efficiency of $P1_{vir}$ is 10^{-6} per phage. In the second step, the recipient strain is co-incubated with the donor lysate. While most of the recipient cells will be infected with $P1_{vir}$ and lyse, a small fraction will be infected with transducing particles. Some of the recipient cells infected with the transducing particles will integrate the donor DNA into their chromosome. This event is facilitated by RecA mediated homologous recombination. The recipient cells that incorporate the donor DNA are called transductants. They can be selected for and purified on the appropriate selective media. The following protocol outlines the procedure for performing a generalized $P1_{vir}$ phage transduction:

To prepare the donor lysate:

1. Prepare an overnight culture of the donor strain. Inoculate a single colony of the donor strain (e.g. MG1655-*ΔargR*) into 5 ml of LB media supplemented with 5 mM $CaCl_2$ and appropriate antibiotics. Incubate overnight at 37°C, with agitation, 200 rpm.
2. The following day, subculture 1/100 into 10 ml of antibiotic-free LB media supplemented with 5 mM $CaCl_2$. Incubate at 37°C, with agitation, 200 rpm, until an OD600 of 0.3.
3. Divide the 10 ml culture into 2 equal 5 ml volumes. Label one "control" and other "phage".
4. Add 10^7 PFU of $P1_{vir}$ to the "phage" culture.
5. Incubate both "phage" and "control" cultures at 37°C, with agitation, 200 rpm, until lysis in the "phage" culture is evident.
6. Continue incubating for an additional 1 h to ensure complete lysis in the "phage" culture. The "control" culture should remain turbid.
7. To the lysate add 100 µl of chloroform (lyses any cells that were not lysed by phage infection).
8. Centrifuge at 5,000 x g for 15 min to pellet the cell debris.
9. Transfer the supernatant to a fresh tube taking care no to dislodge the pellet of cell debris.
10. Add another 100 µl of chloroform to the supernatant and mix by inversion.
11. Date and titre the lysate.

To titre the donor stock lysate:

1. Make a dilution series of the donor stock lysate in ddH_2O e.g. 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}.
2. Inoculate of single colony of MG1655-*ΔargR* in 10 ml of LB media supplemented with 5 mM $CaCl_2$. Incubate at 37°C, with agitation, 200 rpm, until an OD600 of 0.4.
3. For each lysate dilution (e.g. 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) add the following to a borosilicate glass tube: 100 µl of the lysate dilution and 100 µl of cells. Label each tube with the appropriate lysate dilution. Include a "no phage" control (a tube with only 100 µl of cells).
4. Vortex the cells+lysate (and appropriate controls) tubes gently.
5. Incubate the tubes in 37°C water bath for 10 min to allow the phage to preabsorb into the cells.
6. Add 3.5 ml of soft agar to each tube. Vortex briefly and pour the mixture onto pre-warmed LB agar plates.
7. Once the soft agar has solidified, incubate the plates overnight at 37°C right side up.
8. Count the plaques the following morning and estimate the titre of the lysate stock. A good titre for transductions should have approximately 10^{10} PFU/ml. The "no phage" control should be confluent.

To perform a generalized $P1_{vir}$ phage transduction:

For each recipient strain follow the procedure described below:

1. Prepare an overnight culture for the recipient strain. Inoculate a single colony of the recipient strain into 5 ml of LB media supplemented with 5 mM $CaCl_2$ and appropriate antibiotics. Incubate overnight at 37°C, with agitation, 200 rpm.
2. The following day, centrifuge at 5,000 x g for 15 min to pellet the cells.
3. Re-suspend the cell pellet in 250 µl of LB media supplemented with 5 mM $CaCl_2$.
4. Label three 15 ml polyethylene tubes "virus control (V)," "cell control (C)," and "cell+virus (CV)".
5. Add 100 µl of recipient cells to the C and CV tubes.
6. Add 100 µl of donor lysate (e.g. prepared from MG1655-*ΔargR*) to the V and CV tubes. For a successful transduction the lysate should have a titre of approximately 10^{10} PFU/ml.
7. Gently mix suspensions by tapping the sides of the tube (do not vortex). Incubate the suspensions in a 37°C water bath for 10 min to allow the phage to preabsorb into the cells.

