Mutations in *atpG*, encoding the γ subunit of ATP synthase cause lowered expression of *pckA* in *Escherichia coli*

A thesis submitted to the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
in the Department of Microbiology and Immunology
University of Saskatchewan

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Fall 2002

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Abstract

Phosphoenolpyruvate carboxykinase [E.C.4.1.1.49] (Pck) catalyses a key reaction of gluconeogeneis in *Escherichia coli*. It converts the Krebs cycle intermediate oxaloacetate (OAA) to phophoenolpyruvate (PEP), which is a part of glycolysis. Transcription of *pckA*, the structural gene for Pck is regulated by catabolite repression and the transcription increases 100 fold in early stationary phase, by an unknown mechanism.

This study used mini Tn*10-ATS* (Kan^R) to isolate mutants that affect the expression of *pckA* in stationary phase. Using penicillin selection, $Succinate^-$, Kan^R mutants defective in Pck were isolated. The mutants had lower growth yields and lower Pck specific activity than the wild type. Experiments with a slow growing *recA* strain and a *pckA*::Tn*10* mutant indicated that the lowered Pck levels were not correlated with low growth yields. Four independent mutants were isolated which had Kan^R tightly linked to the Suc^- phenotype but not to *pckA* as determined by P1 transduction. These mutants fermented maltose and arabinose, which indicated that the mutations were not in the *cya* or *crp* genes. PCR with IS*10* and REP (Repetitive Extragenic Palindrome) primers was done to amplify presumptive mini Tn*10* insertions. However, no homology to the transposon could be found and it was hypothesised that the mutants contained spontaneous Kan^R mutations, which resulted in Pck^- ($Succinate^-$) phenotype.

Since it was known that mutations in the *atp* genes, encoding ATP synthase had Suc^- Kan^R phenotypes, P1 transduction was used to test for the linkage to genes close to the *atp* operon. Three of the mutations were tightly linked to *ilvD* and to *rbs*, while the other appeared to have multiple Kan^R and could not be mapped further. The mutants were found to have low levels of ATP synthase as well as Pck and transductants regained both enzyme activities and kanamycin sensitivity. Plasmids

containing all or just the F_1 portion of the *atp* operon complemented the phenotypes (Suc^-, Pck^-, Kan^R and ATP $synthase^-$) of all the mutants.

DNA fragments encoding the F_1 region of ATP synthase of the mutants were sequenced after PCR amplification. In two mutants there was a two base pair "GC" deletion in *atpG* resulting in a truncation of 28 amino acids at the carboxyl terminus end of the γ subunit of ATP synthase. The other mutant had a "T" deletion in *atpG*, which led to a 40 amino acid truncation at the carboxyl terminus of the γ subunit of ATP synthase. Complementation of the Suc^- phenotype with plasmid pBWG15 expressing the γ subunit confirmed that the mutations were in *atpG* in the mutant HG205.

This study led us to identify a role of the *atpG* gene in *pckA* expression in stationary phase. The *atpG* mutations could affect expression of *pckA* in different ways. First, gluconeogenesis is an energy consuming process. Low ATP levels (low energy state) in the mutants could lead to low Pck levels. Second, *atpG* might affect *pckA* at a genetic level. Third, the γ subunit is a gate for proton flow and links ATP synthesis to proton translocation. The presence of the faulty γ subunit could make ATP synthase a proton pore, which results in collapse of the pH gradient. This could have some effect on the expression of *pckA*. Fourth, intracellular pH might also affect the synthesis or activity of Pck. There are a number of genes, *e.g. ompF*, *lamB*, *mar* operon, whose expression changes as a function of pH. Lastly, ATP synthase might interact with a protein kinase or is a protein kinase itself and affects the phosphorylation of a protein that activates the expression of *pckA*.

From the results of this work, *atp* genes seem to play an important role in the expression of Pck activity. The *atp* genes could regulate Pck activity at the transcriptional, translational or protein levels. This opens up a new area of investigation of the stationary phase regulation of *pckA*.

Acknowledgments

First of all, I would like to thank my supervisor, Dr. Hughes Goldie for giving me an opportunity to work in his lab for my Ph.D and allowing me to work independently. Thank you for being such a nice person and showing compassion when I needed it the most. I would like to thank my advisory committee, Drs. Wei Xiao, Bill Roesler, Harry Deneer, Sidney Hayes and Harold Bull. Thank you for all the positive criticism, excellent advice and encouragement during the duration of my program. I can say with confidence that I had the best committee that a student could hope for.

I will always cherish the time that I spent with fellow graduate students with special mention to Brian Novakovski, Rob Walters, Landon Pastushok, Monique Horbay, Yu Zhu, with whom I have had numerous discussions, science and other wise. Thank you Todd Hryciw for being such a good friend and always reminding me that I was a strange Indian. I would like to thank Dr. Juthika Menon, a friend who helped me a great deal when I first came to Saskatoon. A special thank you to my friend Dr. Mahua Ghosh for putting up with all my atrocities and for a friendship I will always cherish. A heart felt thank you to Sujatha akka and family for all the love, care and being there for me always. I would like to thank all the staff and the faculty of the Department of Microbiology and Immunology for making the department such a pleasant place to work.

I greatly appreciate the financial support provided by College of Graduate Studies and Research, the College of Medicine Graduate Scholarships and the Arthur Smyth Graduate Scholarship.

Lastly, I would like to express my gratitude to my parents whose faith and confidence in me made me the person I am today. Thank you Achan for always reminding me, "You can be what you want to be. Have a vision and work towards it and success will be yours". To my dearest Amma for all her support and my lovely Kuttu who in all her light heartedness made things seem easier than they were.

To my parents

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List of Abbreviations

Amp	ampicillin
ANSA	1-Amino-2-naphthol 4-suphonic acid
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
C/EBP	CAAT/enhancer binding protein
Cam	chloramphenicol
cAMP	cyclic adenosine 3'-5'-monophosphate
Cra	catabolite repressor/activator
Crp	cAMP receptor protein or catabolite gene activator protein
Csr	carbon storage regulator
CTAB	hexadecyltrimethylammoniumbromide
cya	adenylate cyclase gene
Δ	deletion mutant (null mutation)
DCCD	1,3,-dicyclohexylcarbodiimide
DNA	deoxyribonucleic acid
dNTP	2' –deoxyribonucleotide 5' triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediminotetraacetic acid
EMP	Embden Meyrhoff-Paranas
Fbp	fructose-bis-phosphatase
g	gravitational force
GTP	guanosine 5'-triphosphate
Hfr	High frequency recombination
Kan	kanamycin
kb	Kilobase
Kda	Kilo dalton
LB	Luria Bertani medium
mini Tn*10*-ATS	mini transposon-altered target specificity
min	minute
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
nt	nucleotide
OAA	oxaloacetic acid
PBI	Plant Biotechnology Institute
Pck	phosphoenolpyruvate carboxykinase
PEPCK-C	cytoplasmic phosphoenolpyruvate carboxykinase
PCR	polymerase chain reaction

PEP	phosphoenolpyruvate
PFU	plaque forming units
pH_i	pH inside
pH_o	pH outside
Pi	inorganic phosphate
PMF	proton motive force
Ppc	phosphoenolpyruvate carboxylase
ppGpp	guanosine tetraphosphate
Pps	PEP synthase
PTS	phosphotransferase system
Pyr	pyruvate
REP	repetitive extragenic palindrome
RNA	ribonucleic acid
RNP	RNA polymerase
RpoS	RNA polymerase Stationary phase sigma factor
Rsd	regulator of Sigma D
σ^D	*rpoD* gene product
σ^S	*rpoS* gene product
Suc	succinate
TBE	Tris borate EDTA
TCA	tricholoro acetic acid
TE	Tris–ethylene diamino tetraacetic acid
Tet	tetracycline
Tris	Tris (hydroxymethyl) amino methane
u.v.	Ultraviolet light

CHAPTER ONE

1.0 Introduction

Overview

A century ago it came to light that microbes adapt to their environment by expressing new enzymes. The earliest findings were in yeast where it was found that the enzymes for galactose metabolism are expressed when cells are exposed to galactose or lactose (Dienert, 1900). In 1938, when Karstrom coined the term "enzyme adaptation", the focus on regulatory mechanism took a new turn. In 1942, Jacques Monod initiated his studies on "adaptation" to lactose by *E. coli.* The 1950s saw the beginning of genetic approaches to study gene regulation. End product repression was discovered where a small molecule added to the medium shut off the expression of the enzyme responsible for its biosynthesis. The 1960s witnessed the elaboration of metabolic pathways and detailed analysis of regulatory pathways involved in metabolism and bacteriophage development and lysogeny.

By 1970, numerous novel regulatory mechanisms starting from regulation of arabinose catabolism (Irr and Englesberg, 1970) to repression of translational initiation at the RNA level had been discovered (Zubay *et al.*, 1970). The advent of technology shifted the focus of research to regulatory protein-DNA interactions. Regulatory problems like heat shock control, SOS response to DNA damage,

differential gene expression between aerobic and anaerobic conditions, stationary phase gene expression, *etc* was all examined in detail. Many regulatory proteins have been purified, and protein-DNA interactions have been studied in detail. The latter studies have revealed innovative findings in gene regulation and DNA structure indicating DNA bending and looping (Matthews, 1992). The field of gene regulation that began with the study of the regulation of small molecules in gene expression has now ended up largely as the study of protein-DNA interactions. Much remains to be learned about the ways that proteins interact with DNA, the ability of proteins to alter the DNA structure and the interaction between regulatory proteins and RNA polymerase when bound to DNA. Similar issues exist at the RNA level in terms of the effect on translation, mRNA degradation, protein assembly and degradation.

How sugars are transported into a bacterial cell under different environmental conditions still remains an important field of investigation. Carbohydrates are taken up by bacteria through active transport systems (Krulwich, 1990). The energy source for transportation may be transmembrane electrochemical gradients of protons (carbohydrate-H^+ symporters) (Kaback, 1990) or of sodium ions (carbohydrate-Na^+ symporters) (Dimroth, 1990), hydrolysis of ATP (Ames, 1990), or vectorial transport (Meadow *et al.*, 1990 and Saier and Chin, 1990). Microorganisms excrete metabolic products into the medium if the substrates (precursors) are in excess or if toxic metabolites are accumulated. This active transport may involve anion exchange (Maloney *et al.*, 1990), precursor/product antiport (Poolman, 1990) or ATP hydrolysis. *E. coli* has at least seven types of transport systems for the uptake of the

frequently available sugars. There is at least one type of transport system for the uptake of a carbohydrate that resembles any natural carbohydrate (Lengeler, 1993). This shows the versatility of the transport systems.

Central metabolic pathways generate both stored biochemical energy and metabolic precursors that serve as starting points for the synthesis of the building blocks for the formation of essential cellular constituents of all living cells (Romano *et al.*, 1996). These pathways occur in three domains, the bacteria, the archea and the eucarya (Woese *et al.*, 1990). The central metabolic pathways consist of the Embden-Meyerhof-Parnas (EMP) pathway of glycolysis, the pentose phosphate shunt and the citric acid cycle. In primordial earth, since the atmosphere was probably anaerobic, the first organisms were probably fermentative heterotrophs. Their energy requirement was met by substrate level phosphorylation. The ubiquitous nature of the EMP in bacteria and eukaryotes suggests that EMP was present in the earlier organisms (Hovarth, 1974). Amino acid sequence and crystal studies show that the enzymes in EMP are highly conserved.

Metabolic pathways can be catabolic (substrate degradation for energy for metabolic functions) or anabolic (synthesis of building blocks). Also, there are pathways called amphibolic pathways, which can perform both the catabolic and anabolic functions. Amphibolic pathways are subjected to stringent regulatory mechanisms (Sanwal, 1970). From the number of genes that have been discovered in *E. coli* there must be more than a thousand different metabolic pathways (Bachmann, 1983).

