REGULATION OF CYSTEINE BIOSYNTHESIS IN *ACINETOBACTER BAYLYI* ADP1

by

STEPHANIE THURMOND

(Under the Direction of Ellen Neidle)

ABSTRACT

As part of a large-scale regulatory investigation, cysteine biosynthesis was examined in a soil bacterium, *Acinetobacter baylyi* ADP1. This pathway involves the reductive assimilation of sulfate to meet the essential biological requirement for sulfur. As reported here, there are significant differences in genetic organization, enzymatic steps, and transcriptional regulation in cysteine biosynthesis by *A. baylyi* when compared to well-characterized enteric bacteria such as *Escherichia coli.* To identify the role of a LysR-type regulator, CysB (encoded by ACIAD2597), gene expression was assessed with transcriptional reporters, and mutations in the chromosomal *cysB* gene were generated and characterized. Electrophoretic mobility gel shift assays were used to assess protein-DNA interactions. In addition, growth in defined media was used to characterize the ability of the wild type and mutant strains to utilize a wide range of diverse sulfur sources. These studies have broader implications for biotechnology, metabolic engineering, and novel approaches to drug development.

INDEX WORDS: Cysteine biosynthesis, *Acinetobacter*, sulfur assimilation, LysR-type transcriptional regulator, CysB

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LIST OF ABBREVIATIONS

LTTR	LysR-Type Transcriptional Regulator
APS	Adenosine 5'-phosphosulfate
PAPS	Phosphoadenosine 5'-phosphosulfate
OAS	O-acetylserine
NAS	N-acetylserine
MESNA	2-mercaptoethane sulfonate
EMSA	Electrophoretic mobility shift assay
ORF	Open reading frame
DBD	DNA-binding domain
EBD	Effector-binding domain
Km	Kanamycin
Sp	Streptomycin
Sm	Spectinomycin
4MU	4-methylumbelliferone
IPTG	Isopropyl ß-D-1-thiogalactopyranoside

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Sulfur is required for survival in all domains of life. It is an essential constituent in many biologically important compounds, and the ability to utilize environmentally available forms of sulfur is indispensable. In the aerobic biosphere, inorganic sulfur is available in oxidized forms such as sulfate, and incorporation into organic molecules first requires reduction to sulfide. While many anaerobic prokaryotes reduce sulfate during respiration, producing excess sulfide as waste, plants and aerobic bacteria produce only enough reduced sulfur for biosynthetic purposes (1). In vital sulfur-containing cell compounds such as amino acids, lipoic acid, coenzyme A, and glutathione, the sulfur comes predominantly from L-cysteine, which is synthesized in the cell through the uptake, reduction, and assimilation of oxidized sulfur compounds. Cysteine is therefore central to many different cellular processes, and characterizing the biosynthetic pathway is vital to understanding basic bacterial physiology.

Background

Cysteine biosynthesis has been thoroughly examined in two Gram-negative model organisms, *Escherichia coli* and *Salmonella enterica* (2). Studies with these bacteria have resulted in detailed descriptions of the transcriptional regulation of the cysteine biosynthesis pathway, as well as the crystal structures and activities of many of the enzymes involved. Most

of the genes in the pathway are part of a regulon controlled by a LysR-type transcriptional regulator (LTTR) CysB. Cysteine biosynthesis in bacteria outside of the Enterobacteriaceae, including species of *Burkholderia, Pseudomonas, Bacillus,* and *Corynebacteria,* have been investigated to a lesser extent (3-6). These additional studies have revealed innovations in the pathway that may better reflect the diversity of the cysteine biosynthesis pathway among bacteria. However, little is known about cysteine biosynthesis in the Gram-negative *Acinetobacter baylyi* strain ADP1.

ADP1 is a ubiquitous, strictly aerobic bacterium that degrades aromatic compounds in the soil. This organism is ideal for genetic experiments in the laboratory since it is naturally transformable and easy to culture, with a fully sequenced genome available (7). In addition, ADP1 is capable of utilizing a wide range of compounds, making it an interesting model organism for the study of bacterial metabolism, and cysteine biosynthesis in particular. Furthermore, efforts are underway to characterize the structure and function of all 44 of its LTTRs to improve our understanding of this important family of regulators. It appears that one gene, ACIAD2597, encodes a LTTR that regulates cysteine biosynthesis.

The genetic organization and predicted genes of the putative cysteine biosynthesis pathway in ADP1 suggests a pattern of regulation and expression different from the well characterized regulons of *S. enterica* and *E. coli* (Figure 1.1) (2). The purpose of this study was to characterize the cysteine biosynthesis regulon in ADP1 and the role of the ACIAD2597-encoded LTTR. This gene is located at the end of a putative operon involved in the transport of sulfate and thiosulfate (Figure 1.1), and the protein sequence has significant similarity with CysB (see Chapter 2). In current databases, ACIAD2597 is annotated as *cbl* for "CysB-like" (7), a designation used in *E. coli* for a paralog of CysB (8). However, no other CysB paralog is

encoded by the ADP1 chromosome, suggesting the name "CysB" is most appropriate for ACIAD2597.

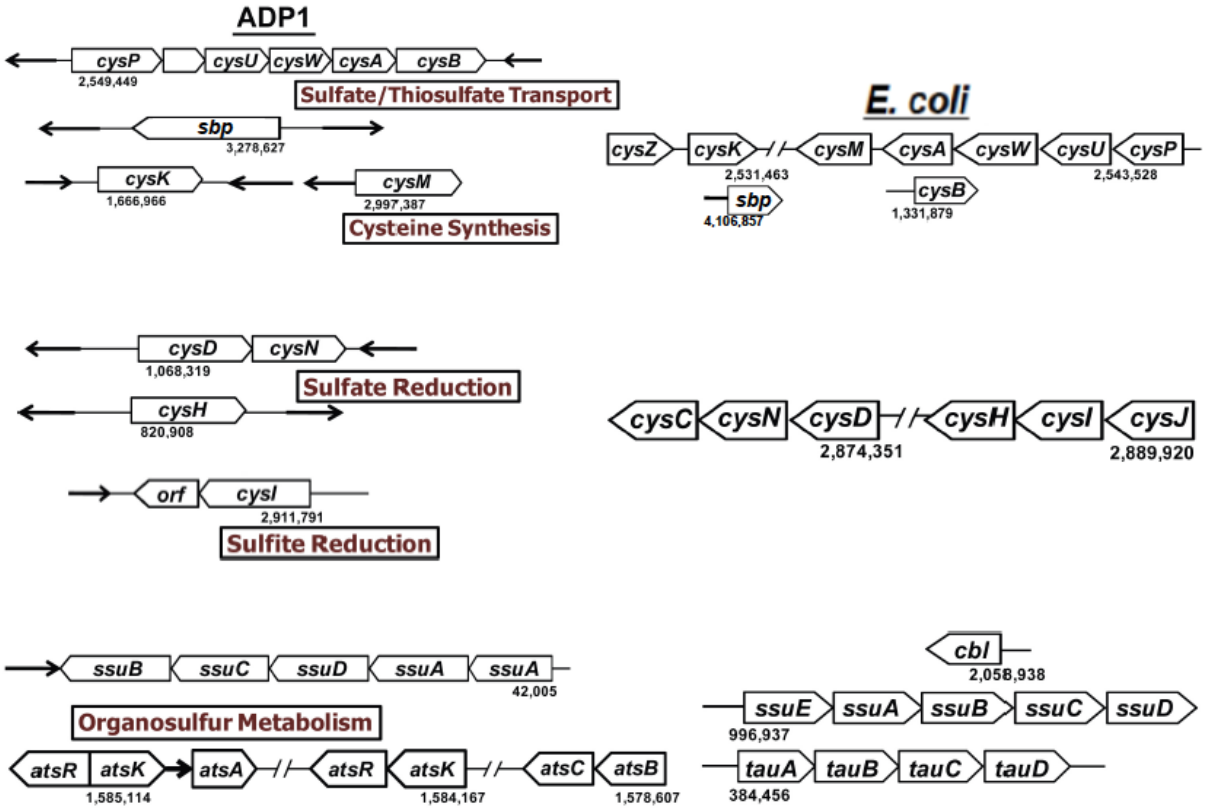


Figure 1.3. ***Cys*** **gene organization in ADP1 and** ***E. coli*****.** The genes of the cysteine biosynthesis pathway are scattered through the genome.

Cysteine Biosynthesis Pathway and Gene Organization

i. Transport

Cysteine is the end product of a multi-step pathway that begins with the transport of oxidized sulfur into the cell. In the case of sulfate and the similarly structured thiosulfate, there are four known transporters in bacteria: carriers belonging to the SulT family; the SulP family; the CysP transporter, belonging to the PiT family; and the CysZ transporter (Figure 1.2) (9). According to the Transporter Classification Database (TCDB), the SulT family is named for the

transport of sulfate and tungstate, but members of this family are capable of transporting several different oxyanions, including thiosulfate (10). In *E. coli* and *S. enterica*, the SulT sulfate-thiosulfate permease consists of the ABC-type transporter CysUWA coupled with the periplasmic permeases SBP (Sulfate Binding Protein) or CysP (thiosulfate binding protein). Despite their names, CysP and SBP have overlapping functions, capable of transporting both sulfate and thiosulfate, and both can function with CysUWA (11). CysU and CysW are membrane proteins of the SulT permease that form a channel for the passage of sulfate and thiosulfate, and CysA is a membrane-associated ATPase (12).

Subunits of the SulT transporter are encoded by operons *cysPUWA* or *sbpcysUWA*. This organization is widespread among bacteria, with the *sbpcysUWA* configuration common in *δ-, ε-*proteobacteria, actinobacteria, firmicutes, and cyanobacteria as the only SulT genes. In contrast, most members of the *α-, β-,* and *γ*-proteobacteria have both *sbpUWA* and *cysPUWA,* or *cysPUWA* with *sbp* unlinked (9). The latter organization is the case in *E. coli, S. enterica*, and ADP1. *B. subtilis* contains another CysP, distinct from the CysP discussed above, that is part of the PiT family (inorganic phosphate transporter). This membrane-associate protein has homology with the *E. coli* PitA (a phosphate permease), but no homology with known sulfate transporters (13). CysP has been shown to be responsible for sulfate, but not thiosulfate, transport in *B. subtilis*, and phosphate transport has not been demonstrated.

CysZ homologs are present in *Pseudomonas* species*, S.enterica, E. coli,* and *C. glutamicum*, but not in ADP1. However, experimental evidence for CysZ function is only available for *E. coli* and *C. glutamicum*, and the results are conflicting. In *E. coli,* CysZ is a high-affinity, high-specificity sulfate transporter that is inhibited by sulfite. It is essential under low sulfate conditions, even with SBP and CysP still present in the genome (14). However,

studies with *C. glutamicum* indicated that CysZ is required for both sulfate and sulfite transport (15).

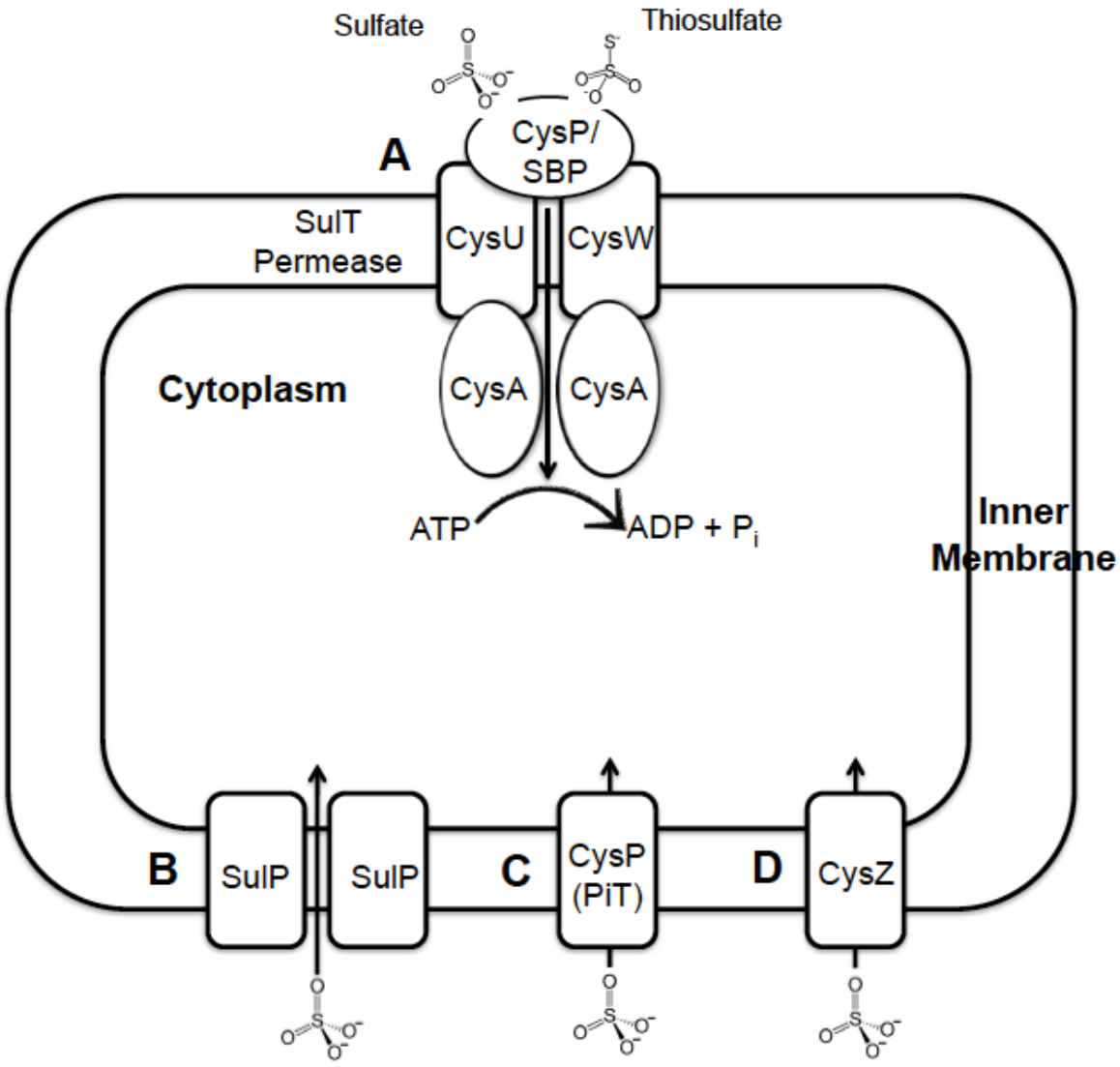


Figure 1.2. Sulfate Transport in Bacteria. A) The SulT permease consists of sulfate- and thiosulfate-binding proteins SBP or CysP, the membrane proteins CysU and CysW, and the ATPase CysA. B) SulP transporter. C) CysP transporter from the Pit family. D) CysZ sulfate:H^+ symporter. Adapted from (9).

While sulfate is the preferred sulfur source for bacteria, aerobic soils contain sulfonates and sulfate esters in much higher abundance (16). *E. coli* is able to use a wide range of sulfonates, and entrance of these compounds into the cell is mediated by the ABC-type transporter SsuABC. The *ssuBC* genes code for membrane ATP-binding proteins, and *ssuA* codes for a periplasmic binding protein. These genes are part of the *ssu* operon, named for "sulfate starvation utilization". Transcription is activated under low sulfate conditions, and repressed when sufficient sulfate is present (17). Transport of sulfonates likely occurs similarly in ADP1 as in *E. coli*. ADP1 contains *ssuABC* homologs located in a putative operon, and, as reported in this thesis, this bacterium is also capable of utilizing a wide range of sulfonates (this study). In fact, ADP1 has two *ssuA* paralogs, ACIAD0037 and ACIAD0038, located directly adjacent to each other that encode proteins with 39% sequence identity (Figure 1.1).