8. Add 12 ml of LB media supplemented with 100 mM sodium citrate to each tube. The sodium citrate prevents the re-infection of potential transductants. Re-suspend the contents in each tube and centrifuge at 5,000 x g for 10 min.
9. Remove the supernatant and re-suspend the cell pellet in another 12 ml of LB media supplemented with 100 mM sodium citrate. Centrifuge at 5,000 x g for 10 min to pellet the cells and, repeat the wash once more.
10. Add 12 ml of LB media supplemented with 100 mM sodium citrate to each tube. Re-suspend the pellet in the media and incubate at 37°C for 30 min on a rotating wheel.
11. Centrifuge each tube at 5,000 x g for 10 min.
12. Decant the supernatant, and re-suspend the pellet in the small volume of media remaining in the tube.
13. Plate 100 µl of the suspension on appropriate selective media to select for transductants. (If you are transducing the *ΔargR* mutation as described above plate on LB agar supplemented with 25 µg/ml of chloramphenicol).
14. Incubate the plates overnight at 37°C.
15. Isolate and purify any transductants that arise the following morning. There should be no growth on the virus control (V) and the cell control (C) plates.

ΔargR mutants fail to grow in minimal media unless supplemented with an exogenous source of pyrimidines

The arginine biosynthetic pathway and the pyrimidine biosynthetic pathway share a common biosynthetic intermediate, carbamoyl-phosphate. Since *ΔargR* mutants, constitutively express arginine biosynthetic genes, there is a constitutive synthesis of arginine *de novo*. This uncontrolled synthesis of arginine requires depleting the cell's carbamoyl-phosphate reserves. A depletion of these reserves will result in a lack of pyrimidine biosynthesis, a pathway that is necessary for growth. The procedure below, describes how *ΔargR* mutants were shown to develop a nutritional requirement for pyrimidines:

1. Prepare overnight cultures: inoculate the *ΔargR* mutants and the appropriate control strains (*argR*$^+$ strains) in a microtitre plate. Fill the wells in the microtitre plate with MM supplemented with the appropriate antibiotics. Inoculate all strains in triplicate. Incubate the microtitre plate overnight at 37°C.
2. The following morning, subculture 1/100 into another microtitre plate with fresh MM.
3. Prepare a series of MM agar plates supplemented with 20 µg/ml of 1. thymine only, 2. cytosine only, 3. uracil only, and 4. a mixture of all 3 pyrimidines. Include a control MM agar plate that lacks pyrimidines.
4. Replica plate the cultures onto the pyrimidine supplemented MM agar plates.
5. Incubate the plates at overnight at 37°C.

6. Assess the growth of each strain the following day. The *ΔargR* mutants should not grow on MM agar plates unless the MM is supplemented with an exogenous source of pyrimidines. The control strains (*argR*$^+$) should grow on all MM plates.

Measuring gene expression by Northern blot analysis

Northern blotting is a technique used to measure gene expression. The messenger RNA (mRNA) produced from a gene of interest is tagged by a radio-labeled probe, allowing one to quantify expression of that gene. This is a multi-step process. First, genomic RNA is extracted from cells. The RNA integrity is assessed (if the RNA is too degraded then the sensitivity of the detection becomes poor) before proceeding to the second step where the isolated RNA is run on a denaturing agarose gel by electrophoresis. Third, the RNA is transferred to a membrane (blotting) and is fixed by UV cross-linking. Next, a radio-labeled probe specific to the RNA sequence of interest is prepared. The membrane-bound RNA is then incubated overnight with the radio-labeled probe. This will allow the probe to hybridize to the RNA sequences of interest. The membrane is then washed to remove unspecific binding of the probe. The membrane is then exposed to a phosphorus screen to assess the intensity of the radio-labeled signal. The intensity of the signal is proportional to the expression of the gene of interest. A more detailed description of this procedure is outlined below. The procedure for measuring the RpoS dependence and growth phase dependence of *argH* by Northern analysis is used as an example.

Growth conditions for RNA isolation (for each strain):

1. Prepare an overnight culture (e.g. use an RpoS$^+$ and RpoS$^-$ strain for assessing RpoS dependent gene expression for e.g. *argH*)): inoculate a culture tube with a single colony. The tubes are filled with LB media with the appropriate antibiotics.
2. Grow each culture overnight at 37 °C and agitate at 200 rpm.
3. The following day, subculture 1/1000 into a culture flask with antibiotic-free LB media.
4. Incubate at 37 °C, agitate at 200 rpm, and monitor growth spectrophotometrically. Collect exponential phase samples when an OD600 of 0.3 is reached and stationary phase samples at an OD600 of 1.5. Collect 5 ml and 2 ml of cultures for exponential and stationary phase samples, respectively. Collect the samples in 15 ml polypropylene conical tubes. Work quickly and place all tubes on ice.
5. Add 2 volumes of pre-chilled RNA*later*™ (Ambion, Inc.) solution to each tube. RNA*later*™ is a bacteriostatic agent that quickly permeates cells to stabilize and protect cellular RNA.