In metabolic pathways, the enzymes that catalyse irreversibe reactions are often under tight regulation (Sanwal, 1970) and are usually located at the beginning of the pathways. The regulation can be achieved by covalent modification of the enzyme structure, by genetic regulation, by enzyme degradation or by allosteric regulation. Control of enzyme levels by degradation is particularly important in stationary phase cells, where cell division does not dilute the enzymes, because the cells grow very slowly (Kolter *et al.*, 1993).

Glucose is the primary sugar that is used by most living organisms as a carbon source. In the absence of glucose the cell resorts to other sources to generate glucose (Brosnan, 1999). The glucose can be generated from fats and proteins and these substrates are called gluconeogenic substrates.

1.1 Gluconeogenesis

1.1.1 Overview

Gluconeogenesis is a term used for *de novo* synthesis of glucose from non-hexose sugars. Gluconeogenesis is universal in all organisms, ranging from bacteria to fungi to plants and animals. Many microorganisms grow on substrates like acetate or propionate, which is converted to glucose via gluconeogenesis. The bacterial cell converts the available nutrients to glucose because glucose is a general precursor of polysaccharides, nucleic acids and other macromolecules. Glucose is also the primary carbohydrate for energy metabolism probably because it is a six-carbon

sugar, which has the lowest proportion of straight chain (non ring) structure of all the aldohexoses (Brosnan, 1999).

Glycolysis is a pathway for the conversion of glucose to pyruvate and is the central pathway in catabolism. Conversion of pyruvate to glucose is the central pathway for gluconeogenesis. Many steps in the pathway are shared between glycolysis and gluconeogenesis. Most of the gluconeogenic reactions are a reversal of glycolysis, but there are three steps that are irreversible in glycolysis, which are the conversion of glucose to glucose-6-phosphate by hexokinase or by the PTS system, conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, and conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (Lehninger *et al.*, 1993). Both glycolysis and gluconeogenesis are independently regulated via reactions that are characteristic for both pathways. The first gluconeogenic bypass is the conversion of pyruvate to phosphoenolpyruvate by phosphoenolpyruvate synthase (Pps). Alternatively phosphoenolpyruvate can also be converted to oxaloacetate via PEP carboxylase (Ppc) and the oxaloacetate to phosphoenolpyruvate through phosphoenolpyruvate carboxykinase (Pck). Conversion of fructose-1,6-bisphosphate to fructose-6-phosphate is carried out by fructose-bis-phosphatase (Fbp).

1.1.2 Gluconeogenesis in *Escherichia coli*

In *E. coli* the gluconeogenic enzymes are phosphoenolpyruvate carboxykinase (Pck), phosphoenol pyruvate synthase (Pps) and fructose-bisphosphatase (Fbp). During glycogenesis (formation of glycogen), phosphofructokinase catalyses the

formation of fructose-6-phosphate and the PEP that is required for the entry into glycolysis is provided by PEP synthase. At the onset of stationary phase, bacteria rapidly convert carbohydrate to glycogen. The *csrA* (carbon storage regulator) gene was identified as a regulatory gene for glycogen synthesis (Romeo *et al.*, 1993). The *csrA* gene was mapped at 58 min on the *E. coli* chromosome. It negatively controls the two structural genes *glgC* and *glgB* for glycogen synthesis as well as the genes expressing gluconeogenic enzymes like phosphoenolpyruvate carboxykinase (Sabnis *et al.*, 1995) (**Figure 1).** However, the effects on Pck and on other gluconeogenic enzymes are not large enough to completely explain stationary phase induction.

A study by Sabnis *et al.* (1995) shows that under conditions of optimum glycogen synthesis in early stationary phase, gluconeogenesis occurs at a fairly restricted time, coincident with glycogen biosynthesis. Perhaps the primary role of gluconeogenesis in glucose grown cells is to enhance glycogen synthesis. The authors have documented growth phase responses for gluconeogenic enzymes, which help the cell to conserve energy. A futile cycle of gluconeogenesis and glycolysis would be prevented or at least minimised in the exponential phase. The conversion of carbohydrate into endogenous glycogen should be favoured as the cells enter stationary phase, and later in the stationary phase, during glyconeogenesis, a futile cycle of glycolysis and gluconeogenesis would be avoided. The decrease in the activities of the gluconeogenic enzymes, which occurs later in stationary phase indicate that enzyme inactivation is also an important determinant of the gluconeogenic capacity of the cell (Sabnis *et al.*, 1995).

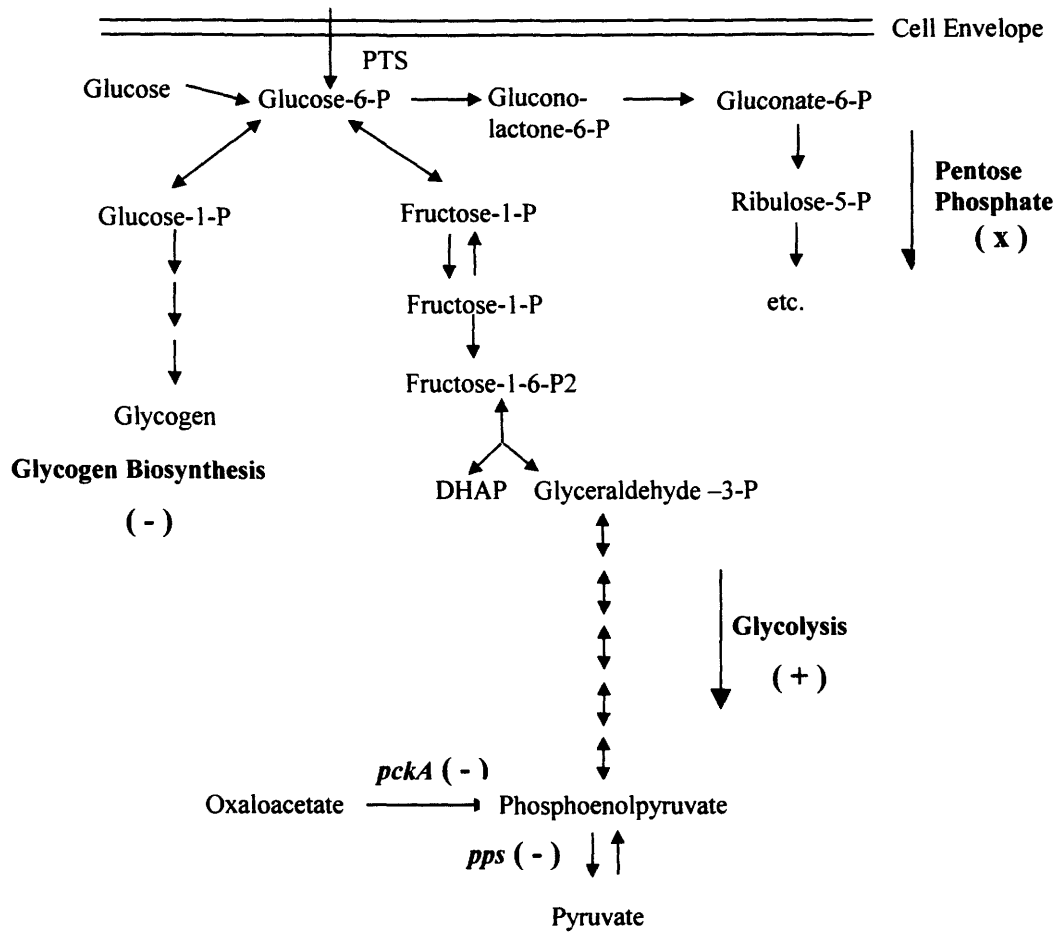


Figure1. Effects of *crsA* on carbohydrate metabolism (adapted from Sabnis *et al.*, 1995).

The circles in the figure indicate the genetic fusions that have been examined. Negative regulation is indicated by (-), positive regulation by (+) and not affected by (x). The pathways that have been investigated are shown in bold.

CsrA plays a negative role in glycogen metabolism and gluconeogenesis but a positive role in glycolysis (Romeo *et al.*, 1993). In the absence of exogenous hexose or in the absence of glucose, gluconeogenic enzymes are present and may be used to form glucose-1-phosphate, which can be converted to a variety of compounds, including ADP-glucose for glycogen biosynthesis. Gluconeogenesis is functionally related to glycogen synthesis because it can provide glucose-1-phosphate for glycogen biosynthesis or for other biosynthetic processes, depending on the environment.

A *csrA*::*Kan*^{R} mutant had low levels of ATP but high levels of ADP and AMP throughout the growth, which led to low energy charge. Also the concentration of fructose-bisphosphate and phosphoenolpyruvate were elevated (Sabnis *et al.*, 1995). This is consistent with the decrease in glycolysis and increase in gluconeogenesis. The elevated levels of PEP in the *csrA* null mutant indicates enhanced synthesis of PEP via Pps and Pck, and also the decreased utilisation by pyruvate kinase, which is a glycolytic enzyme.

Identification of the "reverse EMP" (Romano and Conway, 1996) revealed the ubiquitous nature of the pathway and that its occurance as a gluconeogenic pathway is broader than as a glycolytic pathway (Romano and Conway, 1996). The original function of the EMP was probably gluconeogenesis and not glycolysis. The glycolytic function may have evolved later as the most energy efficient pathway. Fructose-1,6-bisphosphatase is required for growth when succinate, acetate, or glycerol, are present as carbon sources. Fbp catalyses the hydrolysis of fructose-

bisphosphate to form hexose monophosphate required for the synthesis of pentose, aromatic amino acids and polysaccharides. Biosynthesis of sugars from acetate or pyruvate occurs via reactions of the EMP operating in the direction of gluconeogenesis. The pentose phosphate pathway supplies pentoses for the synthesis of nucleic acids.

Carbohydrate metabolism also plays a critical role in anaplerosis *i.e.* replenishing of Krebs cycle intermediates. It is not the carbohydrate metabolism *per se* that is anapleurotic, but the substrates such as pyruvate (through PEP carboxylase and malic enzyme) which are derived from carbohydrates. The existence of anapleurotic pathways requires the existence of catapleurotic pathways, which would tend to deplete the cycle of intermediates. Gluconeogenesis itself may be viewed as a catapleurotic reaction (Brosnan, 1999) whereby the Krebs cycle intermediates are depleted by withdrawing oxaloacetic acid from the Krebs cycle to produce PEP. Glucoenogenesis may occur only when balanced by anapleurotic reactions like synthesis of oxaloacetic acid by PEP carboxylase or by the production of intermediates from gluconeogenic amino acids. The gluconeogenic pathways in prokaryotes are subject to catabolite repression. High levels of cAMP (indicating low amounts of glucose) will activate the transcription of genes encoding gluconeogenic enzymes. Gluconeogenesis is also important in stationary phase when the bacteria have depleted most of the carbohydrate in the medium and they have to rely on gluconeogenesis for the synthesis of glucose from amino acids and fats for survival.

In *E. coli* regulation of *pps* is linked to PEP and depends on the phosphotransferase system (Geerse *et al.*, 1989). Lowry *et al.* (1971) have determined that PEP concentrations are higher in cells growing on gluconeogenic substrates as compared to those growing on glucose. Gluconeogenic growth in *E. coli* is controlled by *pck* and *pps* as determined by Goldie and Sanwal, (1980a) and Chao *et al.* (1993).