While SsuABC transports a variety of sulfonates, *E. coli ssuABC* mutants are still able to utilize taurine (2-aminoethanesulfonic acid), which contains a sulfonate group. Taurine is transported in by the ABC-type transporter TauABC, which is encoded by the genes contained in the *tau* operon (Figure 1.1). Taurine appears to be the only substrate for TauABC, and *tauABC* mutants are unable to utilize taurine while still retaining the ability to use other sulfonates. Similar to the *ssu* operon, the *tau* operon is activated under sulfate starvation conditions (18). ADP1 is able to use taurine as the sole source of sulfur (this study), and there is a homolog of the *E. coli* gene *tauB*, but no homologs of *tauA* or *tauC*. The *tauB* homolog is annotated as *atcC*, and is predicted to code for a sulfate ester permease ATP-binding protein. Notably, *tauB* and *atsC* are homologous to several genes coding for amino acid transporters, including those for glutamate, arginine, methionine, glycine, and the cysteine transporter *cysA*. *AtsC* is part of the *ats* gene cluster in ADP1, which stands for "alkylsulfatase" or "arylsulfatase". This cluster also

contains other genes predicted to be involved in sulfate ester transport: *atsB*, coding for a sulfate ester permease protein, and *atsR*, coding for a periplasmic sulfate ester binding protein. Both of these genes have homologs in *Pseudomonas* species, bacteria that are able to utilize arylsulfonates and sulfate esters, unlike *E. coli* (19, 20). Similar to the gene coding for the sulfonate-binding periplasmic protein *ssuA*, ADP1 has two *atsR* paralogs, ACIAD1601 and ACIAD1593, which code for proteins with 46% sequence identity.

ii. Reduction

Following entrance into the cell, sulfate is activated by phosphorylation before undergoing reduction. The phosphorylation step is catalyzed by an ATP sulfurylase encoded by *cysDN,* which converts sulfate to adenosine 5'-phosphosulfate (APS). In many cases, as in *E. coli* and *S. enterica*, APS is further acted on by an adenylylsulfate kinase CysC, which converts APS to phosphoadenosine 5'-phosphosulfate (PAPS). PAPS is then reduced to sulfite by CysH, a PAPS reductase (21). However, in plants and in many bacteria including ADP1, the *cysC* gene is absent, and APS is acted on directly to produce sulfite. The enzyme responsible for this reduction is frequently annotated as *cysH* (as it is in ADP1), but the gene product has important structural differences with PAPS reductases that allow for the recognition of APS as a substrate (22) (Figure 1.3).

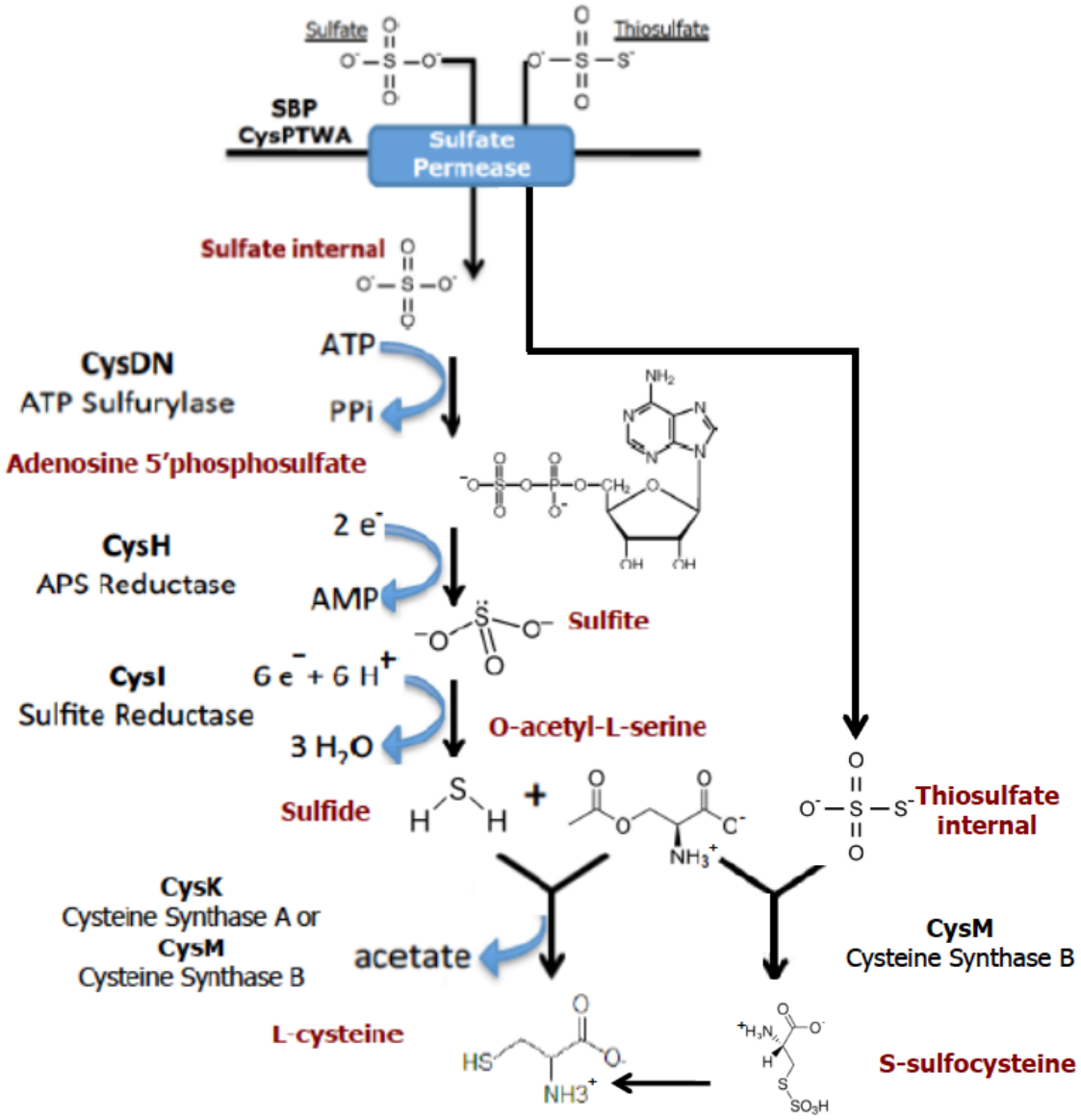


Figure 1.3. Cysteine Biosynthesis Model in ADP1: Sulfate and Thiosulfate Utilization Pathway. External sulfate and thiosulfate are transported into the cell through the permeases SBP and CysP and the ABC-type transporter CysUWA. Sulfate reduction occurs in three steps: phosphorylation by CysDN; APS reduction by CysH; and sulfite reduction by CysI. In the final step of cysteine biosynthesis, sulfide is combined with O-acetylserine (OAS) to produce L-cysteine. Thiosulfate reacts directly with sulfide without any prior reduction to form S-sulfocysteine, which is used to produce L-cysteine.

In *E. coli,* sulfite is further reduced to sulfide by the sulfite reductase CysJI. CysJ is a flavoprotein, and functions within the sulfite reductase complex to transfer electrons from NADPH to the CysI subunit. CysI is an iron-sulfur protein that uses a siroheme cofactor to reduce sulfite to sulfide (23). In plants, CysJ is absent, and a ferredoxin facilitates the transfer of electrons from NADPH to CysI (24). Many bacteria also lack clear orthologs of CysJ, such as *C. glutamicum, Pseudomonas* species*, and B. cenocepacia*. Many of these organisms have genes annotated as *cysJ*, or predicted to be sulfite reductases, but the genes are not located with any other genes involved in sulfur assimilation, and their functional roles have not been proven.

In ADP1, the gene ACIAD0799 is annotated as *cysJ* and is predicted to encode a sulfite reductase. However, the gene product does not have an ortholog in *E. coli, S. enterica,* or any other bacteria with characterized CysJ proteins, so it is not clear if ACIAD0799 is involved in cysteine biosynthesis in ADP1. Additionally, a previous report showed that a *cysJ* deletion does not lead to cysteine auxotrophy (7). If the ACIAD0799 gene product is not providing electrons for the reduction of sulfite by CysI, some other oxidoreductase must be performing this function, or possibly utilizing reduced ferredoxin, as is the case in plants. A gene overlapping with *cysI*, ACIAD2981, is predicted to encode a small protein that may function with *cysI*, but it has no features of known oxidoreductases (Figure 1.1). The ACIAD2981 gene product could be involved in electron transfer, if not directly, then by facilitating interactions with other proteins.

It remains a possibility that a ferredoxin is involved in the transfer of electrons to CysI. In *C. glutamicum*, a gene, *fpr2*, encoding an NADPH-ferredoxin reductase, is clustered with the operon *cysIXHDNYZ*, and its expression is strongly influenced by sulfur availability (15). Additional evidence for ferredoxin involvement in sulfur assimilation comes from a transposon screen of *P. putida* to identify genes involved in siderophore synthesis. Most of the mutants

identified that were unable to produce the siderophore in question had transposon insertions in genes involved in sulfur assimilation, and in a regulatory gene, *finR*. FinR is required for expression of *fprA,* encoding a ferredoxin:NAD(P)H reductase, and *P. putida finR* mutants were cysteine bradytrophs. The *finR* phenotype could be complemented by extra-chromosomal expression of *fprA,* or with *E. coli cysJI*. These results demonstrated functional redundancy between CysJI and FprA, and indicated the essentiality of FprA in sulfur assimilation in organisms that lack CysJ (25). In ADP1, there are two *fpr* paralogs, one of which appears to be transcribed by a regulator similar to FinR, as is the case for *P. putida*. Bioinformatic analysis indicates that FinR, a LysR-type transcriptional regulator, has a conserved C-terminal substrate binding domain with homology to CysL from *B. subtilis* (this study). CysL activates the transcription of the sulfite reductase CysJI (26). These similarities suggest potential involvement of *fpr* and *finR* in cysteine biosynthesis, and these genes were included in the investigation of the pathway in this study.

iii. **Synthesis**

In the final step of cysteine biosynthesis, sulfide is combined with O-acetyl-L-serine (OAS) to form L-cysteine. This reaction is catalyzed by one of two OAS-lyase isozymes, CysM and CysK. For cysteine prototrophy, the function of either of these isozymes is sufficient. CysM has the additional ability to use thiosulfate for the synthesis of S-sulfocysteine, thereby bypassing the need for the sulfate reduction steps (8).

The assimilation pathways for thiosulfate and organosulfur compounds differ from sulfate assimilation in the first steps (Figure 1.3). After transport, thiosulfate is not reduced at all before combining with OAS to produce S-sulfocysteine, a reaction catalyzed by CysM. S-

sulfocysteine is then converted to L-cysteine by an unknown mechanism (27). Taurine and sulfonates are desulfonated after transport by sulfatases and sulfonate monooxygenases, respectively, resulting in the release of sulfite and the corresponding aldehyde. In *E. coli* and *Pseudomonas,* the sulfatase is encoded by *tauD*. ADP1 has two homologs of *tauD*, ACIAD1592 and ACIAD1600, both annotated as *atsK* and clustered with the other *ats* genes (Figure 1.1). In addition to these predicted alkylsulfatases, ADP1 has a predicted arylsulfatase, AtsA, which is homologous to AtsA in *P. aeruginosa*. The *P. aeruginosa* AtsA has been experimentally verified to be an arylsulfatase, and this bacterium is capable of growth on aromatic sulfur compounds as the sole source of sulfur (28). ADP1 is able to utilize aromatic compounds as carbon sources, but the utilization of aromatic sulfur compound has yet to be tested (29).

Desulfonation of sulfonates other than taurine is catalyzed by the alkanesulfonate monooxygenase SsuD (Figure 1.4). Desulfonation results in the release of sulfite, which enters the cysteine biosynthesis pathway described above. SsuD activity requires the co-substrate $FMNH_2$, which is provided by the NAD(P)H-dependent FMN reductase SsuE (17). An SsuD homolog is present in ADP1, and is grouped with the *ssu* genes discussed above. This enzyme is predicted to be $FMNH_2$-dependent, as in *E. coli*. However, no SsuE homolog is present near the *ssuD* gene. As is the case in *B. subtilis,* the oxidoreductase that participates in this reaction is likely located elsewhere on the chromosome (30).

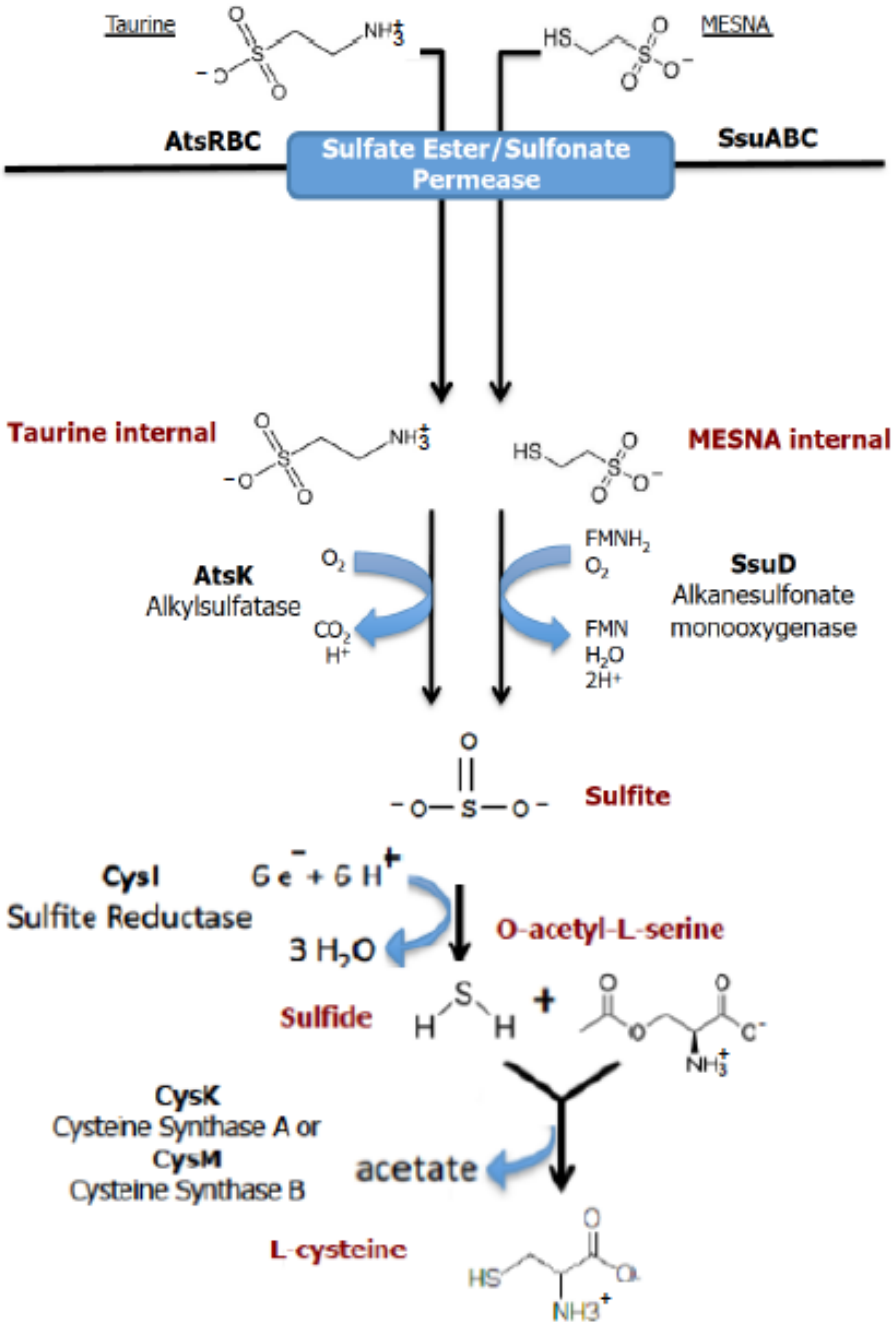


Figure 1.4. Cysteine Biosynthesis Model in ADP1: Organosulfur Utilization Pathway. External organosulfur compounds (taurine and 2-mercaptoethane sulfonate (MESNA) are shown here as examples) are transported through the ABC-type transporters AtsRBC and SsuABC. Desulfonation is catalyzed by AtsK and SsuD and the products enter the cysteine biosynthesis pathway as sulfite.

Other Cysteine Biosynthesis Enzymes

Additional enzymes involved in cysteine biosynthesis have been identified in *E. coli* and *S. enterica*. The PAPS phosphatase CysQ in *E. coli* is required for cysteine prototrophy only during aerobic growth (31). This activity inferred to be required to alleviate toxic build up of PAPS. There is a CysQ homolog in ADP1, and a single-gene deletion study of ADP1 showed that the *cysQ* gene is dispensable on minimal medium (7). It is unlikely that this gene/enzyme participates in cysteine biosynthesis since PAPS is not an intermediate in this pathway.