6. Mix the contents in each tube by inverting the tubes several times. Place the tubes on ice for 10 min, and then pellet the cells by centrifuging at 4°C.
7. For each tube, decant the supernatant, and flash freeze the cell pellet in liquid nitrogen.
8. Store the samples at -80°C or use immediately for RNA extraction (see protocol for RNA extraction using hot acidified phenol).

Extracting RNA using hot acidified phenol (Sigma-Genosys, Inc.):

Make sure all pipette tips and microfuge tube are RNase-free. Use RNaseZap® (Ambion, Inc.) wipes to sterilize glassware and the work area. All aqueous based solutions should be prepared in (diethyl pyrocarbonate) DEPC treated ddH_2O. DEPC is an RNase inhibitor. Add a final concentration of 1% DEPC to inhibit RNases in the solution. Incubate the solution at 37°C overnight, and autoclave on a liquid cycle the following day.

1. For each sample, centrifuge and pellet the cells for RNA extraction as described above. Remove all traces of growth media. For each sample, re-suspend the pellet and add 250 µl of Re-suspension Buffer and 37.5 µl of 0.5 M EDTA. Make sure both solutions are pre-chilled on ice. The final concentrations of the components in the Re-suspension Buffer are as follows: 0.3 M sucrose; 10 mM sodium acetate, pH 4.2.
2. Transfer each sample to a 2.0 ml microfuge tube. Incubate each tube on ice for 10 min.
3. To each sample add 375 µl of Lysis Buffer and vortex vigorously. The contents inside the cells, including the total RNA are released when the cells come in contact with the Lysis Buffer. The final concentrations of the components in the Lysis Buffer are as follows: 2 % SDS; 1% β-mercaptoethanol; 10 mM sodium acetate, pH 4.2.
4. Incubate the samples at 65°C for 3 min in a heating block.
5. Perform a phenol extraction: add 700 µl of pre-warmed 65°C acidic phenol (acidic phenol is buffered with 0.1 M citrate acetate, pH 4.3) to each sample. Vortex the contents vigorously and incubate the samples at 65°C for 3 min.
6. Cool the samples on ice for 3 min. Centrifuge at 12,000 x g for 5 min and separate the phases. Transfer the upper RNA containing aqueous phase to a fresh 2.0 ml microfuge tube. Take care not to transfer any of the lower organic phase. The phenol containing organic phase will trap unwanted proteins and chromosomal DNA.
7. Repeat the phenol extraction twice more (steps 5 and 6).
8. Add 700 µl of a acidic phenol:chloroform:isoamyl alcohol mixture (25:24:1) to the RNA rich aqueous phase. Vortex, and centrifuge at 12,000 rpm for 5 min to separate the phases.

9. Transfer the upper aqueous phase to a fresh 2.0 ml microfuge tube and add an equal volume of a chloroform:isoamyl alcohol mixture (24:1) for a final extraction. Vortex, and centrifuge at 12,000 rpm for 5 min to separate the phases.
10. Transfer the aqueous phase to a fresh 2.0 ml microfuge tube taking care not to transfer any of the organic phase.
11. Add a 0.1 volume of pre-chilled 3 M sodium acetate (pH 5.2) to each sample, and vortex gently. The sodium acetate provides a counter ion to facilitate precipitation of nucleic acids.
12. Add 2 volumes of pre-chilled ethanol to each sample, and vortex gently. The ethanol aids in precipitating nucleic acids.
13. Incubate each sample at -20°C for 1 hr to aid in precipitating nucleic acids.
14. To pellet the RNA, centrifuge all samples at 12,000 x g for 1 hr, at 4°C.
15. Discard the supernatant and re-suspend the RNA pellet in 1 ml pre-chilled 70% ethanol by gently inverting the tube (do not vortex, this may cause shearing).
16. Centrifuge all samples at maximum speed for 15 min, at 4°C, to pellet the RNA.
17. Discard the supernatant by aspiration, taking care not to dislodge the RNA pellet. Air-dry the RNA pellet for 15 min.
18. Re-constitute the RNA pellet in 20-30 µl of RNase-free ddH_2O or TE buffer. If necessary, incubate the pellet in a 37°C water bath to facilitate dissolving.
19. Quantify the concentration of RNA spectrophotometrically by measuring the absorbance at OD260 of an appropriate dilution e.g. 5 µl of sample in 995 µl of RNase-free ddH_2O. RNA concentration in µg/ml is calculated as follows: 40 x dilution factor x sample absorbance at OD260.
20. To determine extent of chromosomal DNA contamination and the integrity of the RNA, run a small quantity of the sample e.g. 1 or 2 µg on a 1.2% non-denaturing agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis). The 16S and 23S ribosomal RNA bands should be clearly visible once stained with ethidium bromide.
21. Store the RNA samples at -80°C to prevent degradation.