Pps competes for pyruvate with pyruvate dehydrogenase (Pdh), an enzyme required for the formation of acetyl coenzyme A, the starting metabolite of the tricarboxylic acid cycle. When the cells grow on succinate, Pck converts oxaloacetate to PEP, and then pyruvate kinase converts PEP to pyruvate (Chao *et al.*, 1993). Pck competes with citrate synthase for the common substrate, oxaloacetate. Mutants with null alleles of *pps* cannot grow on pyruvate (Cooper *et al.*, 1967). Null alleles in both *pps* and *pck* are needed to generate a Suc$^-$ phenotype (*i.e.* inability to grow on succinate), because malic enzyme and Pps can supply PEP in *pck*$^-$ cells (Goldie and Sanwal, 1980a). To control the Pck or Pps expression level, Goldie and Sanwal used a *pps*$^-$ *pck*$^-$ strain and the *pck*$^+$ or *pps*$^+$ gene was expressed from a multicopy plasmid under the control of an inducible promoter. Strains expressing inactive but stable Pps or Pck were used as controls (Liao *et al.*, 1994). The *pck*$^-$ *pps*$^-$ strains were defective for growth on succinate, pyruvate, malate and fumarate but grew well on glycerol or glucose as a sole carbon source (Goldie and Sanwal, 1980a).

Goldie and Sanwal obtained two kinds of revertants from the *pck*$^-$ *pps*$^-$ (Suc$^-$ Pyr$^-$) mutants. One kind, which was Suc$^+$ Pyr$^-$, regained the activity of Pck. The

other kind was Suc^+ Pyr^+, regained the activity of PEP synthase. These results show that the Suc^- phenotype was dependent on the presence of both the *pck*$^-$ and *pps*$^-$ mutations.

1.1.3 Gluconeogenesis in eukaryotes

In eukaryotes, gluconeogenesis supplies glucose as a metabolic fuel. The advantage of glucose as a fuel over fats is that glucose is the only fuel that can synthesise ATP without a requirement for oxygen and that rapid turnover is possible. ATP production is less (2 ATP per molecule of glucose) but this is the only form of energy in RBC (Red Blood Corpuscle), which do not have mitochondria (Brosnan, 1999). Gluconeogenesis is often required in the liver to replenish the blood stream glucose and in renal medulla where the glucose level is low. Another reason for using glucose as a primary carbon source is that the enzymes that bring about the degradation of glucose do not absorb light in the visible region. Oxidative metabolism requires heme compounds, like hemoglobin and cytochromes, which absorb light. Anaerobic glycolysis from sugar can provide ATP for eye lens (Brosnan, 1999) without the absorption of light. One of the main goals of the response to starvation is to provide glucose to the brain. The brain is extremely sensitive to anoxia and glucose is an ideal substrate since it can be metabolized to generate 2 ATP in the absence of oxygen.

Glucose that is formed has other roles too, *e.g.* glucose gets converted to glucose-6-phosphate, which enters the pentose phosphate pathway. NADP reduced to

NADPH provides reducing power to a variety of cytoplasmic events. Apart from pentose phosphate pathway, malic enzyme can provide a route for NADPH formation. In conditions where EMP is the only form of producing the reducing power NADPH, or in cases where EMP is non-functional *e.g.* in glucose-6-phosphate deficiency, there is hemolysis when cells in an organism are exposed to oxidative stress (Beutler, 1983). The pentose phosphate pathway provides pentoses for nucleic acid and nucleotide synthesis. Glucose is also important in glucouronidation, which is involved in elimination of xenobiotics and also in the synthesis of glycoproteins, glycolipids, glycosaminoglycans and in glycolysis.

1.2 Phosphoenolpyruvate carboxykinase [E.C.4.1.1.49]

1.2.1 Phosphoenolpyruvate carboxykinase in *Escherichia coli*

Phosphoenolpyruvate carboxykinase (Pck) catalyses a key reaction of gluconeogenesis in *Escherichia coli*. It catalyses the decarboxylation and ATP-dependent phosphorylation of oxaloacetate (OAA) to form phosphoenolpyruvate (PEP). This is the first committed step of gluconeogenesis in *E. coli* and in most other organisms. In bacteria, such as *E. coli*, Pck is utilized during gluconeogenic growth when sugar levels are low (Goldie and Sanwal, 1980a).

There are two pathways that exist for the generation of PEP from four carbon intermediates in *E. coli*. One pathway involves generation of pyruvate from malate

through NAD-dependent malic enzyme and conversion of pyruvate to PEP by phosphoenolpyruvate synthase (Pps). The other involves conversion of oxaloacetate to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Pck). Double mutants that are deficient in Pps and Pck or in Pck and malic enzyme are unable to grow on the four carbon sugars, whereas single mutants can grow. A *pck⁻ pps⁻* strain was defective for growth on succinate, fumarate, malate, acetate and pyruvate, and grew well on glucose or glycerol as a sole carbon source (Goldie and Sanwal, 1980a).

Cyclic AMP receptor protein (Crp) and cyclic AMP (cAMP) are global regulators in carbon utilisation and play an important role in catabolite repression. Maximum Pck enzyme levels were observed in stationary phase and were repressed by glucose, which makes catabolite repression seem like a very good candidate for regulation (Goldie, 1984). Catabolite repression probably serves the purpose of inhibiting gluconeogenesis when glucose and other carbohydrate carbon sources are available. Stationary phase induction of PEP carboxykinase may be required for the synthesis of carbohydrate storage reserves in the stationary phase. The stationary phase induction of the enzyme requires a regulatory signal in addition to cAMP, since induction does not occur in log phase cells grown on LB medium in the presence of 5mM cAMP (Goldie, 1984). The mechanism of stationary phase induction has not been determined, but fluctuations in intracellular cAMP levels have been ruled out. When cells were grown on minimal media containing 0.4% glucose or on LB containing 0.4% glucose, the Pck levels were lower. On adding 5mM cAMP the activity seen was similar to those grown on LB with no additions (Goldie, 1984). In a

cya⁻ (adenylate cyclase) strain, the levels of Pck were low but were restored when exogenous cAMP was provided. In a Δ*cya* Δ*crp* strain, the Pck levels were not restored, even on addition of 5mM cAMP. This shows that both cAMP and Crp regulate *pckA*. The effect of stringent response on *pckA* induction in a *relA* mutant was studied and it was found that the *pckA* induction was *relA* independent (Goldie and Sanwal, 1980a).

Hfr mapping of the *pckA* locus showed that *pck* was located between 70-76 min on the *E. coli* chromosome. P1 transduction experiments were done with markers in the region of 70-76 min on the chromosome and revealed that *pck* was linked with *asd* (20-30%), *glpD* (45%) and *aroB* (23-26%) at 76 minutes on the map (Goldie and Sanwal, 1980a). The gene order was determined from a three point cross as *aroB-pck-glpD-asd*, and from comparisons of restriction maps, and sequence of *pckA* to those of the *E. coli* genome (Goldie and Medina, 1990; Medina *et al.*, 1990)

1.2.1.1 Studies in *pck-lacZ* transcriptional fusions

To study the regulation of the transcription of *pckA* in *E. coli*, *pck-lacZ* operon fusions were made (Goldie, 1984). One step operon fusions were generated using a Mud-1 phage and the induction of β-galactosidase was determined on different media. Pck is subjected to catabolite repression, and maximum levels of this enzyme are also induced at the onset of stationary phase of growth on rich media (Goldie, 1984). Induction of β-galactosidase in *pck-lacZ* strains grown on different media showed that changes in the levels of β-galactosidase resembled the known variation of PEP

carboxykinase enzyme levels, implying that PEP carboxykinase is regulated at the transcriptional level. The magnitude of the stationary phase induction was shown to be up to 100-fold.

Induction data were obtained for glycerol and succinate carbon sources, and intracellular cAMP levels were correlated with stationary phase induction for LB medium. The *pck-lacZ* fusions were induced during the log phase when cells were grown on minimal medium containing glycerol. It is likely that high cAMP levels in glycerol grown cells contribute to this induction (Pastan and Perlman, 1970). Levels of β-galactosidase were lower when *pck-lacZ* fusion strains were grown on minimal medium plus glucose, in which cAMP levels are known to be lower (Pastan and Perlman, 1970; Peterkofsky and Gazdar, 1974). When the *pck-lacZ* fusion strain was grown in a *pps*$^+$ background, on medium A containing succinate, β-galactosidase levels were 60-70% higher than on medium A containing glycerol and were constantly elevated throughout growth. This could be due to higher cAMP levels in cells grown on succinate or could be due to induction of *pck* by succinate or by other Krebs cycle intermediates. Induction by Krebs cycle intermediates would have a function in the cells since succinate is a precursor of OAA, which is a substrate for Pck.

A deletion of the *cya* gene lowered the expression of a *pck-lacZ* fusion more drastically than addition of glucose to the medium (Goldie, 1984). The effects of both *cya* deletion and addition of glucose were reversed by cAMP. The low residual levels of β-galactosidase in the Δ *cya pck-lacZ* strain may be due to cAMP in the LB

medium, which contains yeast extract. On LB medium, β-galactosidase synthesis was not induced in *pck-lacZ* fusions until the onset of the stationary phase, although cAMP levels were high throughout growth. Also, *pck-lacZ* fusions were not induced during log phase growth on LB medium containing 5 mM cAMP. It is likely that another regulatory signal in addition to cAMP is involved in the induction of Pck synthesis in the stationary phase. Many enzymes *e.g.* those involved in glycogen biosynthesis, proteolysis and Krebs cycle, are induced during the stationary phase. This might be for providing energy storage reserves in stationary phase cells.

This work showed that *pck-lacZ* is regulated by glucose and cAMP (by catabolite repression). Expression of *pckA* was elevated in the presence of gluconeogenic substrates (glycerol/succinate). The expression is also increased in stationary phase, which will be beneficial in glycogen synthesis, since glucose-1-phosphate, which is a precursor for glycogen, can be synthesised from PEP.

In strains overexpressing Ppc and Pck, overexpression of Pck in the presence of glucose causes severe growth retardation for unknown reasons (Liao *et al.*, 1994). It decreases respiration rates even after adjusting for growth rate differences. The same phenomenon was observed in a *ppc*$^-$ mutant background, indicating that those effects are not due to the futile cycles of Pck and Ppc. Wild type cells normally grow slower on pyruvate and succinate than on glycerol. Strains, which optimally express Pps and Pck, can grow at the same rate on pyruvate and on succinate as the wild type *E. coli* growing on glycerol. This suggests that growth of wild type *E. coli* on

gluconeogenic carbon sources is at least partially limited by the supply of PEP (Liao *et al.*, 1994).