In *S. enterica*, the gene *cysG* is required for the synthesis of the CysI siroheme cofactor. *CysG* mutants are cysteine auxotrophs in *S. enterica* and *P. putida* (25, 32). The *cysG* genes in these organisms, as well in ADP1 and *E. coli*, are unlinked to any other sulfur assimilation genes. Studies of this gene demonstrate that it is not part of the *cys* regulon in *E. coli* (21).

Cysteine Biosynthesis Regulation

The regulation of cysteine biosynthesis is best described in *E. coli* and *S. enterica*. In these organisms, the genes involved in the biosynthetic pathway are coordinately controlled by the LTTR CysB. CysB has been implicated in the expression of more than 20 genes involved in cysteine biosynthesis, which are organized into clusters scattered throughout the genome (Figure 1.1). In common with other LTTRs, CysB exhibits negative autoregulation, and can function as an activator or repressor at other promoters in the regulon (33).

Full expression of the *E. coli* and *S. enterica* regulons requires sulfur limitation, the inducer N-acetylserine (NAS), and the regulator CysB (2). Regulation of *cys* gene expression occurs by two mechanisms: end-product inhibition of the serine transacetylase CysE by L-cysteine, and transcriptional regulation by CysB. These two mechanisms are interdependent.

Transcription is activated in the presence of the inducer N-acetylserine (NAS), which is produced from OAS, and transcription is repressed by factors that deplete intracellular OAS. These factors include cysteine inhibition of CysE activity, and sulfide and thiosulfate reacting with OAS to produce cysteine (34) (Figure 1.5). In addition to directly affecting intracellular OAS by reaction, sulfide and thiosulfate also act as antiinducers by inhibiting CysB binding to the *cysP* and *cysJIH* promoters in *S. enterica* (35, 36).

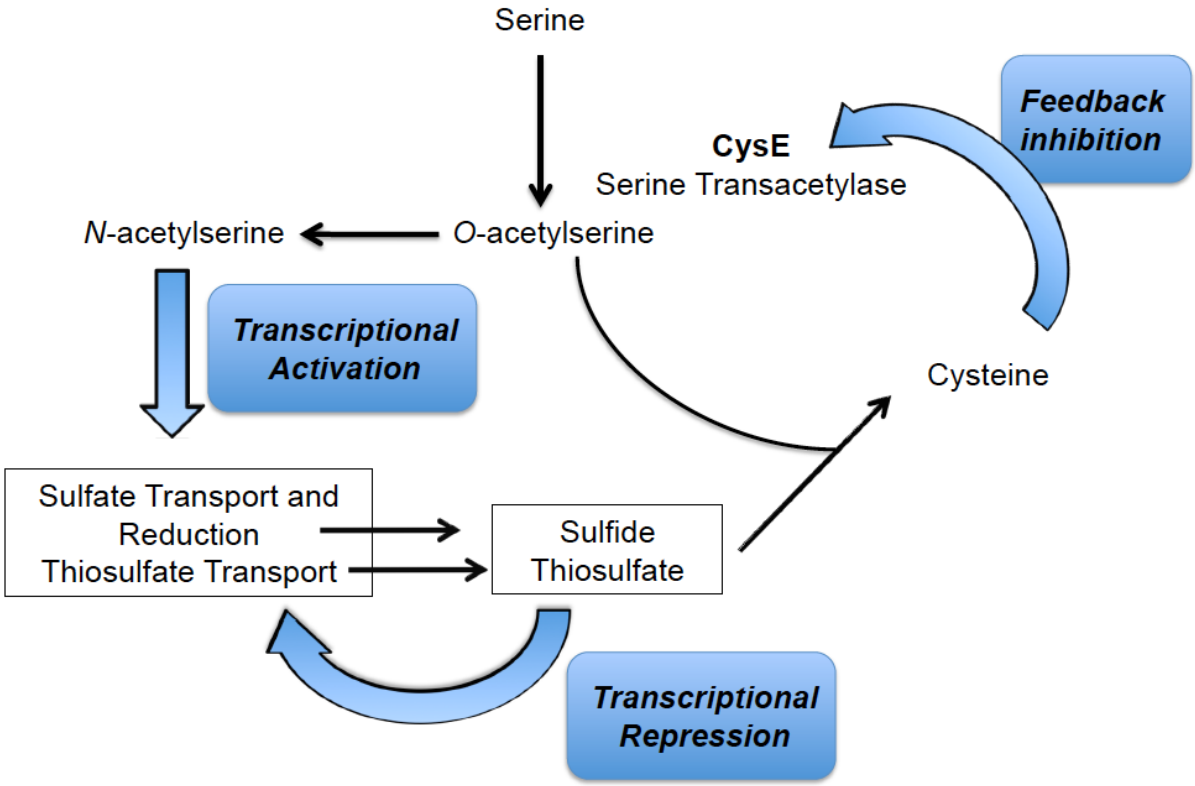


Figure 1.5. Regulation of *cys* gene expression in *E. coli* and *S. enterica* CysB-mediated transcription activation requires the inducer NAS, which is produced from OAS. OAS is synthesized by the transacetylation of serine, catalyzed by CysE. CysE activity is inhibited by L-cysteine, which is formed from OAS and sulfide or thiosulfate. By reacting with OAS and depleting intracellular pools, sulfide and thiosulfate act as antiinducers of *cys* gene expression. Adapted from (2).

Because of the dependence on NAS for CysB-mediated transcriptional activation, *E. coli* and *S. enterica cysB* and *cysE* mutants have very similar phenotypes (2). Additionally, *S. enterica* CysB can bind some *cys* promoters only in the presence of OAS, and at others, binding is greatly enhanced (35, 37). The NAS requirement for *cys* gene transcription has also been demonstrated in *B. subtilis* and *C. glutamicum* (4, 6). However, a study in *B. cenocepacia* showed no effect on *cysI* or *sbp* expression when *cysE* was deleted from the chromosome, indicating OAS-independent expression (3). Furthermore, there was also no demonstrable effect of OAS on CysB binding at promoter regions *cysI, sbp,* or *cysDN*. Currently, no inducer has been discovered for CysB in *Burkholderia*, *Pseudomonas* species, or in ADP1, where cysteine biosynthesis is actively being investigated.

CysB is ubiquitous among Gram-negative bacteria, but many also contain a CysB-like regulator, "Cbl", that shares significant homology with CysB. In *E. coli*, Cbl activates the expression of the *tau* and *ssu* operons, and *cbl* mutants cannot utilize taurine or aliphatic sulfonates (38). *Cbl* itself is positively controlled by CysB (8). It is additionally regulated by APS, which abolishes the activating function of Cbl upon binding to the regulatory domain (39). Cbl in *B. cenocepacia* was renamed SsuR for "sulfate starvation utilization regulator", but is functionally and structurally equivalent to the *E. coli* Cbl. Both control genes that are activated in response to sulfate starvation (18, 40).

In the absence of a "Cbl", many bacteria still contain additional regulators that can control the expression of genes involved in organosulfur metabolism. For example, SsuR in *C. glutamicum* functions similarly to the *E. coli* Cbl and *B. cenocepacia* SsuR, but it is not "CysB-like" structurally (41). Bacteria that contain only one *cys* regulator, such as *S. enterica,* typically do not have the ability to utilize alternative sulfur sources. In ADP1, however, a CysB paralog

has not been identified. Nevertheless, this study shows ADP1 is able to utilize a wide range of compounds as sole sulfur sources, including taurine and sulfonates. The genes for the metabolism of these compounds are clustered on the chromosome, suggesting common regulation (Figure 1.1). It is possible that CysB regulates the expression of these genes in addition to the sulfate assimilation genes.

Overview of This Study

A *cysB* deletion mutant was made by previous lab members Kathryn T Elliott and Cassandra Bartlett and characterized as a cysteine auxotroph. Additional undergraduate students and members of the Neidle, Karls, and Momany labs initiated studies of cysteine metabolism in ADP1. In this study, the growth phenotype of the *cysB*-deleted strain was explored further, and several other genes predicted to be involved in cysteine biosynthesis were deleted. The resulting mutants were grown on different sulfur sources to determine at which point in the pathway the target gene functions. The role of CysB was further examined by making single amino acid substitutions in the effector-binding domain, and by measuring *cys* gene expression under different growth conditions. *In vivo* studies were supplemented by electrophoretic mobility shift assays (EMSAs) to determine CysB interactions with the promoter regions of the *cys* regulon genes. Finally, various sulfur compounds were tested as potential effectors of CysB-mediated transcription by looking for changes in the dissociation constant (K_d) of the CysB-DNA complexes in EMSAs.

CHAPTER 2

REGULATION OF CYSTEINE BIOSYNTHESIS IN *ACINETOBACTER BAYLYI* ADP1

Abstract

ACIAD2597, encoding a LysR-type transcriptional regulator (LTTR) here named CysB, was investigated for its role in cysteine biosynthesis in *Acinetobacter baylyi* ADP1. Homologs of this protein regulate cysteine biosynthesis in the Gram-negative bacteria *Escherichia coli* and *Salmonella enterica.* An *A. baylyi cysB*-deleted mutant was unable to grow on minimal medium without cysteine supplementation, and CysB variants affected expression of genes predicted to be involved in cysteine biosynthesis. CysB binds to the promoter regions of *cysP* and *cysDN*, but not *cysI*. Another LTTR, FinR, binds to the promoter regions of several genes involved in cysteine biosynthesis, including *cysI*. *FinR* is an essential gene, and a *finR* deletion mutant could only be obtained when a gene encoding a ferredoxin:NADPH reductase, *fpr2,* was expressed *in trans*. The *finR* strain with plasmid-borne *fpr2* had inhibited growth on minimal medium that could be alleviated by cysteine supplementation. The *cysB* and *finR* growth phenotypes characterized in this study indicate roles for both regulators in cysteine biosynthesis. These studies also indicate potential ferredoxin:NADPH reductase involvement in sulfite reduction, which suggests sulfur assimilation biochemistry distinct from *E. coli* and more similar to plants.

Introduction

Predicted genes for cysteine biosynthesis in *Acinetobacter baylyi* ADP1 are clustered in several different regions of the chromosome. One regulatory gene, ACIAD2597, resides at the end of a putative operon likely to encode proteins for sulfate and thiosulfate transport. As reported here, the encoded regulator is a homolog of CysB and Cbl from *E. coli,* as well as CysB proteins from other organisms. Based on this similarity and methods that identify orthologs as the best reciprocal matches between genes in different genomes, ACIAD2597 is annotated as *cbl* (7, 42, 43). This designation in *E. coli* was originally based on the "CysB-like" properties of a CysB paralog.

Based on homology, it is difficult to infer the function of the ACIAD2597 regulator in *A. baylyi*. In *E. coli*, CysB controls the majority of the genes involved in cysteine biosynthesis, including positive control of *cbl* transcription (8, 44, 45). Cbl regulates a subset of genes involved in sulfur assimilation that are activated in response to sulfate starvation (18). CysB and Cbl share a high degree of similarity to each other, and this extent of similarity is not shared between ACIAD2597 and any other open reading frames (ORFs) in ADP1. Based on these comparisons, it is unlikely that ADP1 cysteine biosynthesis is controlled by a CysB-Cbl type dual regulatory system, as in *E. coli*.

S. enterica cysteine biosynthesis resembles that in *E. coli*, except that it is controlled by a single regulator, CysB, without the presence of another closely related paralog (34). *S. enterica* is unable to utilize taurine, sulfonates, and other organosulfur compounds as sole sulfur sources, so it is not surprising that there is not a second regulator. In *E. coli* and *S. enterica*, *cysB* is unlinked to any genes involved in sulfur assimilation. In contrast, ACIAD2597 is linked to the *cysPUWA* genes. This genetic context suggests involvement of this regulator in cysteine

biosynthesis, however, this role has not been experimentally explored. The goals of the current investigation include clarification of ACIAD2597-mediated regulation, characterization of the cysteine biosynthetic pathway, and assessment of sulfonate utilization in ADP1.

Structural and biochemical studies of CysB from several enteric bacteria demonstrate that it is a typical LTTR that functions as a homotetramer. A conserved helix-turn-helix motif is located in the N-terminal DNA-binding domain (DBD), which is connected to an effector-binding domain (EBD) by a linker helix (47). LTTRs are notoriously difficult to crystallize, and to date, there is only one published full-length crystallization of CysB, in which only the EBD structure was well ordered and could be characterized (from *K. aerogenes*) (48, 49). An EBD structure from Cbl is also known (from *E. coli*) (50). Additionally, mutational studies of the *cysB* gene in *S. enterica* illuminate the function of some conserved residues in the EBD (51, 52).

One such residue, T149, can tolerate any one of 15 different amino acid replacements, and the corresponding *S. enterica* mutants still retain the ability to grow prototrophically. Nine of these mutants were termed "constitutive" because transcription of the *cys* genes could be activated by the CysB variants without the inducer N-acetylserine (NAS), and regulation was insensitive to the antiinducers sulfide and thiosulfate. A second position, W166, also conferred a partially constitutive phenotype when replaced by an arginine residue. Two of the CysB T149 variants (T149M and T149P) and one with a W166 replacement (W166R), were characterized *in vitro* with electrophoretic mobility shift assays (EMSAs) and transcription run-off assays. The T149 variants mimicked the CysB protein bound to inducer with respect to DNA-binding affinity and DNA bending, and the W166R variant appeared to be locked in a configuration that could not respond to inducer (52). The significance of T149 in effector binding has also been shown in the structure of the CysB EBD from *K. aerogenes*. A model of a sulfate anion in the ligand-

binding cleft predicts direct interaction between T149 and the anion (53). Like CysB in *S. enterica*, *K. aerogenes* CysB responds to the inducer NAS, and it is likely that the EBDs of these two proteins function similarly (54).

Previous lab members Kathryn T Elliott and Cassandra Bartlett characterized a ACIAD2597-deleted *A. baylyi* strain as a cysteine auxotroph, a result that conflicted with a report that this gene is a dispensable gene on minimal medium (7). The cysteine auxotrophy of this strain suggested ACIAD2597 involvement in cysteine biosynthesis, and one of the goals of this study was to determine the role of this regulator in cysteine biosynthesis. In one approach to this objective, *cys* gene expression levels were measured in ACIAD2597-deleted strains and in wild-type ADP1. However, experimental design was complicated by the fact that ACIAD2597 deletion mutants could only be grown with cysteine. Amino acid biosynthesis is predicted to be regulated according the principle of homeostasis, wherein the needs of the cell dictate levels of gene expression, and an amino acid end-product should result in decreased expression of the corresponding biosynthetic genes (55). Accordingly, low *cys* gene expression in the presence of cysteine might make it difficult to compare regulation in the wild-type and *cysB*-deleted strains.

To circumvent this problem, mutations were made in the *A. baylyi* chromosomal ACIAD2597 gene to generate amino acid replacements matching those in the *S. enterica* CysB variants described above. The expectation was that these alterations would result in variants that increase transcription of target genes, thereby allowing prototrophic growth. If these ACIAD2597 variants were able to alter gene regulation in comparison to strains encoding the native ACIAD2597, then a regulatory role for ACIAD2597 could be inferred. As described here, comparative gene expression studies were used to improve our understanding of cysteine biosynthesis and regulation in ADP1.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

ACN strains were derived from *Acinetobacter baylyi* strain ADP1 (56) (Table 2.1). *E. coli* strain DH5α was used as a plasmid host, and was grown in Luria-Bertani (LB) broth. ADP1 strains were grown in a minimal salts media with 20 mM pyruvate as the carbon source at 37°C with shaking (57). For β-galactosidase assays and growth studies, a modified M9 minimal media with no added sulfur was used, with 2 mM $MgCl_2$ in place of $MgSO_4$. The following concentrations of sole sulfur sources were added where indicated: 0.3 mM L-cysteine, 1 mM djenkolate, 1 mM reduced glutathione, and 0.5 mM of all other sulfur sources. Antibiotics were added as needed at the following concentrations: kanamycin (25 μg/mL), ampicillin (150 μg/mL), or spectinomycin + streptomycin (12.5 μg/mL each). To select for the loss of the *sacB* marker, sucrose was provided at 5% w/v and strains were grown at 30°C on agar plates.