Running RNA on a denaturing formaldehyde agarose gel:

Prepare a denaturing formaldehyde agarose gel in 1 x MOPS (3 (N-morpholino) propanesulfonic acid) buffer. First, pre-warm (1) 15 ml of 10 x MOPS and (2) 8.75 ml of a 36% formaldehyde solution to 55°C. Second, dissolve 1.5 g of agarose in 125 ml of RNase-free ddH_2O and allow it to cool to 55°C. Then, mix the MOPS, formaldehyde, and agarose solutions together with 30 µl of a 0.1 µg/ml ethidium bromide stock and set the gel. Final concentrations in the gel should be: 1% agarose, 0.7 M formaldehyde, and 1 x MOPS. The formaldehyde denatures RNA and prevents secondary structures from forming.

The ethidium bromide allows one to visualize RNA under a UV trans-illuminator. Final concentrations of the components in 10 x MOPS buffer are as follows: 0.2 M MOPS, pH 7.0; 20 mM sodium acetate; 10 mM EDTA, pH 8.0.
To prevent secondary structures from forming, RNA samples are prepared as follows before loading them on to the gel:

In Northern blots, typically 5 μg of RNA are run for each sample to obtain a good signal.

Reagents to add to a sample volume of 30 μl (Only stock concentrations are indicated)	**Volume (μl)**
1. Formaldehyde (36%)	5.5
2. Formamide (100%)	15
3. 10 x MOPS	1.5
4. 10 x Loading dye (concentration of components in the 10 x Loading dye are as follows: 50% glycerol; 10 mM EDTA, pH 8.0; 0.25% bromophenol blue. All components are dissolved in RNase-free ddH_2O.	3
5. RNA sample e.g. 5 μg/ml.	1 (add the appropriate volume of sample for a total 5 μg)
6. RNase-free ddH_2O	4 (or add the appropriate volume for a total 30 μl)

7. Place the samples in a 55°C water bath for 15 min to denature the mRNA before loading them onto the gel.
8. Run the gel at 50 V, 400 A for 3 h. Use RNase-free 1 x MOPS as the running buffer.
9. Check the RNA integrity of the samples by exposing under an UV trans-illuminator. The 16S and 23S ribosomal RNA bands should be clearly visible.
10. With a sterile scalpel, trim the edges of the gel and cut out the region that contains your samples. Measure the dimensions of the gel and record them.

Blotting onto a nylon membrane:

Examples of blotting membranes include nitrocellulose and positively charged nylon. The following protocol uses positively charged nylon as the membrane of choice.

1. Cut out a sheet of positively charged nylon that is 3 mm shorter than the gel dimensions.
2. Cut 10 sheets of chromatography paper, each 7 mm shorter that the dimensions of the membrane. The chromatography paper will provide the necessary capillary pressure to draw the RNA onto the membrane during the blotting process.
3. Cut a 3-inch stack of dry paper towels. The paper towels should be 3 mm shorter that the chromatography paper dimensions. The paper towels will also provide the necessary capillary pressure during the blotting process.
4. Soak the gel in 10 gel volumes of RNase-free ddH_2O for 15 min, with gentle agitation. This will remove some of the MOPS in the gel.
5. Transfer the gel into 10 gel volumes of RNase-free 10 x SSC and allow it to soak for 20 min, with gentle agitation. The SSC will serve as a transfer buffer in later steps. The concentration of the components in a 20 x SSC stock solutions are: 3 M NaCl; 0.3 M sodium acetate. Adjust the pH of the solution to 7.0 with concentrated HCl.
6. Transfer the gel into a fresh 10 gel volumes of RNase-free 10 x SSC and soak again for 10 min, with gentle agitation.