1.2.1.2 Cloning of the *pckA* gene

Goldie and Medina (1990) cloned the *pckA* structural gene. A *pckA* mRNA transcript was observed in stationary phase cells but not in log phase cells. The mRNA start site was mapped using the S1 nuclease method and a σ^{70} type promoter was identified at the -10 and –35 positions. N-terminal sequencing identified a translational start of 44 residues. There is a long leader region between the transcription and translation start sites of unknown function. Gel shift experiments have indicated three possible Crp binding sites in the promoter region and two Crp footprints have been obtained by the Cu^{2+}/phenanthraline method (indicated by asterisks). (**Figure 2**, Goldie, unpublished)

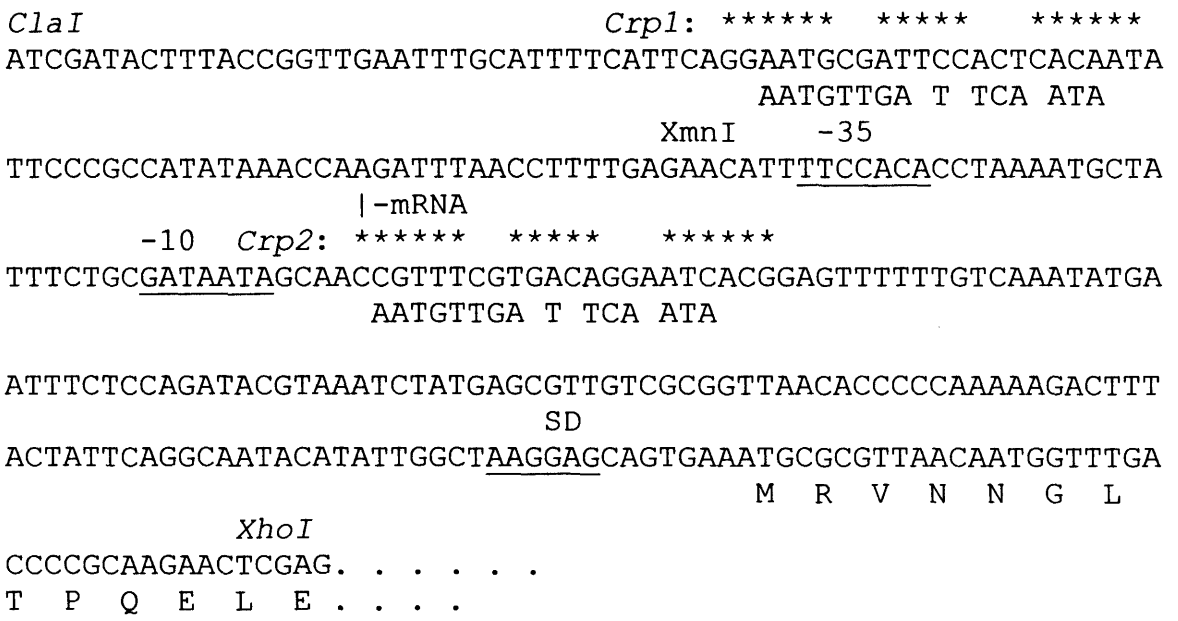


Figure 2. Promoter of *pckA* gene of *Escherichia coli*.

The asterisks indicate Crp footprints and the sequences below the Crp footprints indicate the Crp consensus sequence. The –35 and –10 region of the *pckA* promoter (underlined), the Shine Dalgrano (SD) sequence for ribosome binding and mRNA start have been indicated. The bottom sequence represents the translated the amino acid residues.

1.2.1.3 Activation of Pck enzyme by metal ions

Pck has an absolute requirement for divalent metal cations for activity. Kinetic studies showed the synergistic effect of Mg^{2+} and Mn^{2+} on the activity (Goldie and Sanwal, 1980b), indicating the dual cation function. Furthermore, the x-ray crystallographic structure (Tari *et al.*, 1997) has both metals at the active site. Optimal activity was obtained in the presence of millimolar concentrations of Mg^{2+} and micromolar concentrations of Mn^{2+}. These concentrations are similar to *in vivo* concentrations. *E. coli* Pck is a monomeric, globular protein that belongs to the α/β class of proteins. It has two domains, a 275 residue amino terminal domain and a compact 265 amino acid carboxyl terminal domain. The active site is at the base of a deep cleft between the two domains. Structure and topology of the nucleotide-binding fold are unique. When MgATP binds, Pck undergoes a domain closure via a 20° rotation of the amino and the carboxyl terminal domains towards each other. This traps substrates, excludes solvent from the active site and repositions the important active site groups and metal ions in the active site. ATP is in a *syn* conformation in Pck, the first metabolic enzyme for which this has been discovered.

It was earlier thought that the *E. coli* Pck is allosterically regulated. Goldie and Sanwal (1980b) found that Ca^{2+} or Mn^{2+} could activate Pck in the presence of saturating concentrations of MgATP. To test if Pck was allosterically activated, trypsin digestion of the enzyme was done to remove or destroy any peripheral allosteric site. Goldie and Sanwal (1980b) discovered that the trypsinised Pck cannot be activated by Ca^{2+} but can be activated by Mn^{2+}, which led to the hypothesis that

Ca^{2+} activation might be allosteric. They proposed that Ca^{2+} bound at a surface allosteric site changed the conformation of the enzyme. Using Tb^{3+}, an analogue of Ca^{2+}, and Klotz plots of Tb^{3+} solution fluorescence data, they showed that there were two Tb^{3+} binding sites in Pck and inferred that there were probably two Ca^{2+} binding sites. Hence they concluded that one Ca^{2+} bound at the catalytic site and another one at the surface allosteric site in the absence of MgATP. This allosteric site is removed or destroyed by trypsin digestion. Matte *et al.* (1996) identified two Tb^{3+} binding sites, one coordinated by D269, H232 and K213, which are in the catalytic site, and another one involving E508 and E511 on the periphery, which is removed by trypsin digestion. Sudom *et al.* (unpublished results) found only one Ca^{2+} ion coordinated with the D269, H232 and K213 and the γ-phosphate of ATP. An E508Q, E511Q double mutant was created changing both glutamates in the peripheral sites to glutamines and Pck activities were determined (Pastushok *et al.*, unpublished results). If Ca^{2+} binding at these sites is responsible for allostery of Pck, the mutation should show lowered Pck activity. There was no change in Pck activity seen in the mutant relative to the wild type indicating that Ca^{2+} activation may occur due to binding of a Ca^{2+} ion at the active site in addition to the MgATP complex.

The extensive homology of Pck from *E. coli*, *T. brucei* and *S. cerevisiae* may reflect the fact that all three enzymes are ATP dependent enzymes and may have similar mechanisms. The region surrounding the ATP binding site of *E. coli* Pck is completely conserved suggesting that this sequence is important for function. None of these enzymes exhibits homology to GTP-dependent Pck from rats, chickens or

Drosophila melanogaster in the National Institute of Health database although key active site residues are conserved (Matte *et al.*, 1996).

1.2.2 Phosphoenolpyruvate carboxykinase in eukaryotes

In organisms such as *Ascaris suum* (Christie *et al.*, 1987), *Trypanosoma cruzi* (Cymeryng *et al.*, 1995), Pck is an important enzyme in the glycolytic pathway where it forms OAA from PEP, which in turn enters the citric acid cycle.

Pck in mammals converts OAA to PEP in the presence of GTP. Pyruvate carboxylase converts pyruvate to OAA in the mitochondria, which can be reduced to malate, by malate dehydrogenase (Mdh). Malate is transported to the cytosol and reoxidised by NAD malate dehydrogenase to OAA. The cytosolic Pck converts OAA to PEP. There are two isoforms of Pck, cytosolic and mitochondrial. The cytosolic Pck gene is also expressed in mammalian tissues other than liver and kidney (Hanson and Reshef, 1997). Pck activity is detected in white and brown adipose tissue and the mammary glands (lactating) and small intestine. Yeast Pck is multimeric and absolutely requires adenine nucleotides (Rognstad, 1979). *E. coli* Pck is monomeric and is 59 Kda in size and requires ATP instead of GTP. Rat, human and mouse Pck have more than 90% sequence homology, and the transcriptional regulatory elements within the respective genes are identical and share the same relative positions in the promoter.

In humans and other mammals, Pck is a central enzyme in carbohydrate metabolism, helping to regulate the blood glucose level. Gluconeogenesis in tissues

such as kidney and liver converts lactate and other non-carbohydrate molecules to glucose, which is in turn released into the blood. The importance of Pck to carbohydrate metabolism in humans is such that it has been suggested as a potential drug target in the treatment of non-insulin dependent diabetes mellitus (Valera *et al.*, 1994).

1.3 Stationary phase regulation of genes

1.3.1 Overview

A typical bacterial growth cycle consists of a lag phase, log phase, stationary phase and in some instances a death phase. When nutrients are depleted, the growth rate is zero and at this point the cells enter stationary phase. There is no net increase in cell number at this phase of growth. The physiology of cells in stationary phase is heterogenous and depends on the factors that cause cessation of growth. During the log phase of growth cells normally channel their energy to produce more nucleic acids, proteins and other constituents essential for cell growth and division (Kolter *et al.*, 1993). They have enough energy to combat environmental stress and have increased metabolism. Stationary phase metabolism and regulation have been reviewed by Loewen *et al.* (1998).

The RNA polymerase holoenzyme of *E. coli* consists of the core enzyme composed of $\alpha_2\beta\beta'$ and a σ subunit that directs the core enzyme to initiate transcription at specific promoter sites on DNA (Zillig *et al.*, 1976). The major σ

factor, σ^{70}, also known as σ^{D}, (the *rpoD* gene product) is responsible for transcription of most genes expressed during the exponential cell growth. The *rpoS* gene product, known as σ^{s} or σ^{38}, is the key factor during the transition from the exponential growth phase to stationary phase. Also, the switch of gene expression pattern upon sudden exposure to various stresses is thought to take place by replacement of the σ^{70} subunit on the RNA polymerase. During exponential growth at 37°C, σ^{S} is undetectable but it starts to appear when the cells stop growing. Levels of σ^{S} were found to increase at high osmolarity and low (20°C) temperature (Jighage *et al.*, 1996).

There are four promoters for *rpoS* (McCann *et al.*, 1993). At the onset of stationary phase, all the promoters were induced. In a minimal medium, when cells entered stationary phase, both transcriptional and translational increases in expression of *rpoS* was observed. Translation was increased eight fold and transcription by two fold, which indicated that post transcriptional control was the major determinant in the appearance of σ^{S} (McCann *et al.*, 1993). The half-life of σ^{S} in exponential phase is only 1.5-2.5 minutes while in stationary phase it is 25 minutes (Loewen *et al.*, 1994). Increased transcription, increased translation and increased stability may all play an important role in the increased levels of σ^{S} in the stationary phase as compared to the exponential phase.

Sequences of 33 σ^{S}-dependent promoters have been compared and a consensus sequence CTATACT in the –10 region has been identified, similar to the TATAAT of the σ^{D} sequence (Loewen *et al.*, 1998). In the presence of the consensus

sequence, σ^S can bind to the –10 region of the promoter. Some promoters are recognised by both σ^S and σ^D, while other promoters are recognised by either σ^S or σ^D (Tanaka *et al.*, 1995). σ^D binding requires both the –35 and –10 region, while σ^S requires only the –10 region. It is not surprising that there is considerable overlap in the binding of the two sigma factors given the similarities in the –10 sequence. In the case of the *proU* and the *osmY* promoters, changing the –35 region can affect the expression (Wise *et al.*, 1996). They found that changing the TT to CC favours σ^S directed transcription, but changing the CC to TT in the *osmY* promoter favours σ^D mediated transcription. It is not clear whether the changes caused differences in the affinity of binding by the sigma factor or whether they changed the curvature of the DNA.

Why is it that transcripion takes place from σ^S promoters only in starvation or stationary phase, even though σ^S is present even in exponential phase? The cell does not require σ^S at all times. This may be due to the affinity changes and to changes in the competition of promoters for $E\sigma^S$. There could be other effector molecules that increase the affinity of the $E\sigma^S$ to the promoter. Certain external metabolites, such as acetate and benzoate, are found to trigger σ^S expression (Mulvey *et al.*, 1990; Schellhorn *et al.*, 1992).

1.3.2 Regulators of σ^S

The synthesis and activity of σ^S must be under tight control. The levels of σ^S are modulated both at transcriptional and translational levels (Loewen *et al.*, 1998) **(Figure 3)**.

1.3.2.1 Guanosine pyrophosphate

Many starvation-induced genes were found to be positively regulated by ppGpp during carbon and nitrogen starvation but not during phosphate starvation, indicating a link between ppGpp control and the σ^S-mediated starvation phenomenon (Spector and Cubitt, 1992). This was supported by the fact that a *relA spoT* strain, lacking ppGpp, had phenotypes similar to *rpoS* strains (Gentry *et al.*, 1993). Increasing ppGpp levels by introducing plasmid containing *relA*$^+$ lead to increase in σ^S, which indicated that ppGpp may be a positive regulator of σ^S. During amino acid starvation, the ppGpp levels increase, which would lead to an increase in σ^S.