Plasmid and Strain Construction

Standard molecular biology techniques were used for restriction digests, ligations, *E. coli* transformations, and plasmid purification (58). Overlap extension PCR was used to generate DNA constructs for in-frame gene deletions and allelic replacements, and constructs were cloned into the pUC19 vector (59). ACN strains were generated using previously described methods for transformation of ADP1 (60). To construct allelic replacements in the genome, a counterselectable marker *sacBkm*^{R} was used as follows: the targeted allele was disrupted or replaced with the *sacBkm*^{R} cassette by transformation of ADP1 with a linearized plasmid encoding the cassette, and selection for kanamycin resistance. The *sacBkm*^{R} cassette was then

replaced with the new allele, or gene deletion construct, by a similar transformation, and selection for growth on sucrose. Transformation of ADP1 with expression vectors were performed in a similar manner to transformations with DNA designed for chromosomal integration, except that a higher concentration of kanamycin was used for selection (500 μg/mL) to prevent chromosomal integration.

Growth Phenotype Determinations

Growth curves were constructed for strains used in ß-galactosidase assays in Klett flasks. Strains were grown overnight in modified M9 (no added sulfur) with pyruvate as the carbon source and cysteine as the sole sulfur source. 1:50 dilutions were made into 20 mL of the same media with different sulfur sources and grown at 37°C shaking. Culture turbidity was measured in a Klett meter, and was plotted against time to determine growth phases. Growth phenotypes of *cysB* mutants on different sulfur sources were determined similarly by subculturing cysteine-grown cultures. 1:1000 dilutions were made into 5 mL of the same media with different sulfur sources and grown at 37°C shaking. Ability to utilize different sole sulfur sources was determined by measuring final OD_{600} at 24-72 hours growth after subculture, compared to growth on media with no added sulfur.

ß-Galactosidase Assays

To determine ß-galactosidase activity, strains were grown overnight in modified M9 (see above) with pyruvate as the carbon source and cysteine as the sole source of sulfur. 1:10 dilutions were made into fresh media with the appropriate sulfur source, and the cultures were grown to early log at 37°C shaking. The FluorAce ß-galactosidase reporter kit (BioRad) was

used for the assay, and a TD-360 minifluorometer was used to detect hydrolysis of 4-methylumbelliferyl-galactopyranoside to 4-methylumbelliferone (4MU) by ß-galactosidase. 4MU formation was recorded at various time points to determine the time range of linear activity for ß-galactosidase in each strain. The values reported represent ß–galactosidase specific activity (nmol min^{-1} μl^{-1}) at a time point during the linear phase of the enzyme.

Electrophoretic Mobility Shift Assays (EMSAs)

His-tagged CysB and FinR were purified in Cory Momany's lab by Melesse Nune. Briefly, full-length CysB and FinR with C-terminal pentahistidine tags were expressed in *E. coli* BL21(DE3) cells. Overexpression was induced in autoinduction media as previously described (61). Cell pellets were harvested and suspended in binding buffer (20 mM Tris-HCl , 0.5 M NaCl, 10% glycerol, and 25 mM imidazole, pH 8.0), and lysed with a French press. Supernatant from centrifuged cell lysate was applied to a metal-chelating column charged with nickel, and eluted in elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 10% glycerol, 0.5 M imidazole, pH 8.0). Proteins were further purified by anion exchange and eluted in Q buffer (20 mM Tris-HCl, 10 mM ß-mercaptoethanol, 1 mM EDTA, 10% glycerol, 25 mM imidazole, 1 M NaCl, pH 8.0). For use in EMSAs, proteins were dialyzed into the same buffer, but without NaCl.

EMSA analysis was guided by a predicted consensus sequence for the ADP1 CysB binding site. This sequence was predicted using the Prodoric database as follows. In the Prodoric database, there is a position weight matrix derived from known binding sites of the CysB protein in *E. coli* (62). This matrix was used to evaluate the sequences upstream of genes predicted to be involved in cysteine biosynthesis in *A. baylyi* using the Virtual Footprint program (63).

For EMSAs, purified CysB or FinR was incubated with 2 nM of the indicated *cys* promoter region at 4°C for 1 hour in the following buffer: 6.4 mM Tris acetate (pH 8.0), 20 mM KCl, 1.25 mM ammonium acetate, 0.25 mM magnesium acetate, 10 µM DTT, 0.4 µM EDTA, 10 µM $CaCl_2$, and 2.5 µg/ml BSA. Reactions were run on a 6% polyacrylamide gel in 0.5X TAE for 1 hour at 4°C and stained with SYBR green for 15 minutes. DNA promoter regions were generated by Polymerase Chain Reaction (PCR) using Expand High Fidelity DNA Polymerase (Roche) and purified using a DNA Clean & Concentrator kit (Zymogen). DNA concentration was determined by measuring OD_{260} in a spectrophotomer.

Results

Designation of ACIAD2597 as "*cysB*"

As discussed in the introduction to this chapter, ACIAD2597 is homologous to regulators of cysteine biosynthesis in other bacteria. A sequence alignment reveals that the ACIAD2597-encoded protein is more similar to Cbl than CysB of *E. coli* (~50% similarity; Figure 2.1), and ACIAD2597 is annotated as "*cbl*" in current databases. The designation "*cbl*" does not make sense in *A. baylyi* because there are not two closely related "CysB-like" proteins. Therefore, based on its likely role in cysteine biosynthesis, we designated this gene *cysB*. As shown in the alignment (Figure 2.1), the residues shown to affect regulation in *S. enterica* are conserved in the CysB protein of ADP1.

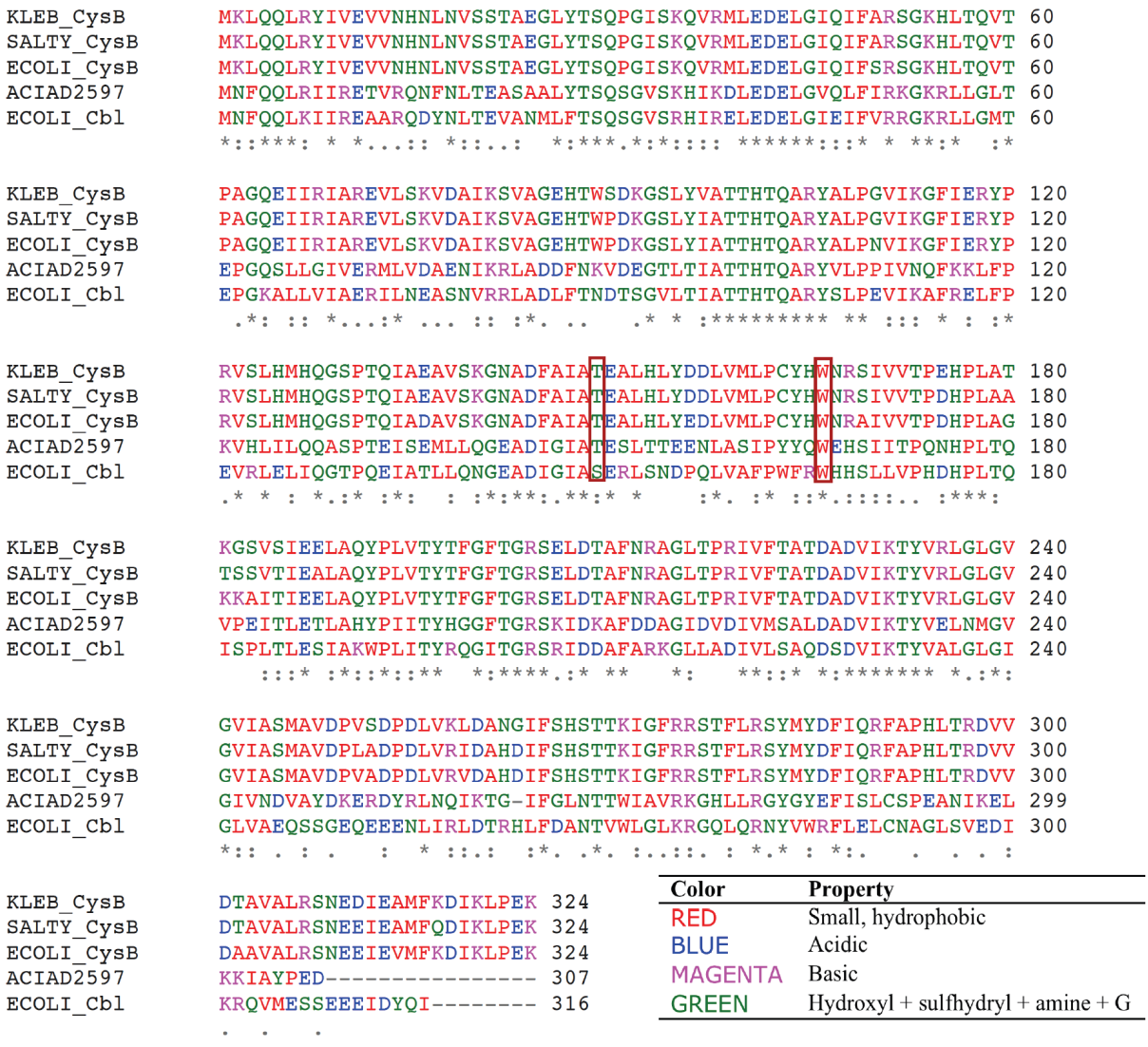


Figure 2.1. Multiple sequence alignment of ACIAD2597 orthologs. *S. enterica* CysB, *K. pneumoniae* CysB, *E. coli* CysB, *E. coli* Cbl, and ADP1 ACIAD2597 amino acid sequences were aligned using ClustalW. Conserved amino acids T149 and W166 are boxed in red. Asterisks indicate fully conserved residues, colons indicate conservation between groups with strongly similar properties, and periods indicate conservation between groups with weakly similar properties (46).

Wild-type ADP1 and *cysB* Mutant Growth Phenotypes

To determine the wild-type range of sulfur utilization and the effects of *cysB* mutations, strains were grown in modified M9 minimal medium with different sole sulfur sources. Without any added sulfur source the wild-type was able to reach a final OD_{600} of ~0.3 in this defined medium, indicating some sulfur contamination from other components. This result is consistent with similar studies of *E. coli* and *S. enterica* (2). As shown in Table 2.1, ADP1 was able to grow in all sulfur sources investigated, except for reduced glutathione. The inability to grow on glutathione may be because ADP1 is not able to transport it; ABC transporters in *E. coli, P. putida, B. cenocepacia,* and *S. enterica* are responsible for glutathione transport, and no homologs of these transporters could be identified in ADP1 (64).

Strains with a mutation in, or deletion of, *cysB* were similarly grown in different sulfur sources. ACN1262 (*ΔcysB;* Table 2.2) was unable to grow on any sole sulfur sources other than L-cysteine, L-cystine, or djenkolate. Cysteine is easily oxidized to cystine, which is comprised of two cysteine molecules bound by a disulfide bond. Current research on cysteine/cystine transport in aerobic bacteria has shown transport to be cystine-specific, and it is likely that most of the cysteine provided in growth media is readily oxidized to cystine (2, 65). The ability of ACN1262 to grow on both cysteine and cystine, then, is not surprising. Djenkolate ([S,S'-methylene bis(cysteine)]), similar to cystine, consists of two cysteine molecules connected by a methylene group. Djenkolate utilization is not unusual in bacteria, but it is considered a "poor" source of sulfur, and results in slow growth and high *cys* gene expression (66, 67). Evidence of a distinct transport mechanism for djenkolate does not exist in the current literature, and it is possible that transport is mediated by the same mechanism as cysteine/cystine.

As previously mentioned, single amino acid replacements were made in the CysB EBD similar to past replacements that were studied in *S. enterica* CysB. Two mutants, ACN1380, encoding CysB(T149M), and ACN1494, encoding CysB(T149P), exhibited similar growth phenotypes to wild-type ADP1. A third mutant, ACN1484, encoding CysB(W166R), however, had a growth phenotype identical to the *cysB* deletion mutant, ACN1262. This result suggests that this strain, unlike its *S. enterica* counterpart, did not encode a functional CysB regulator, (52). ACN1484 was not investigated further in this study.

Table 2.1. Utilization of sulfur sources by wild-type *A. baylyi* and *cysB* mutants. (+) indicates a final OD_{600} greater than the final OD_{600} in M9 medium with no added sulfur (>0.3). (-) indicates a final OD_{600} less than or equal to the final OD_{600} in M9 medium with no added sulfur. NM: not measured. MESNA – 2-mercaptoethane sulfonate; PIPES – piperazine-N,N'-bis(2-ethanesulfonate); MOPS – 3-(N-morpholino)propanesulfonate; MES – 2-(N-morpholino)ethanesulfonate; HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonate); DMSO – dimethyl sulfoxide. Concentrations of sulfur sources provided are listed in the Materials and Methods section of this chapter.

Sulfur Source	Growth for indicated *A. baylyi* strain				
	ADP1 wild-type	ACN1380 CysB(T149M)	ACN1494 CysB(T149P)	ACN1484 CysB(W166R)	ACN1282 No CysB
L-cysteine	+	+	+	+	+
L-cystine	+	+	+	+	+
Sulfate	+	+	+	-	-
Thiosulfate	+	+	+	-	-
Sulfite	+	+	+	-	-
Sulfide	+	+	+	-	-
L-methionine	+	NM	NM	-	-
Taurine	+	NM	NM	-	-
MESNA	+	NM	NM	-	-
PIPES	+	NM	NM	-	-
Cysteate	+	NM	NM	-	-
Djenkolate	+	+	+	+	+
MOPS	+	NM	NM	NM	NM
MES	+	NM	NM	NM	NM
HEPES	+	NM	NM	NM	NM
DMSO	+	NM	NM	NM	NM
Thiocyanate	+	NM	NM	NM	NM
Glutathione	-	-	-	-	-

Table 2.2 Bacterial Strains and Plasmids

Strain or Plasmid	Relevant Characteristics	Source
ADP1 Strains		
ADP1	Wild-type	(56)
ACN1262	*ΔcysB::sacBkm*	CB/KT[a]
ACN1282	*ΔcysB*	CB/KT[a]
ACN1380	*ΔcysB::cysBT149M*	This study
ACN1392	*ΔcysB::cysB5Xhis*	This study
ACN1460	*ΔcysB cysIorflacZkm*$^{R\,b}$	This study
ACN1461	*ΔcysB::cysBT149M cysIorflacZkm*$^{R\,b}$	This study
ACN1463	*cysIorflacZkm*$^{R\,b}$	This study
ACN1484	*ΔcysB::cysBW166R*	This study
ACN1494	*ΔcysB::cysBT149P*	This study
ACN1511	*ΔcysB::cysBT149P cysIorflacZkm*$^{R\,b}$	This study
ACN1543	*ΔcysB::cysBW166R cysIorflacZkm*$^{R\,b}$	This study
ACN1544	*cysDNlacZkm*$^{R\,b}$	This study
ACN1545	*ΔcysB::cysBT149M cysDNlacZkm*$^{R\,b}$	This study
ACN1546	*ΔcysB::cysBT149P cysDNlacZkm*$^{R\,b}$	This study
ACN1553	*SBPlacZkm*$^{R\,b}$	This study
ACN1554	*ΔcysB::cysBT149M SBPlacZkm*$^{R\,b}$	This study
ACN1555	*ΔcysB::cysBT149P SBPlacZkm*$^{R\,b}$	This study
ACN1573	*ΔcysB cysDNlacZkm*$^{R\,b}$	This study
ACN1574	*ΔcysB SBPlacZkm*$^{R\,b}$	This study
ACN1612	*ΔcysP::sacBkm*	This study
ACN1613	*ΔACIAD2244::SpSm*^{R}	This study
ACN1614	*ΔfinR::SpSm*^{R}	This study
Plasmids		
pUC19	Ap^{R}; cloning vector	(68)
pIM1445	Km^{R}; ADP1 expression vector	(69)
pBAC1087	Ap^{R}; *ΔfinR* (731949-732466; 733349-733946)[c] in pUC19	This study
pBAC1098	Ap^{R} Km^{R} Suc^{S}; *sacBkm*^{R} cassette inserted in SalI site of pBAC1087	This study
pBAC1131	Ap^{R} Km^{R} Suc^{S}; *sacBkm*^{R} cassette inserted in PstI site of pUC19	This study
pBAC1134	Km^{R}; pBAC1240 with site-directed mutation of 2554451 CT to TG[c]	This study
pBAC1135	Km^{R}; pBAC1240 with site-directed mutation of 2554501 T to A[c]	This study
pBAC1136	Km^{R}; pBAC1240 with site-directed mutation of 2554501 T to A[c]	This study
pBAC1149	Ap^{R}; *ΔcysB::cysB5Xhis* (2553478-2554927; 2554928-2555526)[c] in pUC19	This study
pBAC1150	Ap^{R}; *ΔcysB::cysBT149M* (2553478-2555526; 2554451 CT to TG)[c] in pUC19	This study
pBAC1151	Ap^{R}; *ΔcysB::cysBW166R* (2553478-2555526; 2554501 T to A)[c] in pUC19	This study