Set up the transfer apparatus as follows:

1. Fill a glass trough 2 inches deep with RNase-free 20 x SSC. This will serve as the transfer buffer.
2. Soak 2 sheets of chromatography paper in the buffer and spread it across a glass plate over the middle of the trough. The glass plate will support the gel during transfer. The ends of the chromatography paper should be in contact with the 20 x SSC buffer. The 2-sheet thick chromatography paper will serve as a wick to transfer the buffer from the trough to the gel.
3. Remove any air bubbles that are trapped between the solid support and the wick by "rolling them out" with a pipette.
4. Transfer the gel onto the center of the wick support, well side up.
5. Remove any air bubbles that are trapped between the wick and the gel.
6. Carefully place the nylon membrane on top of the gel. Make sure that it covers all sample-carrying lanes, and it does not droop over the sides of the gel.
7. Remove any air bubbles that are trapped between the gel and the membrane.
8. Soak 1 of the 10 chromatography paper sheets (that are 7 mm shorter than the membrane) and carefully place it on top of the membrane. Make sure it does not droop over the sides of the membrane.
9. Remove any air bubbles that are trapped between the membrane and the sheet of chromatography paper.
10. Carefully place the remaining 9 sheets of chromatography paper on top of the membrane. Make sure none droop over the sides and make contact with the gel.

11. Place the 3-inch stack of dry paper towels on top of the stack of chromatography paper. Again, make sure none of the paper towels droop over the sides and make contact with the gel.
12. Put a glass plate on top of the stack of paper towels. Add a weight on top of the glass plate to facilitate the necessary capillary pressure to draw the RNA from the gel onto the membrane.
13. Cover the entire transfer apparatus in Saran Wrap to reduce evaporation of the 20 x SSC transfer buffer.
14. Blot over night.

Fixing the transferred RNA onto the membrane:

The RNA can be fixed to the membrane by baking for 2 h at 80°C. A much quicker alternative is to UV cross-link the nucleic acids to the membrane. This procedure is outlined below:

1. De-stack the gel, and discard of the paper towels and chromatography paper.
2. Cut a small triangular-piece off the top left corner and with a pencil, lightly mark the wells on the membrane. This will facilitate identifying the orientation of the samples in later steps.
3. Carefully peel the membrane away fro the surface of the gel and place it on a sheet of blotting paper, RNA side up.
4. Place the membrane into a UV cross-linker irradiate the blot at 254 nm for 1 min 45 sec at 1.5 J/cm^2.
5. Soak the blot in RNase-free ddH_2O for 10 min to remove excess salts from the 20 x SSC buffer.
6. Store the blot at -80°C in between two sheets of overhead film, or proceed with the appropriate pre-hybridization steps.

Amplifying the probe for detecting *argH* transcript levels:

The length of a typical probe for detecting bacterial transcripts by Northern analysis ranges from 150 bp to 250 bp. The probe has to be exclusively complementary to the mRNA of interest to reduce non-specific binding to other RNA species. The primers indicated in Table B6 will be used to PCR amplify a probe specific for quantifying *argH* transcript levels.

Primer	Sequence (oriented 5' to 3')	Melting Temperature
Forward	CTGCGCTTTGATTACCGTCT	60.4°C
Reverse	GCAGCTTTTTGCCTAACTGG	60.0°C

Table B6: Primers used to amplify a probe used for quantifying *argH* transcript levels by Northern blotting. The melting temperatures of each primer are also indicated.

The resulting probe will be complementary to region which spans from bp 67 to bp 311 of the *argH* coding sequence. The total length of the probe will be 244 bp.

1. Add the necessary components to a PCR thermo-tube as indicated in the protocol "PCR amplifying DNA" (see above). Use genomic DNA extracted from MG1655 as the template for the amplification, and the primers indicated in Table B6.

2. Program the PCR thermocycler with the PCR conditions indicated in Table B7:

PCR Step	Temperature	Duration	Number of Cycles
Initial denaturation	95°C	2 min	
Denaturation	95°C	30 sec	30
Annealing	55°C	30 sec	
Extension	72°C	2 min	
Terminal extension	72°C	7 min	

Table B7: PCR Thermocycler settings for amplifying a probe for quantifying *argH* transcript levels.