Many σ^{70}-dependent genes are also under the regulation of ppGpp and are expressed in stationary phase. Kvint *et al.* (2000) demonstrated that, even in the presence of high levels of σ^S, there is still a requirement for ppGpp in the formation of Eσ^S (σ^S associated with the RNA polymerase core enzyme). The phenomenon of how ppGpp inhibits some promoters while acting, as a positive regulator at others is not completely understood. The inhibition might be via the dissociation of the ppGpp programmed RNP from the stringent promoters, because ppGpp drastically reduces the stability of the open complex with RNP. Zhou and Jin (1998),

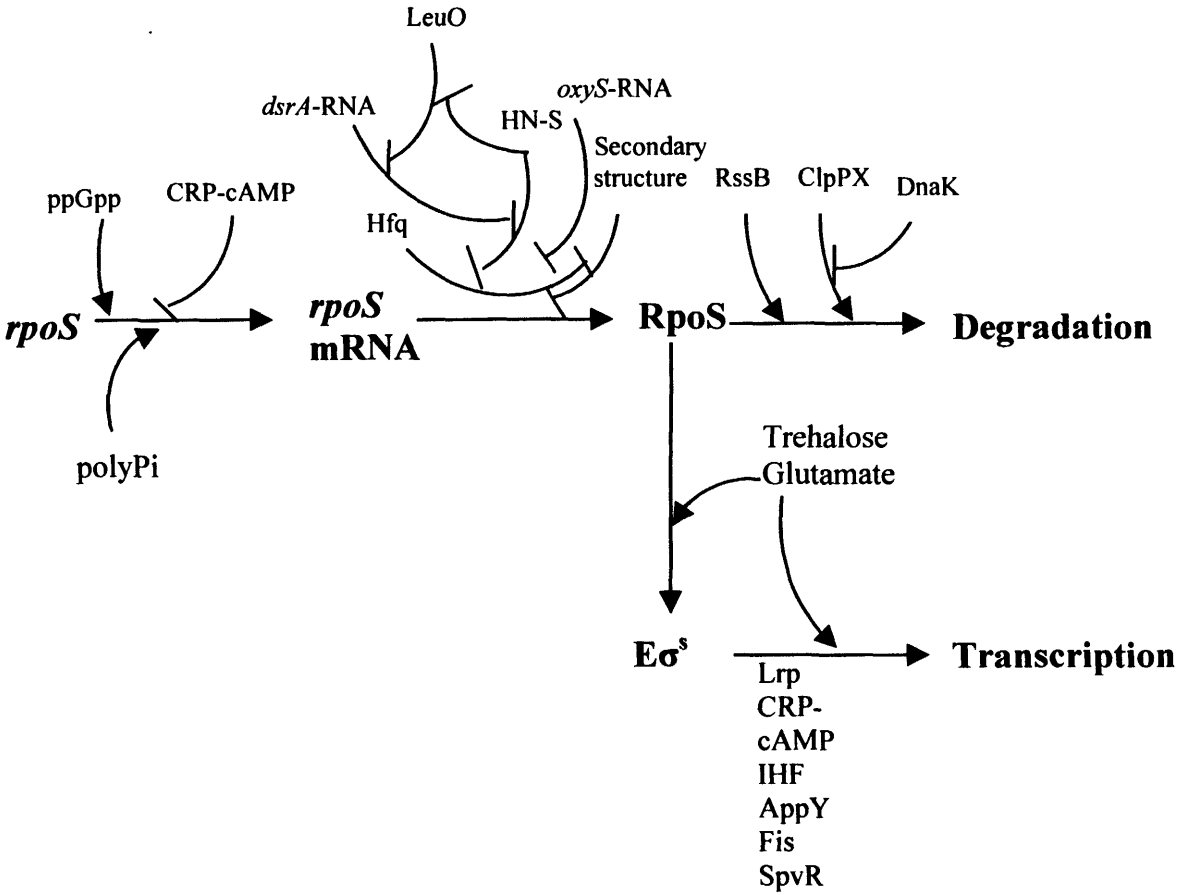


Figure 3. Regulation of RpoS at transcriptional and translational level (adapted from Loewen *et al.*, 1998).

Various effector molecules that affect *rpoS* have been identified. The effector molecules at transcriptional and translational level have been indicated in the figure. Activation is indicated by → and inhibition, by —|

proposed a model in which recruitment of the RNP to the promoter is the rate-limiting step in promoters positively regulated by ppGpp. Therefore, they are very sensitive to the concentration of RNA polymerase. In the presence of high levels of ppGpp, the RNP is dissociated from the stringent promoters and becomes available for promoters with decreased ability to recruite RNP. Therefore, increased transcription from σ^S promoters in cells expressing a mutant β RNP might be due to high levels of RNP. The ability of σ^S to activate specific promoters may be directly or indirectly affected by ppGpp. There is little effect of σ^S in *in vitro* systems, which indicates that some other signal is required for the proper initiation of transcription.

The number of tRNA molecules also decreases in stationary phase, which may be due to scavenging for nutrients (Jakubowski *et al.*, 1992). This may result in the formation of temporary empty acceptor sites in the ribosome, which initiate *relA*-independent synthesis of ppGpp. Carbon starvation may lead to the *relA* independent synthesis of ppGpp. This may be due to activation of the *spoT* gene product and, therefore, ppGpp may affect other cellular processes, including the increased expression of σ^s. Several tRNA synthetases have been shown to bind metabolic intermediates and to generate cyclic compounds (Jakubowski *et al.*, 1992). For example isoleucine-valine and lysine tRNA synthetases bind homoserine and form homoserine lactone (Jakubowski *et al.*, 1992). This is important because acetylated homoserine lactone derivatives are important as high cell density signaling molecules in bacteria (Fuqua *et al.*, 1994). Homoserine itself is shown to be involved in the induction of *rpoS* expression (Huisman *et al.*, 1994).

1.3.2.2 Histone like protein H-NS

H-NS is a DNA binding protein and its expression is auto-regulated during the log phase of growth. The repression is removed in stationary growth and σ^S-independent expression of H-NS increases 10-fold (Derch *et al.*, 1993; Falconi *et al.*, 1993). It is postulated that H-NS might be binding to promoters and preventing σ^{70} RNA polymerase from recognizing them. The *proU* promoter is another example of the relationship between σ^S and H-NS. H-NS promotes *proU* supercoiling and *proU* expression is controlled by σ^S in stationary phase (Manna *et al.*, 1994). IHF (Integration Host Factor), in contrast to H-NS, binds to DNA and bends it. The levels of IHF increases about six fold in stationary phase. IHF is not the major nucleoid component in growing cells but becomes the second most-abundant nucleoid component (next to Dps) in stationary phase cells (Talukder *et al.*, 1999).

1.3.2.3 DsrA

DsrA is a small, untranslated RNA that regulates the expression of RpoS and H-NS at the translational level. The three stem-loops of DsrA correlate with the regulatory function. The first loop is involved in the translational regulation of RpoS, the second loop is involved in the regulation of H-NS and the third one has the transcriptional terminator (Sledjeski *et al.*, 2001).

Hfq is a protein that is required for the replication of the genome of phage Qβ. Hfq is an important protein for the efficient translation of σ^S in *E. coli*. Sledjeski, *et al.* (2001), showed that an Hfq mutant is defective for DsrA-mediated regulation of

RpoS and H-NS. This effect can be partially overcome by over-expressing DsrA. Hfq does not affect transcription. It was found that purified Hfq binds DsrA *in vitro*. Experiments by Sledjeski *et al.*, 2001 indicate that Hfq is a protein cofactor which functions by altering DsrA, forming an active RNA protein complex. DsrA-mediated regulation of *rpoS* was severely reduced in a Hfq mutant. Hfq probably binds to the first loop of the DsrA and unwinds it. DsrA can then bind to the 5' region of the *rpoS* mRNA to prevent the formation of the inhibiting secondary structure. Hfq could also bind to DsrA to put it in proper conformation for σ^S or H-NS expression. In a mutant of Hfq, *hfq-1*, the DsrA is truncated at the 5' end, which shows a definite involvement of Hfq in the formation of a functional DsrA (Sledjeski *et al.*, 2001).

Expression of *leuO* causes reduced accumulation of σ^S *via* affecting the translational expression of RpoS by repressing the synthesis of the DsrA RNA (Fang *et al.*, 2000). Mutations in *leuO* should increase the accumulation of σ^S at low temperatures. Another molecule that modulates the translation of *rpoS* by modulating Hfq is oxyS RNA (Zhang *et al.*, 1998).

1.3.3 Anti-σ factor

The role of anti-σ factor in the control of σ subunit activity is becoming increasingly evident. A novel protein, Rsd (Regulator of Sigma D) is found to be associated with σ^{70} during transition from exponential to stationary phase (Jishage and Ishihama, 1998). The intracellular level of Rsd increases during transition of growth from exponential to stationary phase. The Rsd gene was identified at 90 min

on the *E. coli* chromosome. *In vitro* experiments show that Rsd complexes with σ^D but not with σ^N, σ^S, σ^H, σ^E or σ^F. *In vitro* transcription studies indicate that Rsd protein interferes with the engagement of σ^{70} in transcription. Jishage and Ishihama (1998) proposed that Rsd, with σ^{70} binding activity plays a role in controlling the σ^{70} function in stationary phase *E. coli*. There are various lines of evidence to show this: (1) Rsd is formed during growth transition from exponential to stationary phase in parallel to the shut off transcription from σ^{70} promoters, (2) some σ^{70} is still present as a complex with Rsd during stationary phase, (3) purified Rsd forms a complex with σ^{70} but not with the other sigma factors, (4) the binding site of Rsd is located downstream from the promoter –35 binding region and (5) Rsd interferes with the σ^{70} mediated transcription of some promoters.

SprE is thought to control the levels of σ^S. SprE binds to σ^S and channels it to the ClpX (Zhou *et al.*, 2001). ClpX unwinds the protein and presents it to ClpP for degradation. The SprE is now released from the complex. SprE and RssA were thought to constitute an operon. SprE is also known as RssB in *E. coli*. The levels of SprE are high in stationary phase. It has been shown that phosphorylation of SprE increases the degradation of σ^S (Ruiz *et al.*, 2001). It is not known how SprE is phosphorylated *in vivo*; therefore SprE is an orphan response regulator.

It is also observed that cells harbouring *sprE*::IS1 allele (have high levels of σ^S due to an increase in RpoS stability) grow poorly on succinate and acetate, for unknown reasons (Pratt and Silhavy, 1998). Probably, σ^S is an indicator of stress conditions and succinate and acetate are non-fermentable carbon sources, which

require energy for metabolism. They are not utilised efficiently. In fact, acetate is a chemotactic repellant (Khan *et al.*, 2000). A *rpoS*::Kan^R mutation can restore the growth of the cells on succinate and acetate substrates in *sprE*::1S1 strains, showing that the inability to grow on these substrates is indeed due to high levels of σ^S in stationary phase (Pratt and Silhavy, 1998).

Growth on succinate and acetate can also be restored in a *crl*::Cam (curly subunit). The effect of *rpoS*::Kan was always greater than *crl*::Kan when both are present together (Pratt and Silhavy, 1998). But, the effect was not more drastic than the effect of *rpoS*::Kan^R alone. These results indicate that the *crl* plays a global role in the stationary phase regulation and that *crl* and *rpoS* function in the same pathway. They also found that in the presence of a *crl* null allele, the activity of the σ^S is decreased and the levels of the σ^S protein are actually increased. Probably Crl is a DNA binding protein, which regulates the transcription of *rpoS* controlled genes. It could also bind to the σ^S itself and modulate the association with core RNP to control the amount of RpoS holoenzyme in the cell.