pBAC1154	Ap^R Km^R; *lacZkm*^{R} cassette inserted in SalI site of pUC19	This study
pBAC1156	Ap^R; *ΔcysB::cysBT149P* (2553478-2555526; 2554450 A to C) [c] in pUC19	This study
pBAC1199	Ap^R; *cysNcitN* region (1070170-1071654) [c] in pUC19	This study
pBAC1203	Ap^R; pBAC1199 with XhoI cut site inserted after 1070899 [c] by SDM	This study
pBAC1204	Ap^R Km^R; *lacZkm*^{R} cassette inserted in XhoI site of pBAC1203	This study
pBAC1205	Ap^R; *SBP* region with XhoI cut site (3277966- 3279393; XhoI site inserted after 3278627) [c] in pUC19	This study
pBAC1207	Ap^R Km^R; *lacZkm*^{R} cassette inserted in XhoI site of pBAC1205	This study
pBAC1228	Ap^R $SpSm^R$; $SpSm^R$ cassette inserted in HindIII site of pUC19	This study
pBAC1229	Ap^R; *ΔcysP* (2548515-2551415) [c] in pUC19	This study
pBAC1230	Ap^R Km^R Suc^S; *sacBkm*^{R} cassette inserted in SalI site of pBAC1229	This study
pBAC1231	Ap^R; *ΔACIAD2244* (2215659-2214127) [c] in pUC19	This study
pBAC1232	Ap^R; *ΔfinR* (731949-734853) [c] in pUC19	This study
pBAC1233	Ap^R; *ΔACIAD0747* (731949-734853) [c] in pUC19	This study
pBAC1234	$SpSm^R$ cassette inserted in HindIII site of pBAC1231	This study
pBAC1235	$SpSm^R$ cassette inserted in HindIII site of pBAC1232	This study
pBAC1236	$SpSm^R$ cassette inserted in HindIII site of pBAC1233	This study
pBAC1237	Km^R; *ACIAD0747* (733437-734198) [c] in pIM1445	This study
pBAC1238	Km^R; *ACIAD2244* (2214826-2215605) [c] in pIM1445	This study
pBAC1239	Ap^R; *ΔSBP* (3277631-3280565) [c] in pUC19	This study
pBAC1240	Km^R; ACIAD2597-pentahistidine expressed from a modified pET28b vector (70)	Momany Lab

a. Generated by Cassie Bartlett and KT Elliott.
b. *lacZkm*^{R} cassette inserted immediately after stop codon of target gene without any deletion of wild-type sequence.
c. Position in ADP1 genome according to GenBank accession CR543861.

Assessing *cys* Gene Expression with Chromosomal *lacZ* Transcriptional Fusions

To determine if CysB regulates cysteine biosynthesis, chromosomal *lacZ* fusions were constructed at various loci predicted to be involved in this pathway. A promoterless *lacZ*-Km^R cassette was fused to the ends of *sbp, cysI-orf,* and *cysDN* in the chromosomes of strains that encoded either the wild-type CysB, CysB(T149M), CysB(T149P), or no CysB. It was expected that wild-type regulation would lead to high expression of the *cys* genes in minimal medium

during early- to mid-log phase when the growing population would need to reduce the provided sulfur source to generate cysteine. This growth phase was selected for comparative studies of *cys* gene expression in different genetic backgrounds. As described in the Materials and Methods section, strains were subcultured after overnight growth from minimal medium with cysteine to a fresh defined medium, and gene expression was assessed by measuring LacZ (ß-galactosidase) activity in permeabilized culture samples after they reached early-log phase.

As noted earlier, we expected that *cys* gene expression would be higher when cells need to reduce sulfate in order to produce cysteine as compared to conditions where cysteine is provided to the cells exogenously. Consistent with this expectation, *cysI* expression in the *cysI*::*lacZ* strain ACN1463, when assessed for LacZ activity, was approximately two-fold higher in medium provided with sulfate as the sole sulfur source in comparison to the same medium supplemented with cysteine (Figure 2.2; p-value<0.05). Since this strain has an otherwise wild-type genetic background, it is not clear which regulator(s) are responsible for this media-dependent difference in gene expression. To evaluate whether CysB is involved in regulating *cysI*, LacZ activity was measured in ACN1461, a strain that is isogenic to ACN1463 except that it encodes a CysB variant, CysB(T149M). As shown in Figure 2.2, the presence of this variant compared to wild-type CysB resulted in increased *cysI* expression by 2-fold in both media (p-values<0.05). The observation that a single amino acid replacement could alter *cysI* gene expression suggests that CysB plays a role in the transcriptional regulation of this gene.

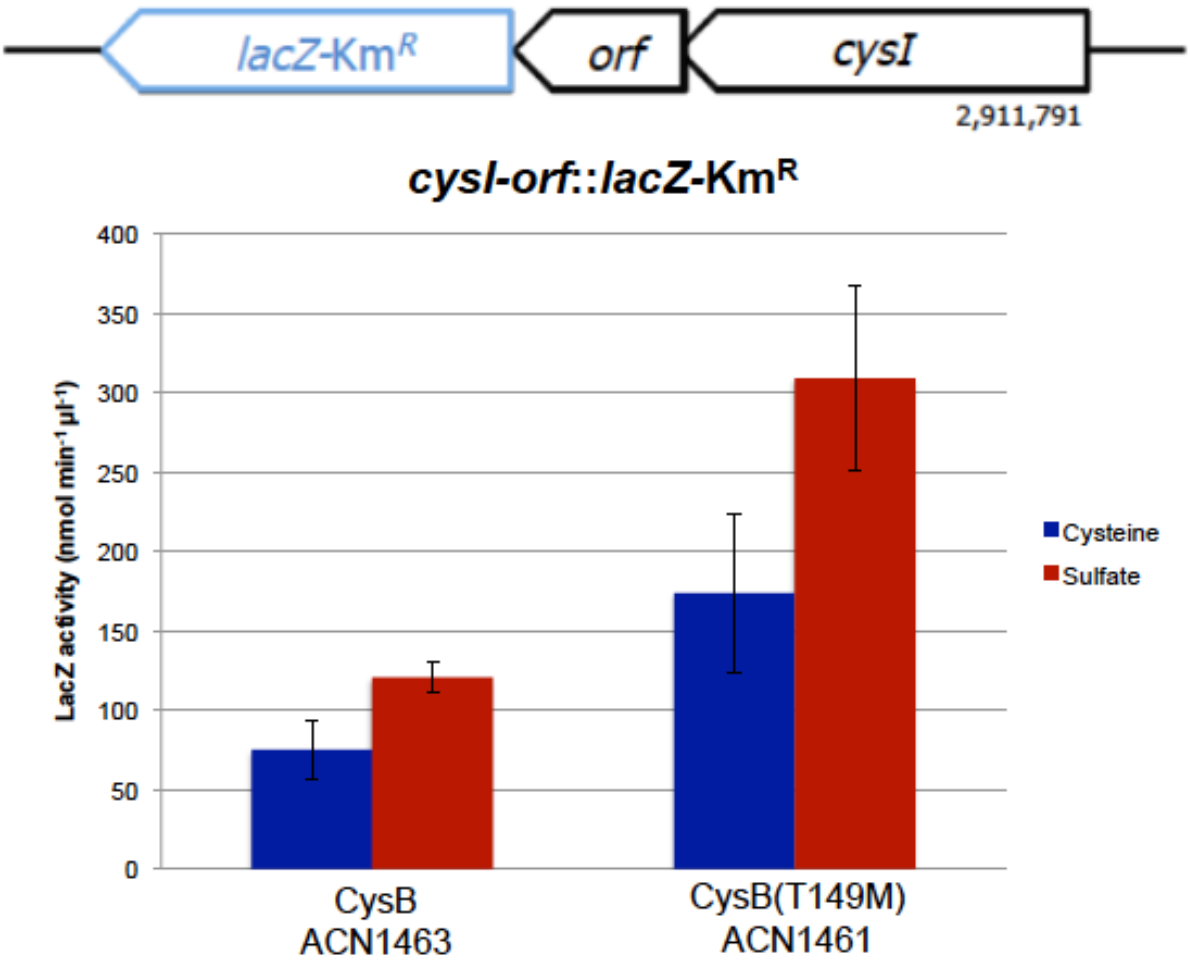


Figure 2.2. *cysI-orf* expression. The values indicated represent LacZ activity as a result of a chromosomal transcriptional fusion with *cysI-orf*, and are the average of at least four independent experiments.

In contrast to the elevated *cysI* expression seen in sulfate-grown compared to cysteine-grown cells, *cysDN* expression in the *cysDN*::*lacZ* strain ACN1544, as measured with LacZ activity, did not appear to be affected by different sulfur sources (Figure 2.3). LacZ activity in ACN1544 was assessed in three independent experiments, however, the comparison between acitivty in cysteine and sulfate media was not statistically significant (p-value=0.56). This strain expresses wild-type CysB, and it was anticipated that, if CysB regulates both *cysDN* and *cysI*, *cysDN* expression would be similarly affected by different sulfur sources. Given the high p-value calculated for this comparison, different sulfur sources do not appear to affect *cysDN* expression.

Expression at this locus was similarly determined with LacZ activity in strains expressing the CysB variants CysB(T149M) and CysB(T149P) to evaluate whether CysB plays a role in regulating *cysDN*. These variants were similar to each other in their effects on *cysDN* expression, and will be discussed together. LacZ assays with the strains expressing CysB(T149) variants revealed elevated activity in sulfate-supplemented medium compared to activity in the corresponding strain expressing wild-type CysB, in the same medium (Figure 2.3). These assays were performed twice, and statistical evaluation of the 2-fold increase in activity between CysB(T149P)-expressing cells and sulfate-grown CysB-expressing cells was signficant (p-value<0.05). The smaller increase (<2-fold) observed between sulfate-grown CysB(T149M)-expressing cells and wild-type CysB-expressing cells, however, was not statistically significant (p-value=0.06). These initial results suggest that *cysDN* is upregulated in the presence of CysB(T149P), in contrast to the situation with wild-type CysB, when sulfate is provided as the sole sulfur source. CysB(T149M) may similarly upregulate *cysDN* in sulfate-supplemented medium, but further studies are needed to confirm this result.

The increased LacZ activity observed in sulfate-grown strains expressing the CysB(T149) variants compared to the strain expressing wild-type CysB was not seen in cysteine-supplemented medium. *CysDN* expression, as measured with LacZ activity, was not significantly changed by the presence of CysB(T149) variants compared to wild-type CysB in cysteine-supplemented medium (Figure 2.3). Therefore, under these conditions, the role of CysB in regulating *cysDN* expression is unclear.

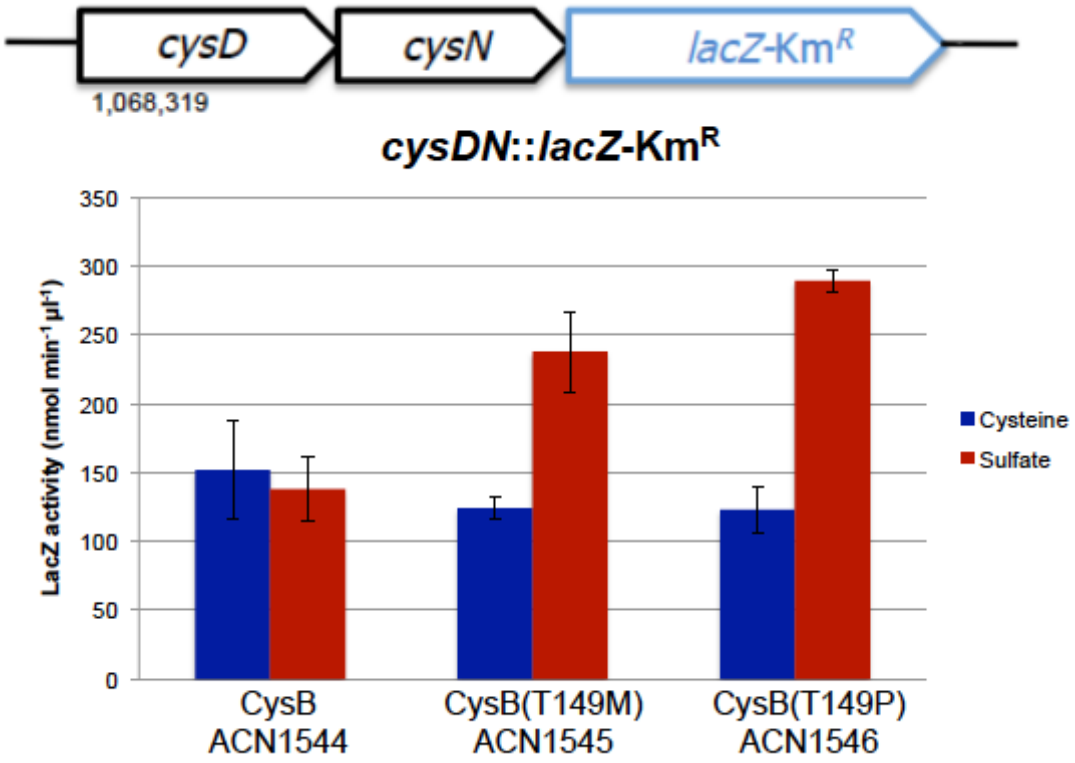


Figure 2.3. *cysDN* expression. The values indicated represent LacZ activity as a result of a chromosomal transcriptional fusion with *cysDN,* and are the average of at least two independent experiments.

Sbp expression was also analyzed using a *sbp*::*lacZ* fusion in strains expressing the three *cysB* alleles individually. Similar to the situation in the *cysDN*::*lacZ* strain encoding wild-type CysB, *sbp* expression in ACN1553 (encoding wild-type CysB), as measured with LacZ activity, did not appear to differ significantly when the growth medium was supplemented with cysteine versus sulfate (Figure 2.4). LacZ assays with this strain were performed twice, and statistical analysis indicated that the difference between LacZ activity in cysteine-grown and sulfate-grown cells was not significant ($p=0.40$). However, the high standard deviation in the cysteine-grown ACN1553 LacZ activity suggests that additional experiments are needed to obtain a more accurate comparison.

Expression at the *sbp* locus was, however, affected by different sulfur sources in a strain that encodes CysB(T149M) variant (ACN1554; Figure 2.4). Sulfate-grown ACN1554 exhibited 10-fold greater LacZ activity when compared to cysteine-grown ACN1554, and when compared to ACN1553 in either growth condtion (p-values<0.05). In contrast, the strain encoding the CysB(T149P) variant (ACN1555) appeared to express *sbp* at similar levels to the strain expressing wild-type CysB, when grown with cysteine (Figure 2.4). In sulfate-grown cells encoding CysB(T149P), however, LacZ activity was more than 3-fold less than activity in sulfate-grown cells encoding wild-type CysB (p-value=0.05). These results suggest that CysB plays a role in regulating *sbp*, and that the two CysB EBD variants affect this regulation differently.