3. To confirm that amplification was successful, run the PCR mixture on an agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis). In subsequent steps the PCR amplified probe will have to be extracted from the gel. To maximize the probe yield obtained from the gel extraction procedure load as much as possible into the well.

Extracting the PCR amplified probe from an agarose gel:

1. Use a sterile scalpel and excise the band that corresponds to PCR amplified probe from the agarose gel. Try to trim away as much of the agarose as possible.
2. Collect the gel fragment that holds the band of interest in a pre-weighed 1.5 ml microfuge tube.
3. Weigh the microfuge tube and record the weight of the agarose gel fragment for subsequent steps.
4. Use a commercially available gel extraction kit e.g. QIAEX II Gel Extraction Kit (150) (Qiagen, Inc.; CAT# 20021) to isolate the PCR amplified probe from the agarose gel. Follow the manufacturer's directions to complete the gel extraction.
5. Store the purified probe in an appropriate buffer at -20°C or proceed with radio-labeling (see below).

Radio-labeling the gel-extracted purified probe with ^{32}P isotope:

For detecting mRNA transcripts that are from low to medium abundance by Northern analysis, the probe used should have a radio-labeled specific activity >2 x 10^8 cpm/μg. Normally, the probe is PCR amplified with one of the dNTP in the PCR mix being radio-labeled e.g. dCTP*. The radio-labeled dCTP* will be incorporated into the probe in subsequent PCR amplifying cycles. This procedure is described in detail below:

1. PCR amplify the *argH* probe. Use genomic DNA isolated from MG1655 as template for the PCR reaction, and the primers indicated in Table B6. For a 50 μl reaction add the following to a PCR thermo-tube:

Note 1: Radio-labeled dCTP* will be used as the radio-labeled nucleotide of choice.
Note 2: A control reaction with no radio-labeled dCTP* will be run in parallel.

Reagents to add for a 50 µl PCR reaction (Only stock concentrations are indicated)	Volume (µl)	
	Radio-labeled probe	Non-radio-labeled control
Template DNA (20 µg/µl) to be amplified e.g. genomic DNA from MG1655	1	1
Forward primer (25 µM)	2	2
Reverse primer (25 µM)	2	2
A mixture of non-radio-labeled dATP, dTTP, and dGTP (10 mM for each deoxyribonucleotide)	1	1
Radio-labeled dCTP* (3000 Ci/mmol = 10 µCi/µl)	5	0
Non-radio-labeled dCTP (0.1 mM)	1	0
Non-radio-labeled dCTP (10 mM)	0	1
10 x PCR buffer stock (concentration of components in the 10 x PCR buffer stock obtained from Fermentas are as follows: Tris-Cl (pH 8.8), 750mM; $(NH_4)_2SO_4$, 200mM; Tween 20, 0.1%).	5	5
$MgCl_2$ (25 mM)	3	3
Taq DNA polymerase (1 unit/µl)	2	2
ddH_2O	add the appropriate volume for a total 50 µl)	add the appropriate volume for a total 50 µl)

2. Program the PCR thermocycler with the PCR conditions indicated in Table B7 (see above).
3. To confirm that amplification was successful, run a sample of the non-radio-labeled control PCR mixture on an agarose gel (see protocol for Visualizing DNA by agarose gel electrophoresis). If the amplification was successful proceed with step 4, otherwise repeat from step 1 (you may try altering PCR conditions or use different primers, to obtain the desired PCR amplified product).
4. Use a commercially available probe purification kit e.g. ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, Inc.; Product # 27-5335-01) to purify the PCR amplified radio-labeled probe. Follow the manufacturer's directions to complete the probe purification. Remember to save a small aliquot (2-5 µl) of your PCR reaction mix before completing this step. It will be used to determine the percentage incorporation of the radio-labeled dCTP* into the probe in the subsequent step.

5. To quantify the percentage incorporation of the radio-labeled dCTP* into the probe: Fill 2 scintillation vials with 10 ml of scintillant. Add 2-5 µl of your PCR reaction mix before probe purification to the first vial, label the vial "before purification". Add the same volume of the purified probe to the second vial, label the vial "after purification".
6. Invert the vials several times to mix their contents. Read the cpm of each vial in a scintillation counter. Determine the percentage incorporation using the following formula:

$$\text{Percentage incorporation} = \frac{\text{cpm of probe "after purification"}}{\text{cpm of PCR mix "before purification"}} \times 100$$

Ideally, for a probe to yield a strong signal the percentage incorporation should be at least 50%. To prevent excessive radioactive decay of the incorporated isotope, proceed immediately (or as soon as possible) to the hybridizing procedure (see protocol for Pre-hybridization and hybridization conditions).