1.3.4 Effect on glycogen synthesis

Mutants of *rpoS* are defective in glycogen synthesis (Loewen and Hengge-Aronis, 1994). The *glgCAP* operon for glycogen synthetic enzymes is induced in stationary phase, but *glgCAP* is not under the control of σ^S. Therefore, some other σ^S dependent genes must be involved in glycogen synthesis. The *glgS* gene is regulated by both cAMP and σ^S from two separate promoters and under conditions of maximal

glycogen synthesis; *glgS* expression depends entirely on σ^S (Loewen and Hengge-Aronis, 1994). GlgS protein is glycosylated *in vivo* and acts as a primer for glycogen synthesis.

Transcription of *rpoS* is high in a Δ *cya* Δ*crp rpoS*::*lacZ* fusion strain (Lange and Hengge-Aronis, 1991, 1994). Transcription of *rpoS* decreases upon addition of cAMP. The level of the σ^S was increased in a Δ *cya* strain and decreased in this strain upon addition of cAMP. A signal produced during stringent response is also produced during starvation and plays an important role in stationary phase by influencing the transcription of *rpoS* confirmed by studies with transcriptional fusions (Lange *et al.*, 1995). Inorganic polyphosphate is another component that influences the σ^S levels and causes an increase in σ^S transcription (Shiba *et al.*, 1997). Therefore ppGpp, cAMP-Crp and polyphosphate are all modulators of σ^S levels.

1.4 Catabolite Repression

1.4.1 Overview

Catabolite repression is a relatively new term given to a phenomenon discovered almost a century ago. This was initially called adaptation, diauxie or glucose effect. Dienert in 1900 showed that yeasts grown on glucose do not break down galactose but those grown on galactose metabolise glucose. When grown in the presence of both the sugars as carbon sources, yeast metabolise glucose first and subsequently galactose. In 1938, Karstrom showed that bacteria grown on glucose

could not break down galactose, lactose, arabinose or maltose. The analysis of this phenomenon, known as adaptation was first done by Monod in 1942. He classified the sugars into various categories: (A) the ones that are not diauxic in association with glucose and (B) others that are diauxic. The "A" class of sugars included glucose, mannose, fructose, saccharose and mannitol. The other sugars classified as "B" such as maltose, arabinose, sorbitol, lactose, *etc.*, have diauxic growth in the presence of small amounts of glucose. A typical diauxic growth is charcterised by an initial lag phase followed by the log phase where there is utilisation of the easily metabolisable sugars, followed by another lag phase and then a log phase where the class B sugars are utilised. Monod drew the following conclusions from the study. Each cycle in diauxic growth represents the utilisation of one kind of sugar. The first cycle represents the utilization of A class sugars, which are easily metabolisable and that are acted upon by constitutive enzymes, and the second cycle represents the utilisation of the B class sugars, which are acted upon by adaptive enzymes. The second lag phase that is seen during diauxic growth can be decreased if the ratio of B to A is increased or if the cells are adapted to the new sugar. Diauxic growth is due to the inhibitory action of the A class sugars on the synthesis of the enzymes for the utilisation of the class B sugars.

Any compound that can serve efficiently as a source of intermediary metabolites and of energy may reduce the rate of formation of glucose sensitive enzymes relative to the rate of formation of other proteins. Mangasanik (1961) coined a new name for the glucose effect: catabolite repression. The catabolites,

which are formed rapidly from glucose, accumulate in the cell and repress the formation of enzymes whose activities would increase the already large intracellular pools of these compounds. In 1965, Makman and Sutherland identified cAMP in *E. coli* and showed that glucose starved cells accumulate large amounts. In 1968 it was discovered independently by Perlman and Pastan, and Ullmann and Monod that cAMP is an antagonist of the glucose effect. Ullmann and Monod showed that cAMP relieves both catabolite repression and inducer exclusion. After the discovery of the cAMP receptor protein, Crp (also known as Catabolite gene Activator Protein or CAP), (Zubay *et al.*, 1970) and the identification of the genes encoding adenylate cyclase (*cya*) and Crp (*crp*), it was established that the cAMP-Crp complex promotes gene expression of catabolic operons. The effect of cAMP in relieving the inhibitory effects of glucose has often been associated with the positive control mediated by cAMP-Crp (Catabolite Repressor Protein).

Pastan and Adhya (1976) showed that *cya* and *crp* mutants were defective in utilising sugars such as lactose, maltose, glycerol, etc, because the enzyme for the utilisation of the sugars could not be synthesised. The genes that could not be expressed were known to be subject to catabolite repression (Saier, 1996). Activation of these genes was mediated by cAMP-Crp complex. However, there could be additional regulatory controls.

When cAMP binds to Crp, a conformational change in the protein occurs (Saier and Ramseier, 1996). This cAMP-Crp complex can now bind to specific sites at or near target promoters and bring about activation of transcription. Many of the

promoters that control catabolic functions are under the control of Crp. Crp also controls the expression of some membrane proteins not involved in metabolite transport and Crp also regulates genes involved in starvation and stress responses. The levels of cAMP can be suggested as a global signal for the presence or absence of glucose, but the presence of certain metabolites may also be required for operon-specific expression.

1.4.2 Mode of action of cAMP-Crp

Crp can bring about activation or repression in various different ways (Botsford and Harman, 1992). Simple activation occurs when cAMP-Crp complex binds to a sequence upstream of the promoter activating transcription *e.g. lac* operon. Crp can bind to the regulator of the operon to activate it, which in turn activates transcription *e.g* the *mel* operon. Co activation, where both the cAMP-Crp complex and the activator of the operon should be present to cause the expression of the operon also occurs (*malB* locus). Crp can also act as a repressor, such as the *cya* promoter P2 (Aiba, 1983). Experiments with *cya-lacZ* strains show that cAMP-Crp complex negatively regulates the *cya* expression at the P2 promoter (Fandl *et al.*, 1990). Another mechanism of Crp mediated transcriptional repression is at the *deoP2* promoter (Short and Singer, 1984). Therefore, the Crp has multiple mechanisms in activation and can also act as a repressor. The *crp* gene is autoregulated by cAMP-Crp, which binds to sites downstream of a transcription start site. This promotes RNA polymerase to bind to a site further down from the *crp* promoter (Busby and

Kolb, 1996). The repression is due to the RNA polymerase binding at the second promoter, P2.

Binding of cAMP-Crp to DNA at the Crp-dependent promoters causes bending of DNA that brings about structural change near the promoter (Botsford and Harman, 1992). This has been observed at the *lac* promoter. DNA bending is likely to promote protein-protein interactions important in Crp-dependent promoter acivation. Also DNA footprinting experiments have been done with the *lac* promoter (Botsford and Harman, 1992). Crp* mutants which do not require cAMP-Crp interaction have been isolated which can bring about activation at the cAMP-Crp dependent promoters in the absence of cAMP (Harman *et al.*, 1983). The cAMP-Crp complex and the RNA polymerase form a tight complex at the promoters. In the absence of RNA polymerase, the cAMP-CRP fails to protect the DNA from DNase I cleavage (Straney *et al.*, 1989). Studies with Crp*, Crp*-cAMP complex, have shown that both of these can activate the promoter, but in the absence of RNA polymerase, it still cannot protect the DNA from DNase I cleavage. This shows that the activation domain of the Crp is different from the RNA polymerase binding domain.

Most *in vitro* work is done using linear deproteinated DNA. DNA supercoiling favours the cAMP-Crp complex and the *lac* repressor interactions *in vivo* and *in vitro* (Borowiec *et al.*, 1987 and Whitson *et al.*, 1987). Similarly, histone-like proteins also increase the cAMP-Crp *lac* repressor interaction (Feldheim *et al.*, 1990).

1.4.3 Effect of the PTS on catabolite repression

It is known that the phosphotransferase system (PTS) plays a very important role in the mechanism of catabolite repression. Mutants in E1 (*ptsO*) or Hpr (*ptsH*) or both cannot grow on maltose, lactose, glycerol, melobiose, etc. (Lengeler, 1996). It was also found that the *pts*$^-$ phenotype is similar to the *cya*$^-$ phenotype and prevents the utilisation of non-PTS sugars. The effect on non-PTS sugars can be reversed on addition of extracellular cAMP. The regulatory molecule, IIA^{Glc}, can exist in a phosphorylated ($P\text{-}IIA^{Glc}$) and in an unphosphorylated form (Lengeler, 1996). In its phosphorylated form, $P\text{-}IIA^{Glc}$ is probably involved in the activation of the enzyme adenylate cyclase (Cya) and hence through modulation of the intracellular cAMP levels, regulates the *crp* modulon. Suppressor mutations have been isolated which represses the *pts*$^-$ *cya*$^-$ mutation (Lengeler, 1996).

When cells are grown on non-PTS sugars like lactose, melobiose or maltose, the $EIIA^{Glc}$ is phosphorylated and brings about the activation of adenylate cyclase (Lengeler, 1996). This leads to elevated cAMP levels and increased gene expression. When glucose is added to the medium, phosphorylation of the PTS and IIA^{Glc} is lowered and adenylate cyclase is inactive resulting in low cAMP levels. The levels of cAMP and Crp are important in catabolite repression. In the absence of Crp, the cAMP levels are very high. It has been found that even in the absence of glucose, expression of β-galactosidase from *lac* fusions of genes for utilising non-PTS sugars are reduced in the presence of low Crp levels (Ishizuka *et al.*, 1993). When cAMP measurements were made, they did not account for the β-galactosidase expression.

The effect of glucose could not be completely reversed by the addition of cAMP. But the effect of glucose was abolished when Crp levels were increased (Ishizuka *et al.*, 1993).

When catabolite repression is severely increased by nitrogen source limitation or by other growth conditions, addition of cAMP to the bacterial cultures has little effect and catabolite repression persists (Guidi-Rontani *et al.*, 1980). Catabolite repression can occur in the absence of cAMP in bacteria, which normally produce the nucleotide. Indeed, the mutants deficient in adenylate cyclase or Crp (*Δcya ΔCrp*) are still subject to some catabolite repression (Guidi-Rontani *et al.*, 1980, 1981). Bacteria, which do not synthesise cAMP, such as *Bacillus subtilis,* have genes regulated by catabolite repression (Fisher and Magasanik, 1984). In yeast, catabolite repression is not associated with variations in cAMP levels and high extracellular cAMP concentrations do not relieve catabolite repression (Eraso and Gancedo, 1984).

1.4.4 cAMP independent catabolite repression

Dessein *et al.* (1978) showed that there was a cAMP independent mechanism of catabolite repression in *E. coli.* Similarly, experiments conducted with *B. subtilis* and other bacteria, led to the suggestion that these organisms might possess multiple, cAMP-independent mechanisms of catabolite repression (Fisher and Magasanik, 1984).

The catabolite repressor/activator (Cra) protein of enteric bacteria was initially characterised as the fructose repressor, FruR (Saier and Ramseier, 1996). Mutants

defective in the *cra* gene exhibited a pleiotropic phenotype, being unable to grow with gluconeogenic substrates as the sole carbon source (Chin *et al.*, 1987 and Geerse *et al.*, 1986).

The *cra* gene is located 3 min on the *E. coli* chromosome. The Cra protein has homologies to various repressor proteins. It has an N terminal helix-turn-helix domain that is involved in DNA binding (Saier and Ramseier, 1996). The Cra binding sites are located downstream of or overlap the promoters of the genes where Cra acts as a repressor. Repression by Cra is reversed by sugar catabolites like fructose-1-phosphate or fructose-1,6-bisphosphate. In operons that are activated by Cra, the Cra binding site is located upstream of the promoters. Ligand binding displaces Cra from the DNA so that it causes repression. Catabolite activation is generally observed whenever Cra represses transcription, while catabolite repression is observed whenever the protein activates transcription. When the Cra binding site overlaps or follows the RNA polymerase binding site, it represses transcription (Saier and Ramseier, 1996).