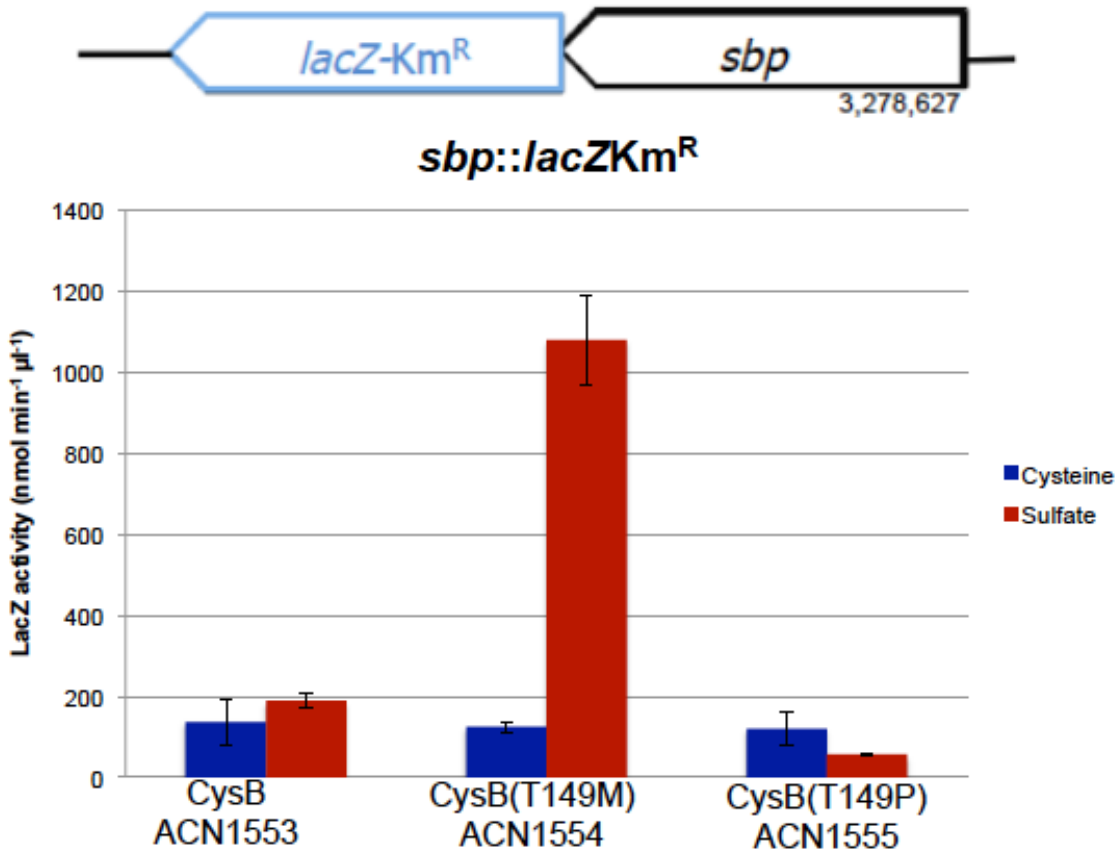


Figure 2.4. ***sbp*** **expression.** The values indicated represent LacZ activity as a result of a chromosomal transcriptional fusion with *sbp,* and are the average of at least two independent experiments.

If CysB activates the expression of *sbp, cysDN,* or *cysI-orf*, we would expect LacZ activity in *cysB*-deleted strains with *lacZ* fusions of these genes to be very low. As discussed in the introduction to this chapter, *cysB*-deleted strains can only be grown in cysteine-supplemented medium, which was expected to result in downregulation of cysteine biosynthesis genes, in the presence or absence of CysB. As shown in the initial data above, it was possible to measure LacZ activity in strains grown in cysteine-supplemented medium that encoded wild-type CysB. Given the level of this activity, it seemed worthwhile to compare LacZ activity in strains encoding wild-type CysB and strains lacking the *cysB* gene.

As shown in Figure 2.5, LacZ activity in the *cysB*-deleted strain ACN1574 was 2-fold lower than the LacZ activity in the strain expressing wild-type CysB (ACN1553), indicating decreased *sbp* expression without CysB. However, this difference was not statistically significant ($p=0.31$), and, as discussed above, there is large standard deviation in LacZ activity data from ACN1553. Therefore, while the decreased expression in the absence of CysB might suggest a role for this regulator in activating *sbp* expression, additional repetitions of these experiments are needed to confirm whether or not there are statistically significant differences in gene expression.

Similar to the *sbp*::*lacZ* results, the strain with a *cysDN*::*lacZ* fusion and a *cysB* deletion generated 2-fold less LacZ activity than the strain with the same *lacZ* fusion and an intact *cysB* gene (Figure 2.5). Statistical analysis revealed that in this case, the decrease in activity was significant ($p<0.05$). Because gene expression is higher in the presence of CysB, it appears to play a role in transcriptional activation of this gene.

In the strain with a *cysI-orf*::*lacZ* fusion and a *cysB* deletion, LacZ activity could not be detected (ACN1460; Figure 2.5). This result could indicate that CysB is required for *cysI-orf*

expression. However, because this result differs from that with the other genes, this experiment will be repeated with independently constructed strains to ensure that the results are reproducible.

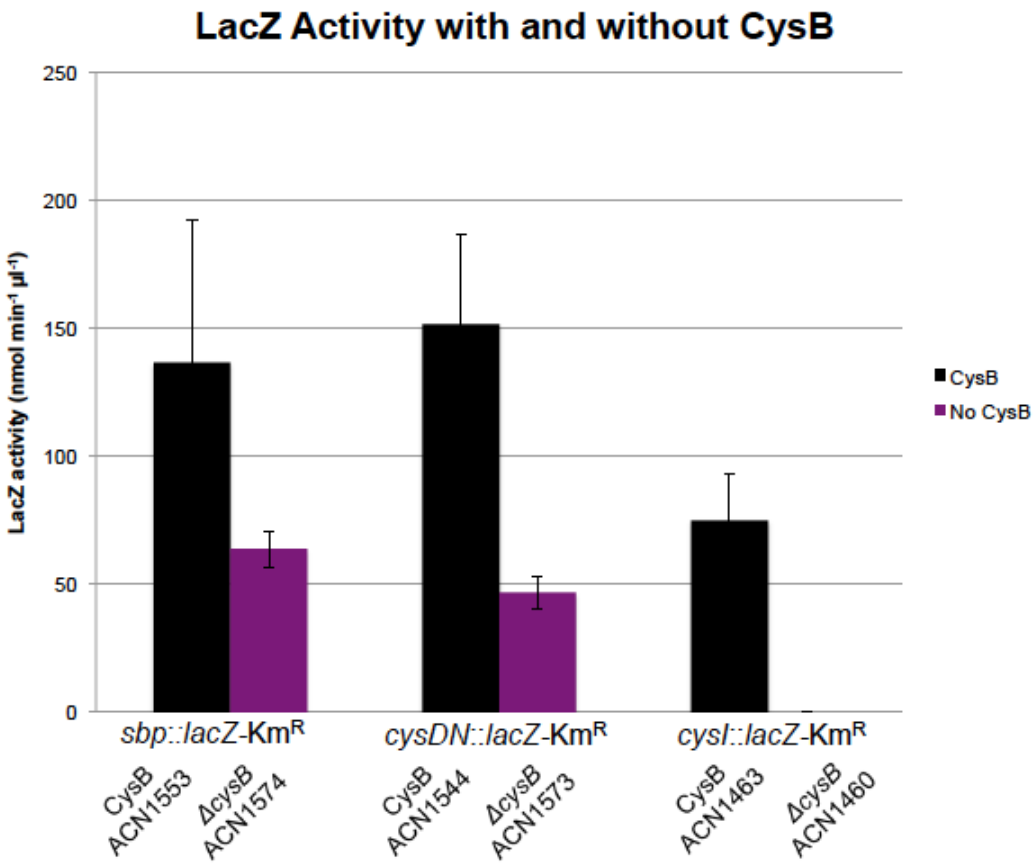


Figure 2.5. *cys* gene expression with and without CysB. Assays were performed with cysteine-grown cells. The values indicated represent LacZ activity as a result of chromosomal transcriptional fusions with *sbp, cysDN,* and *cysI-orf*, and are the average of at least two independent experiments.

Deletion and Complementation Experiments with *finR* and *fpr*

As described in Chapter 1, the protein that facilitates the transfer of electrons to CysI has not yet been identified in ADP1, and the possibility of Fpr, a putative ferredoxin:NADPH reductase, fulfilling that role was discussed. The *fpr* gene is downstream of, and oriented divergently to, *finR*, which is predicted to encode a LTTR that has some structural similarity with *B. subtilis* CysL. Additionally, there is a *fpr* paralog elsewhere in the chromosome (Figure 2.6B). All three of these genes were identified as essential on minimal medium in a single-gene deletion survey of ADP1, and that phenotype was confirmed in this study (7). Efforts were made to delete *finR* on rich medium (LB) and on minimal medium supplemented with several different sulfur sources, all of which were unsuccessful.

Because of the location of *finR* in the ADP1 chromosome, and previous work with *P. putida* demonstrating the regulation of *fpr* by FinR, it was hypothesized that the essentiality of *finR* in ADP1 may be due to the requirement for *fpr* transcription (71). If *fpr* were expressed from a FinR-independent promoter, it might be possible to eliminate the need for *finR*. To this end, strains were constructed with a plasmid-borne *fpr* gene expressed from a non-native promoter. Subsequently, attempts were made to inactivate the chromosomal copy of *finR* by allelic replacement with engineered DNA that could be selected by an inserted drug marker. Both *fpr* paralogs (ACIAD0747 and ACIAD2244: *fpr* and *fpr2*, respectively) were cloned separately into an IPTG-inducible expression vector, pIM1445 (69), which can be maintained in *A. baylyi*. The resulting plasmids, pBAC1237 and pBAC1238, were individually transformed into wild-type ADP1, and transformants were selected using a high concentration of Km (500 µg/mL). *A. baylyi* strains transformed with pIM1445 have increased resistance to Km compared to strains that contain a single copy of our drug resistance cassette (Km^R) in the chromosome

(data not shown). Selection with a high amount of Km was used to maintain the plasmid at multi-copy levels, thereby reducing the chance of isolating drug-resistant strains in which the plasmid integrates into the chromosome via homologous recombination between a chromosomal copy of *fpr* and a plasmid-borne copy. To ensure that the expression plasmids were producing each of the Fpr paralogs at sufficient levels for prototrophy, control strains were constructed in which each *fpr* gene was deleted from the chromosome and complemented *in trans* by an appropriate plasmid carrying the deleted gene.

To disrupt *finR*, ADP1 cultures with pBAC1237 or pBAC1238 *in trans* were transformed with linearized DNA containing a $SpSm^R$ cassette flanked by DNA with homology to the upstream and downstream regions surrounding *finR* (Figure 2.6A). Transformants were selected on LB agar with Sm, Sp, high concentrations of Km, and IPTG. Additionally, all deletions were attempted in ADP1 cultures with the empty vector pIM1445. If *fpr* expression were required for *finR* disruption, we would expect negative results from these controls.

The *finR* gene was successfully removed by allelic replacement when pBAC1238 was in the cell. In the resulting mutant, ACN1614(pBAC1238), *finR* is replaced with a $SpSm^R$ cassette, and *fpr2* expression is induced from the expression plasmid pBAC1238. There is an additional native genomic copy of *fpr2* (Figure 2.6B). Growth of this strain is inhibited on minimal medium, and this phenotype is alleviated with exogenous L-cysteine.

None of the other attempts to disrupt *finR* were successful. To date, a *finR* deletion mutant has not been isolated in *A. baylyi* containing the expression plasmid pBAC1237 (*fpr* expression). However, a *fpr* deletion mutant also has not been obtained in the same background, possibly indicating insufficient expression from pBAC1237. Additionally, efforts to complement *Δfpr* with *fpr2*, and vice versa, have been unsuccessful.

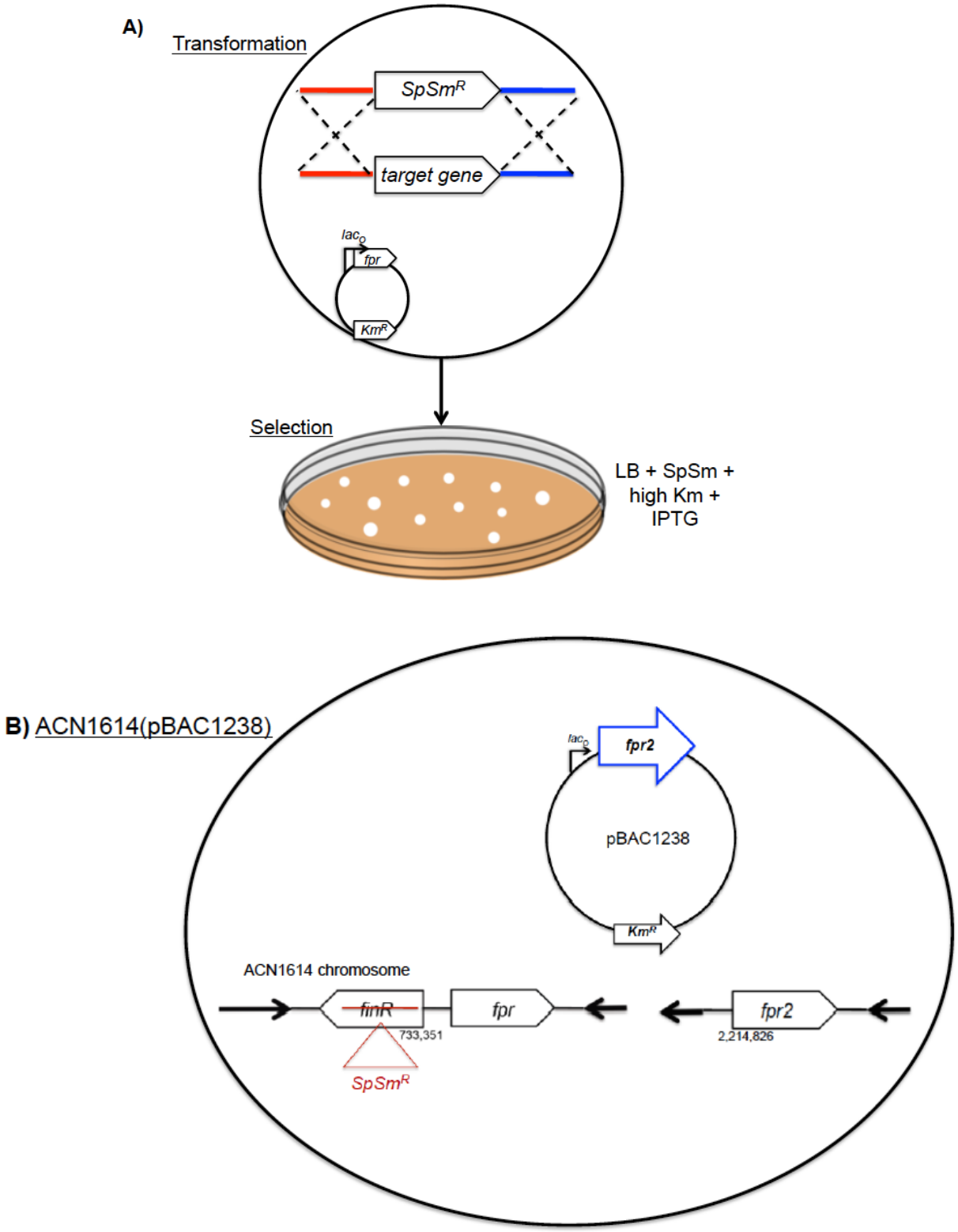


Figure 2.6. *finR* and *fpr* mutants. A) Strain construction schematic for *finR* and *fpr* allelic replacements with $SpSm^R$ in the presence of expression plasmids conferring kanamycin resistance. B) Genetic map of ACN1614(pBAC1238).

EMSAs with CysB and FinR

While the results described above suggest roles for CysB and FinR in the regulation of cysteine biosynthesis, direct interactions of these regulators with the operator-promoter regions of specific genes had not been demonstrated. To investigate such interactions, EMSAs were done with purified proteins and PCR-generated DNA. Based on genetic context, and the nearby presence of a predicted CysB binding site (Figure 2.7), it seemed likely that CysB would regulate *cysP* expression. A 447 bp fragment was generated that included 297 bp of DNA upstream of the *cysP* coding sequence and part of the structural gene (Table 2.3). Unpublished results indicate that the transcriptional start site is 24 nt upstream of the *cysP* start codon (results from students in MIBO4600L, Nicole Laniohan, and Melissa Tumen-Velasquez). As shown in Figure 2.8A, increasing amounts of protein affected the mobility of the DNA, indicating the formation of a protein-DNA complex that is consistent with the ability of the CysB protein to bind the *cysP* promoter region. The potential role of FinR in regulating *cysP* was examined with the same *cysP* promoter region as used with CysB. EMSA analysis revealed not only that FinR can retard the mobility of free *cysP* promoter DNA, but it does so at lower concentrations than CysB (Figure 2.8B).

A potential CysB binding site was also located upstream of *cysDN* (Figure 2.7). A 525 bp fragment was generated that included 457 bp upstream of the *cysD* start codon and part of the coding sequence (Table 2.3). Increasing amounts of CysB caused an upward shift in DNA mobility, indicating CysB binding to the *cysDN* promoter region (Figure 2.8C). FinR binding at the same region was also examined, and, as with the *cysP* promoter region, the DNA shifted with lower amounts of FinR than with CysB (Figure 2.8D).