Pre-hybridization and hybridization conditions:

Blots are washed in a hybridization buffer to facilitate binding of the probe to mRNA sequences of interest. A preparatory step includes washing the blot in the hybridization buffer for at least 2 h at 68°C. This is a pre-hybridization step, which reduces non-specific binding of the probe to surfaces of the membrane that are not cross-linked to mRNA. After pre-hybridization the probe is added directly to the hybridization wash and incubated with the blot. This will allow the probe to bind to the mRNA species of interest. All washes are typically performed at 68°C to prevent precipitation of the SDS component in the hybridization buffer. Below is a more detailed description of this procedure:

Note: At no point in the procedure should the blot dry out!

1. Carefully insert the blot into an appropriate hybridization tube. Make sure the RNA side is not against the sides of the tube. The RNA side of the blot must be in direct contact with the hybridization buffer.
2. Add 10 ml of hybridization buffer into the tube. Concentrations of the ingredients in the hybridization buffer are as follows: 0.5 M phosphate buffer, pH 7.2; 7% SDS; 10 mM EDTA. All solutions are made in RNase-free ddH_2O. Pre-warm the hybridization buffer to 68°C before adding it to the blot this will prevent the SDS from precipitating.
3. Incubate the blot at 68°C in an appropriate shaker or rotisserie device for 2 h.
4. Denature the radio-labeled probe by adding 10% v/v of 3 N NaOH. Incubate the mixture at room temperature for 6 min.

5. If the percentage incorporation of the radio-labeled nucleotide in the probe is high (i.e. >50%) add 20 µl to the denatured probe into the hybridization buffer. If the incorporation is < 50% add a larger volume (30 µl – 50 µl).
6. Incubate the blot at 68°C in an appropriate shaker or rotisserie device for 20 h. This will allow the probe to hybridize to the blot.

Eliminating radio-labeled probe that is non-specifically bound to the blot:

1. Prepare the following stringency washing solutions in RNase-free ddH_2O: Low stringency wash - 2 x SSC, 0.1 % SDS; Moderate stringency wash – 1 x SSC, 0.1 % SDS; High stringency wash – 0.25 x SSC, 0.1 % SDS.
2. Remove the blot from the hybridization tube, and place it into a plastic tray. Fill the tray an inch deep with the Low stringency wash - 2 x SSC, 0.1 % SDS. Incubate the blot at 30°C for 15 min with gentle agitation. This will remove traces of the hybridization buffer, and wash-off loosely bound probe from the blot.
3. Decant the Low stringency wash. Repeat the wash twice more.
4. Decant the Low stringency wash and immerse the blot in an inch deep of the Moderate stringency wash - 1 x SSC, 0.1 % SDS. Incubate the blot at 30°C for 15 min with gentle agitation. This will remove non-specifically bound probe from the blot.
5. Decant the Moderate stringency wash. Repeat the wash once more.
6. Decant the Moderate stringency wash and wash the blot one final time with the High stringency wash – 0.25 x SSC, 0.1 % SDS. Incubate the blot at 30°C for 15 min with gentle agitation.

Developing the signal using a phosphorous imaging screen:

1. Cut a sheet of overhead film that is 1 cm larger than the dimensions of the blot. This will serve as a support for the blot to simplify subsequent manipulations.
2. Carefully remove the blot from the High stringency wash and place it in the center of the overhead film, RNA side up.
3. Remove any trapped air bubbles between the overhead film and the blot by carefully "rolling then out" with a pipette.
4. Seal the blot with a sheet of Saran Wrap.
5. Remove any trapped air bubbles between the blot and Saran Wrap. Avoid making wrinkles in both the Saran Wrap and in the blot.
6. Tape the sealed blot, RNA side up, on an appropriate cassette support.
7. Position a phosphorus screen over the blot and close the cassette shut. Allow a signal to develop overnight.
8. Use the appropriate scanning apparatus to develop the resulting image on the phosphorous screen.

9. Evaluate the signal intensity of the probe that develops on the phosphorous screen. If the signal intensity is too weak, extend the incubation time accordingly, and re-develop.

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