The *fru* operon in *E. coli* is regulated by the Cra protein and partly by the cAMP-Crp complex, which plays a secondary role. In the absence of cAMP, fructose is one of the few sugars that can be utilized efficiently (Saier and Ramseier, 1996). Fructose is the only sugar that feeds directly into the Embden Meyerhof glycolytic pathway without isomerisation or epimerisation. It is possible that the primeval role of glycolysis was as a fructose pathway and that Cra has assumed the role in regulating the carbon flow.

Mutations in *cra* were isolated by screening for cells that expressed the protein products of the *fru* operon at constitutive levels. The effect of Cra null mutations could not be overcome by the addition of cAMP, which shows that the Cra action is independent of the cAMP-Crp system (Chin *et al.*, 1987). The effect of the null mutations was similar to the glucose-mediated effect (Chin *et al.*, 1989; Geerse *et al.*, 1989). In *cra*::Tn*10* insertion mutants, the levels of many enzymes like phosphofructokinase, enzyme I of the PTS and several enzymes II of PTS were all elevated but the key gluconeogenic enzymes Pps, Pck, and fructose-1,6-bisphosphatase were lowered as compared to the levels in the wild type cells. (Saier and Ramseier, 1996). However, Goldie (unpublished) has been unable to reproduce the results for Pck, in spite of several attempts. It seemed reasonable to propose that Cra mediates responses to carbon availability by transcriptionally activating the expression of genes that are normally subject to catabolite repression, while repressing the expression of genes that are subject to catabolite activation. The effects of CsrA (Sabnis *et al.*, 1995; Romeo, 1993), on gluconeogenesis observed were the reverse of Cra and rather small (two to four fold). These two proteins probably act independently. This shows that there are many different processes that influence carbon metabolism.

1.4.4.1 Effect of Cra on carbon flow

The *pps* gene is subject to positive control by Cra, while *pykF* and *gapB* are subject to negative control (Saier, 1996). Cra exerts an activating effect on *ppsA*

expression regardless of the carbon source used. However, this activating effect is much more substantial when cells are grown with pyruvate (gluconeogenic substrate) than when they are grown with glucose (glycolytic substrate). In the absence of Cra, the activity is very low (Saier and Ramseier, 1996). The operator-promoter regions of the *ppsA* and *pykF* genes lack detectable Crp binding sequences, and in these two genes, Cra plays a dominant and clear-cut role in transcriptional regulation in response to carbon source availability.

Sugars and sugar derivatives were oxidized more readily in the *cra* mutant in wild type cells, while gluconeogenic substances were oxidized less readily (Saier, 1996). This observation led to the generalized conclusion that Cra controls the direction of carbon flux and primarily functions to select a fermentative pathway or an oxidative pathway of carbon metabolism, depending on physiological conditions. Cra generally represses transcription of genes encoding glycolytic enzymes (key enzymes in the EMP shunt) but activates transcription of genes encoding biosynthetic and oxidative enzymes (enzymes in the glyoxylate shunt, gluconeogenic pathway and electron transfer). Cra modulates the activity of adenylate cyclase (Crasnier *et al.*, unpublished results). It is therefore clear that Cra does not act alone to exert its multiple effects but interacts with other pleiotropic regulators to create a network of transcriptional effects that serve to coordinate various bacterial sensing devices. The Cra mediated catabolite repression and activation probably arose at a time when bacteria were first imposing distinct transcriptional control mechanisms upon their carbohydrate catabolic and anabolic capabilities (Saier and Ramseier, 1996).

Carbon utilisation in *E. coli* is a very complex process. In some cases it involves the use of PTS where the IIA^{Glc} phosphorylation is important for the activation of adenylate cyclase and activation by Crp. However, catabolite repression also takes place in a Δ*crp* mutant lacking cAMP-Crp complex and also in gram-positive organisms that lack intracellular levels of cAMP. Carbon utilisation should be linked to gluconeogenesis, stationary phase regulation, ATP and PEP synthesis, acetyl CoA synthesis and also to nitrogen metabolism, since many stringent response molecules are produced as a result of amino acid starvation and stress (Spector and Cubitt, 1992). When nutrients are limited, the cells respond through the carbon starvation-induced systems (Lengeler *et al.*, 1996), which might be linked to the global regulator, cAMP-Crp. Lastly, a small stimulus might cause a large number of systems to function together in response to feast or famine, and they all work towards a common goal of search for food and the optimal utilisation of the available carbon sources.

1.5 ATP synthase in *Escherichia coli* [E.C.3.6.3.14]

1.5.1 Overview

ATP synthase is present ubiquitously in mitochondria, in chloroplasts and in bacteria. The bacterial proton-translocating ATP synthase couples the electrochemical potential difference generated across the membrane due to H^+ translocation to ATP synthesis from ADP and Pi. It also plays a reverse role in

degradation of ATP to ADP and Pi to generate the proton motive force (PMF) across the membrane for driving cellular functions like transport and motility. The membrane bound ATP synthase of *E. coli* has a central role in energy transduction in aerobic as well as anaerobic conditions. During aerobic conditions, the PMF generated during respiration is used for ATP synthesis to maintain a solute gradient (Jensen and Michelsen, 1992). The generation of ATP from ADP can occur via oxidative phosphorylation, mediated by the ATP synthase and powered by the PMF. During anaerobic conditions, ATP is derived from substrate phosphorylation (*e.g.* the EMP pathway) and ATP hydrolysis is used to maintain the PMF. Wild type *E. coli* can use both oxidative phosphorylation and substrate level phosphorylation, whereas the *atp* mutants are dependent solely on substrate level phosphorylation (Jensen and Michelsen, 1992). In photosynthetic bacteria and in strict anaerobes, the ATP synthase functions as ATP synthase in oxidative phosphorylation. In fermentative bacteria, the ATP synthase functions as ATP-utilising H^+ pump and the PMF generated is used for transport and motility (Jones *et al.*, 1983; Kasimoglu *et al.*, 1996).

The growth rates of mutants having *atp* deletions are reduced by 74-79% in comparison to the wild type, whereas the growth yield is reduced by 55-58% (Jensen and Michelsen, 1992). They show that the growth rate and growth yield are dependent on the levels of biosynthetic reactions at a given time. Mutants in *atp* (earlier called *unc*) cannot grow on nonfermentable substrates such as succinate, and have lower growth yields on limiting concentrations of glucose (Cox and Downie,

1979). Thorbjarnardottir *et al.* (1978) isolated *atp* mutants that are resistant to aminoglycoside antibiotics probably due to defects in antibiotic transport. The also have reduced ability to accumulate amino acids.

1.5.2 Gene order and subunit composition

The *atp* operon maps at 84.6 min on the *E. coli* chromosome. The order of genes in the operon is *atpB, atpE, atpF, atpH, atpA, atpG, atpD* and *atpC*, encoding the subunits a, c, b, δ, α, γ, β and ε, respectively. A promoter sequence 73 base pairs upstream of *atpB* (Gay and Walker, 1983) has been identified and confirmed by the study of Tn*10* insertions (Von Meyenburg *et al.*, 1982) and by DNase I footprinting studies. Porter *et al.* (1983) showed that the promoter 73 base pairs upstream of the open reading frame is strongly active both *in vivo* and *in vitro* and it was concluded to be the *unc* promoter since the other promoter like sequences upstream of the *atpB* were either inactive or only weakly active. They also concluded that the open reading frame preceeding the *atpB* is a part of the *atp* operon and they named it *atpI*. There is not much work published on the genetic regulation of the *atp* operon.

The ATP synthase enzyme is bound to the inner membrane in bacteria. After detergent solubilisation, the ATP synthase is obtained as $\alpha_3\beta_3\gamma_1\delta\varepsilon a_1 b_2 c_{12}$. The molecular mass of ATP synthase is 520,000 daltons (Capaldi and Schulenberg, 2000) and the enzyme consists of two components, F_1 and the F_0. The F_1 ($\alpha_3\beta_3\gamma\delta\varepsilon$) is the water soluble portion and is on the cytoplasmic side of the membrane, which includes the catalytic portion, $\alpha_3\beta_3\gamma$. The δ subunit is on the outside of the F_1 and extends

from the F_1 region to the F_0 through the stalk (Hazard and Senior, 1994). The ε subunit plays an important part in the rotor and interacts with the α, β and the γ subunits (Capaldi and Schulenberg, 2000). F_0 is the transmembrane proton channel and is composed of $a_1c_{12}b_2$. The evidence that the enzyme contains eight subunits, all of which are required for the activity, came from the identification of eight complementation groups by mutational analysis (Downie *et al.*, 1979).

The integrity of the F_0F_1 complex is important for the coupling of ATP synthesis respiration to PMF dependent reactions (Simoni and Postma, 1975). Removal of F_1 led to uncoupling via a specific increase in H^+ permeability, which was due to F_0 left in the membrane and can be reversed by binding of F_1 or by DCCD treatment. Therefore the removal of F_1 from the membrane resulted in the blockage of respiration-dependent PMF driven reactions like solute transport and motility. This has been used as a direct measure of membrane permeability and also suggests that F_0 is the H^+ conductor (Mitchell, 1973).

1.5.2.1 Subunit composition of F_1

The ATP synthase protein was sequenced by Walker *et al.* (1984) and the structure was determined in 1994, for which he received the Nobel Prize. The catalytic subunit of ATP synthase is $\alpha_3\beta_3\gamma$ of the F_1. The two large proteins in the F_1, α and β, are conserved among species. There are some well established facts about the ATP synthase (Berden *et al.*, 2000). There are six nucleotide-binding sites at each of the three αβ subunits of the F_1 portion of the enzyme. The catalytic sites are

located on the β subunits. The catalytic mechanism of ATP synthase implies the catalytic involvement of the three catalytic sites. These catalytic sites show negative cooperativity of binding and positive cooperativity of catalysis (Boyer, 1993). Tight binding of a nucleotide at a catalytic site is an intermediate step in catalysis. On the removal of the other loosely bound nucleotides, one of them always remain bound to the catalytic site (Van Dongen *et al.*, 1987) and it always remains bound as long as the other catalytic sites are empty. The noncatalytic sites are located in the α/β interface in addition to the catalytic sites and may be liganded to MgAMP-PNP in a functional ATP synthase, and interacts with amino acid residues of the α subunit and to a lesser extent to the amino acids in the β subunit (Abrahams *et al.*, 1994).

It has been shown that ATP hydrolysis is coupled to a 120° rotation of the γ subunit as ATP is hydrolysed at each of the catalytic sites. The rotation prompts the γ subunit to deform the α and β subunits, which gives enough energy to the enzyme to remove one ATP from the enzyme complex. The likely reason that has been put forth for the movement is migration of the protons through the F_0. Garcia and Capadi, (1998) have shown that on crosslinking the γ to the β subunit, the catalytic ability of the enzyme is lost, although ATP affinity is still retained. From this, it can be concluded that the rotation of the γ subunit might be solely responsible for cooperative binding of ATP to the catalytic sites.

A model that has been widely accepted for energy coupling by F_0F_1 ATP synthase is called the Binding Change Mechanism (Boyer, 1993). This model explains that the energy requiring step is not the synthesis of ATP, but the highly

regulated cooperative binding of substrates and release of products (Boyer *et al.*, 1973; Kayalar *et al.*, 1977). The ATP binds to the first catalytic subunit to be hydrolysed to ADP and Pi. But the ADP and Pi are not released until another nucleotide binds to the catalytic site. Therefore, at no time during the reaction are any of the catalytic sites empty. It is thought that the ATP affinity changes are coupled to the proton transport due to the rotation of the subunits in the F_0F_1. Rotation of the γ subunit in F_1 is thought to change the affinity of binding of the substrates by deforming the catalytic sites and the rotation of the c subunit in the F_0 is responsible for completing the proton transport (Vik *et al.*, 1994).