In *E. coli*, the *ssu* gene cluster is regulated by Cbl, and in other bacteria, it is regulated by CysB (17, 72). Alternatively, a regulator distinct from CysB, such as SsuR in *C. glutamicum,* controls these genes (73). The *ssu* genes in ADP1 are similarly clustered (Figure 1.1). CysB and FinR were separately tested for binding at this locus using a 522 bp DNA fragment that included 439 bp upstream of the *ssuA* start codon (Table 2.3). As shown in Figure 2.8E, CysB was able to bind the *ssu* promoter region at concentrations of 250 nM and above, and FinR was able to produce a DNA shift at concentrations of 100 nM and above.

Upstream of *cysI-orf*, two sequences were found that resemble the predicted CysB binding site (Figure 2.7). These sites, and the LacZ data discussed above, suggested a potential role for CysB regulation of *cysI*. A 397 bp DNA probe that included 306 bp upstream of the *cysI* start codon, and part of the *cysI* coding sequence, was generated for EMSA analysis. Furthermore, an undergraduate, Maliha Ishaq, determined the *cysI* transcriptional start site to be 29 nt upstream of its coding sequence (unpublished data). However, CysB interaction with the *cysI* promoter region could not be detected in EMSAs (data not shown). FinR is also a candidate for *cysI* regulation because of the potential role for Fpr in the electron transfer reaction needed for sulfite reduction. As shown in Figure 2.8F, FinR shifts the *cysI* promoter region at concentrations of 5 nM and above.

Given the surprising affinity FinR demonstrated at all promoter regions examined in Figure 2.8 compared to CysB, a DNA probe unrelated to sulfur metabolism was used to examine the potential for non-specific binding. A 397 bp DNA fragment upstream of *catB*, a promoter where regulation by another LTTR, CatM, has been established, was used in an EMSA with increasing amounts of FinR (Figure. 2.8G) (74). FinR at concentrations of 50 nM and 100 nM generated a shift in *catB* DNA mobility. This result suggested FinR may bind promiscuously at

moderate concentrations, and subsequent EMSAs with FinR will be performed with lower titrations of the protein to help rule out non-specific interactions. Additional controls will address specificity by using labeled promoter DNA and unlabeled competitor DNA.

Table 2.3. Primers used to generate promoter regions for EMSAs

Promoter Region	Forward and Reverse Primers [a]	Genome position [b] and fragment length
cysDN	F 5' – GACATTTTGTGCTTTAAACGG – 3' R 5' – CGAATAATATGAATACTCTCAGCT – 3'	1067862-1068386 525 bp, *cysD* start codon at position 457
sbp	F 5' – AATCTTTCGCTGCGTTGG – 3' R 5' – TCTGTAGGCCAAATGTATGC – 3'	3279553-3279869 317 bp, *sbp* start codon at position 241
cysI	R 5' – GTCAAGCCACACCGAT – 3' F 5' – GGTTTGATCACGGAACTG – 3'	2911723-2912097 375 bp, *cysI* start codon at position 306
cysK	F 5' – TTAACAACTAAGAAATATGAACTTGCC – 3' R 5' – ACCTAAAACCGTTGCACC – 3'	1666657-1667100 444 bp, *cysK* start codon at position 309
cysP	F 5' – TGCTAAATGACGATGCAT – 3' R 5' – CTGTTTCCAGTATGCACC – 3'	2549152-2549598 447 bp, *cysP* start codon at position 297
ssu	F 5' – AATTTGAACCATGAAGATGAAATG – 3' R 5' – TAGTGCCCGACAAGAG – 3	43979-44500 522 bp, *ssu* start codon at position 439
catB	F 5' – GCCTGCTTCAGTCACTTTAG – 3' R 5' – CTAATGTCTGCGTCTGCATC – 3'	12845-13241 397 bp, *catB* start codon at position 306

a. Primers designed by Melissa Tumen-Velasquez.
b. Position in ADP1 genome according to GenBank accession CR543861.1.

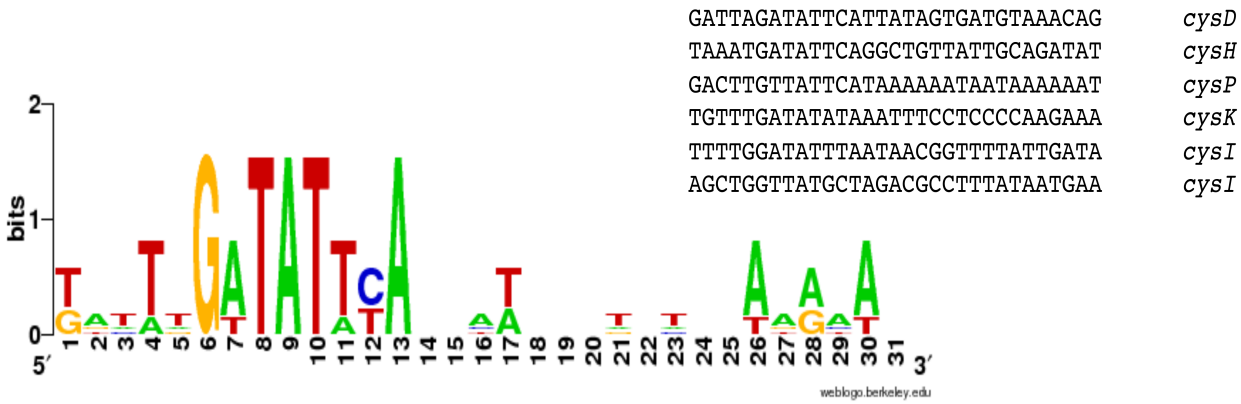


Figure 2.7. Predicted consensus sequence for CysB binding in ADP1. *A. baylyi* sequences that displayed a significant match to the *E. coli* sequences (see Materials and Methods) were aligned with each other and used to derive an *A. baylyi* consensus sequence with the WebLogo program (62, 63, 75). The potential binding sites detected upstream *of cysD, cysH, cysP, cysK*, and *cysI* in ADP1 are shown together with the LOGO, where the height of each letter indicates the degree of conservation in the aligned sequences.

Not shown in Figure 2.7, two CysB binding sites were also located upstream of the *sbp* coding sequence. However, CysB EMSAs with a 317 bp sequence that included 241 bp upstream of the *sbp* coding sequence did not produce a shift (data not shown). Because of the high sequence similarity of the sites found in this region to the predicted CysB binding consensus sequence, additional EMSAs were performed with a small synthesized DNA fragment containing the CysB binding sites from the *sbp* region (Figure 2.9). CysB was able to bind to this 50mer at concentrations of 400 nM and above.

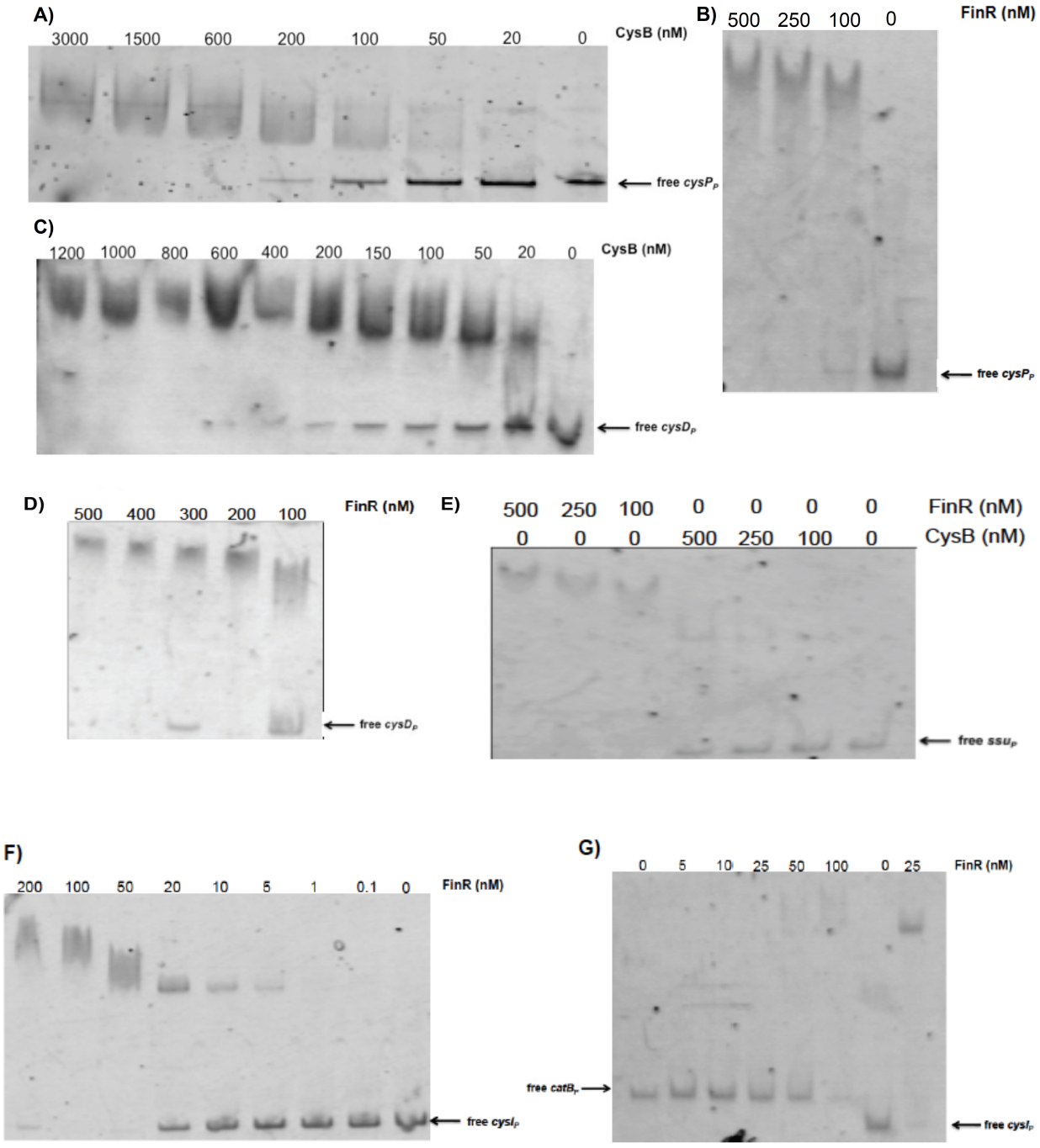
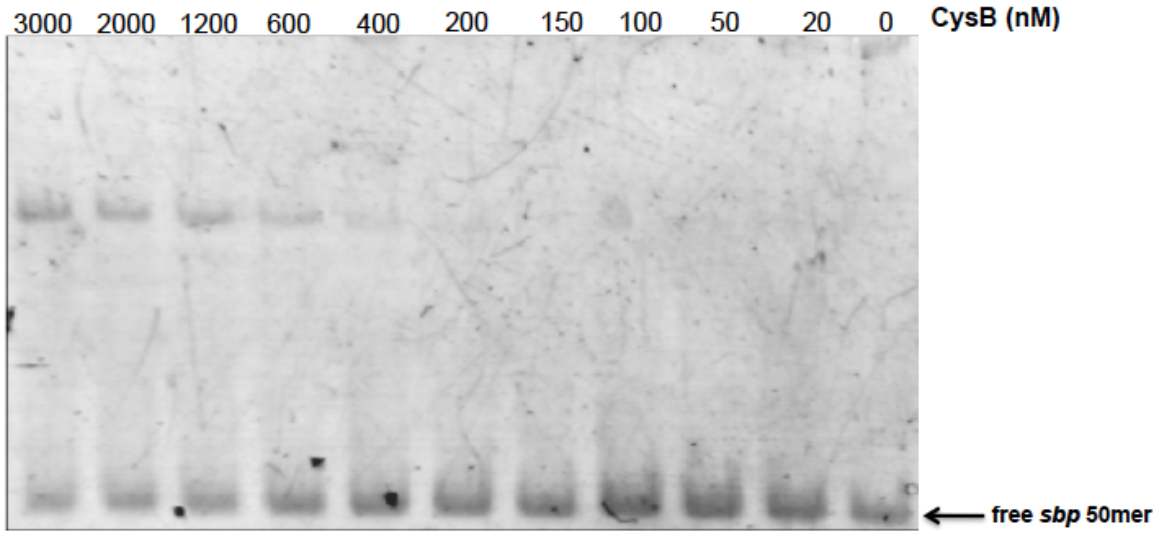


Figure 2.8. EMSAs with CysB and FinR. DNA probes are provided at 2 nM. Protein concentrations are indicated above each lane. A) CysB/*cysP*. B) FinR/*cysP*. C) CysB/*cysDN*. D) FinR/*cysDN*. E) CysB/*ssu* and FinR/*ssu*. F) FinR/*catB*. G) FinR/*cysI*.



5' – TATAGTTATATCAATAACAACATTGCAAATCTGTTTTTGTGATATCAAAC – 3'

Figure 2.9. EMSA with CysB and *sbp* 50mer. DNA probe was provided at 20 nM and the sequence is displayed at the bottom. Protein concentration is indicated above each lane.

Discussion

Collectively the results of these studies indicate that while the general framework of cysteine biosynthesis in *A. baylyi* resembles that in *E. coli* and *S. enterica*, there are several significant differences in genetic organization, regulation, and some of the enzymatic steps. The sulfur source utilization capabilities of ADP1 are dramatically inhibited by the deletion of ACIAD2597, previously annotated as *cbl*, and here named *cysB*. The *cysB*-deleted strain ACN1262 can grow only with L-cysteine, L-cystine, and djenkolate as sole sulfur sources (Table 2.1). Based on the chemical structure of these compounds, they would not be expected to require the CysPUWA transporter for entry. Once these compounds are inside the cell, the reducing environment likely results in the release of cysteine, thereby alleviating the need for the corresponding biosynthetic enzymes. However, the inability of the *cysB*-deleted mutant to grow on minimal medium with sulfate as the sulfur source suggested that the encoded regulator controls expression of the genes that are upstream of *cysB* on the ADP1 chromosome: *cysP*, ACIAD2592, *cysU, cysW*, and *cysA*. These genes may form an operon.

CysB-regulated expression of transport genes

Regulation of *cysP*

A possible binding site for CysB was identified upstream of *cysP*, based on known binding sequences of the comparable regulator in *E. coli*, using the Virtual Footprint program (63). Similar bioinformatics analysis of other genes in this cluster revealed several additional sites that might be recognized by CysB that are upstream of ACIAD2592 (immediately downstream of *cysP*), which encodes a putative esterase, and upstream of *cysW* (data not shown). The validity of such predictions remains to be tested and further work is needed to determine

which genes are co-transcribed. EMSA results demonstrated binding of CysB to the *cysP* operator-promoter region (Figure 2.5). In *E. coli,* CysB-*cysP* binding has been demonstrated with an apparent K_d of 4.72 nM (35, 76). Although quantitative measurements were not attempted with the experiments shown in Figure 2.5, it is clear that in our experiments there was a lower affinity of the CysB regulator for *cysP* DNA than in the published reports for other bacteria.

LTTRs can act as transcriptional repressors and/or activators. We infer a role for CysB as a transcriptional activator because loss of the protein results in cysteine auxotrophy, indicating a loss of biosynthetic enzyme activity. However, there may be multiple binding sites in the same operator-promoter region, some of which are important for repression and others for activation, as is the case in *S. enterica* (77). Further work is needed to localize the position(s) of CysB binding and to determine if different experimental conditions improve the affinity of the protein for this DNA region.

The inducer NAS is required for CysB-mediated transcriptional activation in *E. coli* and *S. enterica*, but it is not required for DNA binding (37). Many LTTRs bind their target sequences without the presence of coinducers (78). However, there are a few reports of LTTRs requiring coinducer for DNA binding, including CmbR, which regulates the *metC-cysK* operon in *L. lactis*. Binding of CmbR to the *metC* promoter requires the presence of OAS in EMSA studies (79). There is currently no known inducer for CysB regulation in ADP1. Several sulfur compounds were tested here for any effects on CysB-DNA mobility in EMSAs. Various concentrations of L-cysteine, L-methionine, sulfate, sulfite, sulfide, thiosulfate, and OAS were used in CysB gel shifts with the *cysP* promoter region and also those of *cysDN,* and *cysI* (not

shown). No meaningful effects were observed in these experiments, but efforts to identify a CysB effector are ongoing.

No studies of gene expression were conducted with *cysP*. Transcriptional fusions were not constructed because of the possibility that insertion of the *lacZ* reporter in *cysP* might prevent transport due to polar effects on the downstream genes. *CysP* appears to be part of a large operon, so placing a transcriptional fusion further downstream may be problematic because the transcriptional unit(s) have not been defined. Future studies will address *cysP* expression using quantitative real-time PCR.

Studies of the CysPUWA transport system in other bacteria suggest that there is overlap in the function of two periplasmic proteins (Sbp and CysP) in transporting thiosulfate and sulfate into the cell. Here we showed that without CysB, neither thiosulfate nor sulfate is able to serve as a sulfur source. Recent results (not presented in this thesis) indicate that a mutant deleted for the *cysP* coding sequence can grow on cysteine, sulfate, thiosulfate, and sulfite. Tests with sulfide have not been done yet. Based on transport studies in other bacteria, our ADP1 model assumes that CysP and Sbp both require the CysUWA proteins to transport both sulfate and thiosulfate.

Regulation of *sbp*

Sbp transcriptional control by CysB was also considered. In *B. cenocepacia*, both CysB and SsuR are capable of binding the *sbp* promoter. Additionally, both proteins were able to upregulate *sbp* transcription *in vitro*, indicating overlapping functions of these regulators at the *sbp* promoter (3). It is worth noting, however, that *sbp* in *B. cenocepacia* is transcribed with *cysUWA*, an operon that is located upstream of *ssuR*. In this case, SsuR-mediated regulation of *sbp* is not surprising. In ADP1, binding sites for CysB were found near the *sbp* gene (Figure

2.9). CysB binding was seen with a 50 bp DNA fragment containing the predicted binding sites, but not with a larger piece of DNA that included these sites, and additional upstream and downstream sequences. Moreover, high concentrations of CysB were needed to shift the 50mer oligonucleotide fragment, so it is not clear if this result is physiologically relevant.

To test the effect of CysB on *sbp* expression, *lacZ* transcriptional fusions were used. It was anticipated that growth in the presence cysteine would result in lowered *cys* gene expression, and growth in the presence of an oxidized sulfur source would result in increased *cys* gene expression. For *sbp*, a slight increase in expression with sulfate compared to cysteine was not found to be statistically significant (Figure 2.4). Expression of this gene may not be strongly regulated by growth conditions. Alternatively, we may not be using conditions in our assays that adequately represent substantial differences between sulfur limitation and surfeit. Two observations suggest that CysB regulates *sbp* expression. First, in a strain encoding a variant with a single amino acid replacement, CysB(T149P), there was significantly increased expression of *sbp* when grown with sulfate compared to that with the wild-type regulator. Secondly, when grown with cysteine, the deletion of *cysB* in an otherwise isogenic strain decreased *sbp* expression (Figure 2.5).

CysB-regulated expression of genes for the reduction of sulfate to sulfite

Regulation of *cysDN*

In ADP1, a CysB binding site was found near *cysDN,* genes that encode an ATP sulfurylase that catalyzes the conversion of sulfate to APS. Consistent with the observance of a potential binding sequence (Figure 2.7), CysB was able to bind to the *cysDN* promoter region at concentrations of 20 nM and above (Figure 2.8C). Assessment of *cysDN* expression with LacZ

activity revealed elevated expression in the CysB(T149M)-encoding strain compared to the wild-type CysB-encoding strain (Figure 2.3). This elevation was also observed in the strain encoding CysB(T149P). The dramatic effect on expression from the *cysDN* promoter resulting from two different individual amino acid replacement in CysB indicates that this regulator can activate transcription. This conclusion is supported by the decrease in expression that results from the deletion of *cysB* when expression is compared to that with the wild-type *cysB* gene in strains grown on medium supplemented with cysteine (Figure 2.5).

Similar to the situation with *sbp*, it was surprising to discover that wild-type expression levels of *cysDN* were comparable in medium provided with sulfate compared to that provided with cysteine. As noted earlier, these genes may not be subject to large scale expression changes depending on the medium and/or our growth conditions may need to be changed in order to signal conditions of starvation versus excess sulfur. Reports in *E. coli* and *S. enterica* show that rapid turnover of CysD and CysN, require active transcription to maintain sufficient levels of enzyme (2). It may be that protein turnover influences transcriptional regulation.

Conversion of APS to Sulfite

In *E. coli* and *S. enterica*, an additional gene, *cysC*, is cotranscribed in a *cysDNC* operon. CysC phosphorylates APS to produce PAPS, which is then reduced by the PAPS reductase, CysH. In ADP1, there is no CysC homolog, and APS is reduced by an APS reductase, also called CysH. A similar bypass of PAPS formation was long known to be characteristic of cysteine metabolism in plants. However, as first demonstrated in *Allochromatium vinosum*, some bacteria encode a pathway like that of ADP1 in which the CysC-encoded enzyme is missing and in which the CysH reductase has distinguishing features characteristic of its use of APS rather than PAPS as a substrate (80). In ADP1, the *cysH* gene is unlinked to any other

genes predicted to be involved in cysteine biosynthesis (Figure 1.1). A CysB binding site was located upstream of the *cysH* coding sequence in ADP1, but interaction at this site was not examined in this study. In *E. coli* and *S. enterica*, *cysH* is part of the *cysJIH* operon, which is regulated by CysB (2) (81).

CysB- and FinR-regulated expression of sulfite reduction genes

Regulation of *cysI-orf*

In *A. baylyi*, *cysI* is not only unlinked from *cysH*, but there is no *cysJ* homolog that appears to be involved in cysteine biosynthesis. Two predicted CysB binding sites were identified upstream of the *cysI* coding sequence (Figure 2.7), but attempts to demonstrate CysB binding with the *cysI* promoter region by EMSA methods were unsuccessful (not shown). To test the effect of CysB *in vivo*, *cysI* expression was assessed using *lacZ* transcriptional fusions. Consistent with the expectation that cysteine biosynthesis genes would be downregulated in presence of cysteine, *cysI-orf* expression was 2-fold higher in sulfate-grown cells than in cysteine-grown cells (Figure 2.2). Additionally, LacZ activity in strains with the *cysI-orf*::*lacZ* fusion and the allele encoding CysB(T149M) was higher than in strains expressing wild-type CysB. This elevation was observed in both cysteine-grown and sulfate-grown cells, and suggests a role for CysB in *cysI-orf* regulation.

In the *cysB*-deleted strain with the same *lacZ* fusion, LacZ activity was undetectable (Figure 2.5). Additionally, a strain expressing CysB(T149P) (ACN1511) exhibited the same inactivity under all growth conditions tested (data not shown). Although these results could correctly indicate that there is no detectable *cysI-orf* transcription, it is also possible that the *lacZ* reporter is dysfunctional. Furthermore, ACN1511 was able to grow prototrophically, indicating

successful production of a functional CysI. Therefore, these results are not conclusive. Expression at this locus should be evaluated further, perhaps with quantitative real-time PCR methods.

FinR and Fpr

Sulfite reduction in ADP1 has more resemblance to the pathway in plants than in *E. coli* and *S. enterica*. In these bacteria, sulfite reductase works with the flavoprotein CysJ. CysJ facilitates the transfer of electrons from NADPH to CysI, which reduces sulfite to sulfide. In plants, a ferredoxin functions in the electron transfer reaction, and there is no CysJ-like flavoprotein. ACIAD0799 is annotated as *cysJ*, but the gene product does not share homology with any characterized CysJ proteins. Another gene, ACIAD2981, overlaps with *cysI*, suggesting coupled transcription and translation and interaction between the gene products. However, the ACIAD2981 gene product does not have any features resembling an oxidoreductase.

A recent report showed that *P. putida* likely utilizes a ferredoxin to transfer electrons from NADPH to CysI (25). Encoded by *fpr*, the production of this protein is dependent on the LTTR FinR. The FinR homolog in ADP1 has some structural similarity with the cysteine biosynthesis regulator in *B. subtilis*, CysL. This observation, coupled with the absence of a known protein facilitating the electron transfer for the reduction of sulfite in ADP1, suggested that FinR may be involved in the regulation of cysteine biosynthesis. FinR binding was examined in EMSAs with the *cysI-orf, cysP, cysDN,* and *ssu* promoter regions (Figures 2.9 and 2.10). FinR was able to bind all of these promoters, with particularly strong affinity for the *cysI-orf* region.

Interpretation of these results remains unclear, however, because, FinR also produced a shift with the *catB* promoter at ~50-100 nM (Figure 2.9B). CatB is a protein involved in the degradation of catechol, a pathway completely unrelated to cysteine biosynthesis, and expression of *catB* is regulated by another LTTR, CatM (74). It is unlikely that FinR binding to the *catB* promoter is non-specific LTTR binding since other LTTRs tested at this site have not been found to bind at this position (78). These EMSAs with FinR will be repeated with a lower titration of the protein. Additional experiments will utilize labeled DNA probes and unlabeled competitor DNA to clarify FinR binding specificity.

If it is confirmed that CysB and FinR both bind to the same promoter regions, they might be able to recognize the same or similar sequences in operator-promoter regions. Similarity in DNA recognition should be reflected in similar amino acids in the N-terminal DNA-binding domain of the regulators. To compare amino acids in this region of the proteins, their sequences were aligned and displayed using the programs MultAlin and ESPript (Figure 2.10) (82, 83).

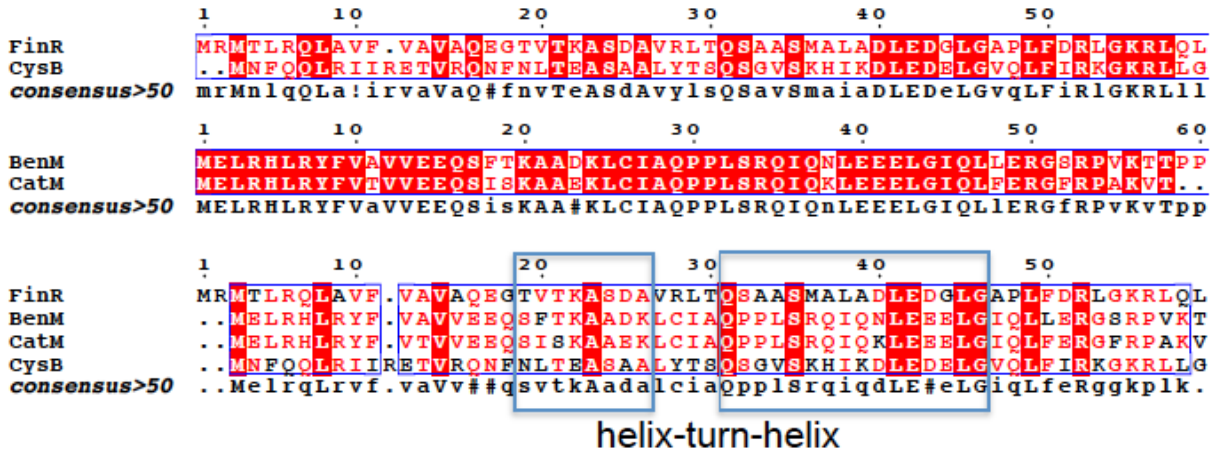


Figure 2.10. Alignment of sequences in the DNA-binding domains of LTTRs from ADP1. Identical residues in the alignments are indicated by capital letters in the displayed consensus sequence. Symbols in the consensus indicate shared properties of the aligned residues. The helices of the helix-turn-helix binding motif are indicated by boxes based on the structure of BenM-DBD (84). The longer helix is the recognition helix.

The alignment shows that FinR and CysB are approximately 40% identical in sequence in their DNA-binding regions. This level of identity does not reveal obvious similarity in DNA-binding properties. In contrast, the comparable regions of two LTTRs that are known to recognize similar operator-promoter regions (BenM and CatM), are nearly identical. These two regulators both bind to the *catB* promoter, another region that FinR was shown to bind. The alignment of all four of these proteins does not reveal any explicit reason that these LTTRs should all recognize the same DNA.

To further evaluate the role of FinR in cysteine biosynthesis, attempts were made to delete *finR*. This gene is essential, so it was necessary to express *fpr2 in trans* before an allelic replacement of *finR* with a drug marker was successful. The resulting strain exhibited inhibited growth on minimal medium that could be alleviated with cysteine supplementation. This result suggests that FinR is involved in cysteine biosynthesis, and that Fpr and/or Fpr2 may be fulfilling the "CysJ" electron transfer function previously uncharacterized in ADP1. This strain still contains the *fpr* and *fpr2* genes in the chromosome, but it is assumed, because of the *finR-fpr* locus organization, that *fpr* is not being expressed in the absence of FinR. Further experiments are needed with this strain to confirm whether the growth phenotype is caused by the *finR* deletion, insufficient *fpr* expression, or both.

Although additional work is needed for conclusive proof of the roles played by FinR and CysB in regulating cysteine biosynthesis in ADP1, these studies provide the first evidence that mutations in the corresponding genes affect a complex metabolic network for sulfate assimilation in this bacterium. The pathway was investigated in terms of genetic organization, gene expression, and sulfur source utilization. These studies contribute not only to our understanding

of metabolic regulation, but also to our understanding of the structure and function of an important family of transcriptional regulators, the LTTRs.

CHAPTER THREE

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Cysteine biosynthesis has been extensively studied the Gram-negative bacteria *E. coli* and *S. enterica* (2). While these organisms provide a model for cysteine biosynthesis in enteric bacteria, they do not represent the immense diversity of the Bacteria kingdom. The research presented in this thesis contributes to the expanding knowledge of sulfur assimilation in bacteria. Cysteine biosynthesis in the soil bacterium *Acinetobacter baylyi* ADP1 was previously uncharacterized, and the work presented here helps elucidate the regulation of this pathway.

ADP1 has exceptionally versatile metabolic capabilities, and this versatility was extended to include a wide range of sulfur compounds. Growth with these compounds, however, was dramatically inhibited by the deletion of *cysB*. Investigation of CysB in EMSAs and *lacZ* transcriptional fusion assays revealed that CysB has a regulatory role in cysteine biosynthesis. Additionally, the inhibited growth without cysteine of a *finR* deletion mutant indicates FinR may be regulating some parts of the pathway. Electron transfer to CysI, a function previously unattributed to any known protein in ADP1, is likely facilitated by the ferredoxin:NAD(P)H oxidoreductase Fpr.

This thesis documents the first experimental evaluation of cysteine biosynthesis in *A. baylyi*. The regulation of this pathway is different from *E. coli* and *S. enterica*, and knowledge

of the variations in cysteine biosynthesis among bacteria contributes to our understanding of complex metabolic processes such as sulfur assimilation.

Future directions

Many aspects of cysteine biosynthesis in ADP1 remain elusive. The regulation of genes in the pathway by sulfur source availability was addressed, and it appears that expression of some genes may be affected differently. These dissimilarities could be indicative of distinct regulatory processes, or they could be consequences of the growth conditions used in the experiments. Comparisons of gene expression at different stages of the growth curve might be informative. Additionally, more sensitive methods for gene expression analysis, such as quantitative real-time PCR, may reveal differences not detectable in LacZ assays.

The roles of CysB and FinR at each step of the pathway need to be interrogated further. Given the overlapping DNA-binding characteristics of these two regulators, it is possible that some loci are controlled cooperatively by both CysB and FinR. If this is the case, EMSAs with both proteins added should result in slower bands than those observed when the proteins are evaluated individually, indicating distinct protein-DNA complexes.

LacZ assays with strains encoding CysB EBD variants displayed altered *cys* gene expression. EMSAs with purified variants compared to the EMSAs with wild-type CysB demonstrated in this thesis should help elucidate the mechanism behind the different levels of expression. The EBD variants used in this study have been shown to have different DNA binding properties in *S. enterica*, indicating distinct conformations (52). This study also demonstrated different responses of the variants to inducers an antiinducers. CysB effectors in

ADP1 have not been discovered, despite efforts described here. However, small molecule candidates have not been exhaustively tested, and EMSA conditions have not been optimized.

Fpr involvement in sulfite reduction is suggested by the *finR* deletion mutant phenotype. If this protein is fulfilling the "CysJ" role in ADP1, the *E. coli* CysJ may be able to complement a *fpr* deletion, as was shown in *P. putida* (25). With the successful allelic replacement of *finR* reported here, analysis of *cys* gene expression in this background may be informative. Additionally, FinR variants could be constructed based on predicted roles of residues located in the DBD or EBD. The crystal structure of FinR currently being worked on the Momany lab will be especially helpful in this endeavor.

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