Subunit β harbours the catalytic sites where ATP is synthesised or hydrolysed. A cylinder of three alternating α and β subunits is formed and the asymmetrically bent shaft of the γ subunit protrudes through it. The γ subunit asymmetry correlates with the various conformations that the catalytic core can assume according to the Binding Change Mechanism model of Boyer (Boyer, 1993). There are lines of evidence for ATP-driven rotation of the γ subunit with the F_1 shaft. Yoshida's group attached flourescently-labeled actin filament to the γ subunit (Noji *et al.*, 1997) and the F_1 was immobilised by engineering polyhistidine tags to the β subunit. The ATP-dependent rotation of the γ subunit was viewed directly through a video microscope (Kinosita *et al.*, 1998).

The δ subunit is very critical for the correct binding of F_1 to F_0. The δ subunit is elongated and highly helical. Hazard and Senior (1994) proposed a hairpin-like structure for the δ subunit, extending from the F_1 region to the F_0 through the stalk on

the outside of F_1. Mutagenesis, proteolysis and in vitro assembly studies indicate that the δ subunit makes contact with α and β subunit and also with the *b* subunit of the F_0 portion (Weber *et al.*, 1997).

Subunit ε, component of the F_1 interacts with the α and the β subunits at the C-terminal domain and interacts with the γ subunit at the N-terminal β subunit sandwich domain and interacts with the *c* subunit of the F_0 portion at the bottom of the N-terminal domain (Capaldi and Schulenberg, 2000). The ε subunit plays an important part in the rotor.

The rotation of the ε subunit was observed by fluorescent-labeled actin filament tagging and viewing with a video scope (Kato-Yamada *et al.*, 1998). The ε subunit is seen to be scrambled between the α_3 and the β_3. When ATP synthesis drives the rotation of the γ and the ε, there are changes in the interaction between the γ and ε subunits. However, cross-linking ε and γ and ε and *c* does not prevent the functional ATP hydrolysis capability. The first cross-linking of ε and *c* was done by Zhang and Fillingame (1995). It was found that this did not affect the ATP hydrolysis at all, but blocked the H^+ pumping. They obtained dimers between some *c* subunits. Later, the experiments were repeated by Capaldi and Schulenberg (2000) and they cross-linked ε and *c* and 80% of the cross-links retained minimal ATP hydrolysis without affecting H^+ pumping. This is strong evidence for the co-rotation of the ε and the *c* subunits.

There is no assembly of F_0 over the F_1 in the absence of ε (Abrahams *et al.*, 1994) which shows that it plays a very important structural role. Position of the ε in the central stalk and the movements during ATP driven proton translocation are key to the coupling mechanism. Subunit ε also influences the conformation of the γ subunit. Cross linking studies have shown that the conformational and the positional changes in the γ are lost when the ε is removed (Turina *et al.*, 1994)

In the presence of ADP, ε binds to the β subunit via a cysteine. In the presence of ATP, ε binds to the α subunit via the corresponding cysteine (Capaldi and Schulenberg, 2000). Therefore, this nucleotide dependent shift of ε subunit could represent rotation of ε from one αβ to another with conversion of ATP to ADP. During ATP hydrolysis and ATP formation, interaction between ε and α and β has to be broken and reformed while keeping the interaction with γ and *c* intact. The breaking and reforming of the interaction could be the rate-limiting step in the reaction.

F_0 and F_1 are linked to each other through two stalks of 45 Å length (Capaldi and Schulenberg, 2000). The inner stalk consists of ε and γ and the outer stalk is made of hydrophilic regions of the *b* subunit of the F_0 and the δ subunit of F_1. The central stalk transmits energy from the proton channel to the catalytic subunit while the peripheral stalk acts as a stator or scaffold, holding the a subunit while the mobile unit that comprises of the γ, ε and the *c* subunits rotates. F_0 consists of a_1b_2 and $c_{9\text{-}12}$. The *c* subunits form a cylinder and makes contacts with subunit *b* and the subunit *a* to

$\alpha_3\beta_3$ via the δ subunit. Subunit *b* is proposed to be a stator that keeps the $\alpha_3\beta_3$ to the F_0 together while the subunits *c*, γ and ε rotate as a unit (Capaldi and Schulenberg, 2000).

1.5.2.2 Subunit composition of F_0

Subunit *a* is a part of the F_0. It is highly hydrophobic and consists of six alpha helices. Subunit *a* interacts with the $\alpha_3\beta_3$ via the δ subunit (Dimorth, 2000). There is direct interaction of subunit *a* with subunit *c* shown by the fact that an "*ac*" complex that is competent in proton transport can be isolated (Dimorth, 2000). In *E. coli* the inhibitor of ATP synthase DCCD, acts by modifying the Asp61 of the *c* subunit of the F_0. This abolishes proton translocation and ATP hydrolysis (Dimorth, 2000). This indicates a coupling between the two protons that move down an electrochemical gradient and interact with the carboxyl side chain of the *c* subunit via protonation and deprotonation of the Asp61 carboxylate. This brings about a conformational change in the *c* subunit and the message is relayed through the F_0F_1 stalk to the catalytic region of the ATP synthase (Dimorth, 2000).

Subunit *b* is present in a stoichiometry of two in the F_0 ATP synthase. The hydrophobic part of the subunit *b* is important for association of F_1 with F_0 (Deckers-Hibestreit *et al.*, 2000). They found that the formation of subunit *b* dimers seem to be important for the interaction with the F_1 portion and it has been shown that four C terminal residues are involved in direct binding to F_1 via δ. Like subunit *b*, δ is also required for the binding of F_1 to F_0. Also, membranes of mutant strains expressing

truncated δ have low ATP synthase activity, indicating the inability of F_1 to bind to the membrane in the absence of δ. Subunit *b* can tolerate deletions up to 11 amino acids in the region of (bD50-bI75) while maintaining coupled enzymatic activity. This indicated that subunit *b* is probably not rigid but has an inherent flexibility, possibly having a dynamic role in coupling.

ATP-driven rotation of the *c* oligomer within the F_0F_1 assembly was shown by attaching flourescein labeled actin to the *c* subunit. This suggests that γ, ε and *c* together rotates during ATP hydrolysis (Sambongi *et al.*, 1999).

Proton translocation through the F_0 region drives the rotation of the oligomeric *c* subunit, which in turn drives the rotation of the γ subunit. Genetic and protein chemistry studies have shown that ε and γ subunits play a very critical role in the functioning of the enzyme (Fillingame *et al.*, 2000). A polar loop is thought to entail the *c*, ε, δ, γ and the *b* subunits, where the ε-c interaction is important at the base of the stalk and the γ-β, ε-β interaction is important at the stalk-F_1 interface. The ε and γ subunits are thought to play a much more important role in the catalytic function of the enzyme than previously expected.

1.6 Rationale and research objectives:

Phosphoenolpyruvate carboxykinase is one of the key enzymes in gluconeogenesis. It is a monomeric enzyme and has a molecular weight of 60 KDa. The enzyme catalyses the first committed step of gluconeogenesis, the carboxylation of oxaloacetate (OAA) to phosphoenolpyruvate (PEP) which can then enter glycolysis. Expression of *pckA* is regulated by cAMP (Goldie and Sanwal, 1980). There are three Crp binding sites that have been identified on the *pckA* promoter (unpublished results). Expression of *pckA* increases about 100 fold in stationary phase in a *pck-lacZ* fusion (Goldie, 1984) in a cAMP independent fashion. It has been seen earlier by Goldie, 1984 that the enzyme is specifically induced during the onset of stationary phase in spite of the presence of 5mM cAMP in the medium, which indicated the presence of an unknown signal in stationary phase that was affecting the regulation of *pckA*.

The work presented in this thesis attempted to study the regulation of *pckA* in stationary phase by isolating regulatory mutants to formulate a model for regulation.

The goals of the work are as follows:

(1) Isolate mutants using mini Tn*10*-ATS to identify genes regulating *pckA* expression.

(2) Characterisation of the mutants for growth, Pck activity and genetic mapping.

(3) Determine the nature of the mutation in our isolated mutants.

(4) Construct a model for stationary phase regulation of the expression of *pckA*.

CHAPTER TWO

2.0 Materials and Methods

2.1 Media and Reagents

Growth media, yeast extract, tryptone and agar were obtained from DIFCO Laboratories, Detroit, MI. Ampicillin (100 μg/mL), tetracycline (20 μg/mL) and kanamycin (25 μg/mL) was added as required. Vitamin B1 (thiamine) was added to medium A at 0.01% concentration. Minimal Medium A, LB medium (without glucose), λ medium and R medium were prepared as described by Miller (1972). Petri plates contained medium plus 2% agar, except R plates, which had 1.2% agar. Restriction enzymes were purchased from New England Biolabs Inc. (Beverly, MA). and used according to the manufacturer's instruction. Taq polymerase and deoxynucleotides were purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). The biochemicals and organic chemicals were purchased from Sigma (St. Louis, MO) and inorganic chemicals from BDH Inc. (Toronto, ON.). Ticarcillin was obtained from SmithKlein Beecham, Pharma Co. (Oakville, ON). Other antibiotics were purchased from Sigma (St. Louis. MO). Kits required for

molecular biological techniques were obtained from QIAgen Inc. (Valencia, CA). Oligonucleotide primers were obtained from GIBCO BRL Custom Primers (Grand Island, NY) and Invitrogen Canada Inc. (Burlington, ON).

2.2 Transposon mutagenesis

Mini Tn*10* elements are generally short (400-3000 bp) and have been engineered to carry a wide assortment of resistance genes. Mini Tn*10*-ATS (**Figure 4**) has an altered target sequence, which allows it to insert almost at random in *E. coli* DNA (Kleckner, *et al.*, 1991). The transposase gene is deleted from the Tn*10*, which increases transposon stability at regions of the chromosome where it integrates. For the mini Tn*10*-ATS derivative 103, used in this study for carrying out mutagenesis, the two ends are perfect inverted repeats of a 70 bp segment carrying the outside end of IS10 Right (generated by cleaving IS10R right with *BclI* and converting the *BclI* site to *Bam*H1 site). Mini Tn*10*-ATS was obtained on a λ phage, Strain No. NK1316 (P_{am} 80 λ phage vehicle) (Kleckner *et al.*, 1991)

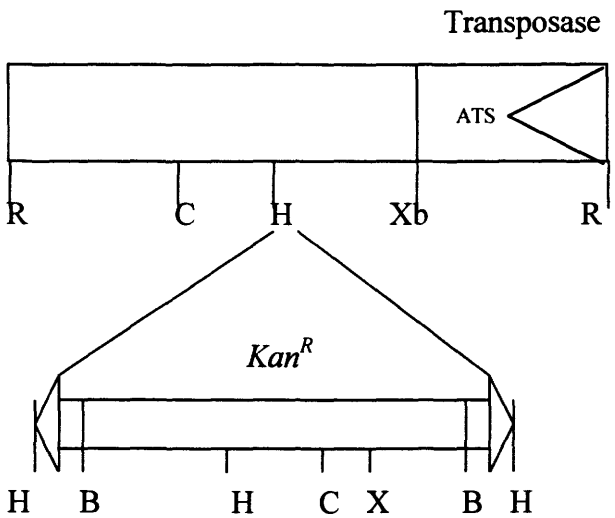


Figure 4. Structure of Mini Tn*10*-ATS in λNK1316.

A 1.8 Kbp HindIII (H) containing the mini Transposon ends of Tn*10* and the Kan^R gene from Tn903, was cloned into a fragment of Tn*10* containing the mutated ATS (Altered Target Specificity) Tn*10* transposase (Kleckner *et al.*, 1991). The entire *EcoR1* fragment, with the mini transposon and transposase in *cis*, is carried by λNK1316.

2.2.1 λNK1316 lysates

Day 1

MM294A was inoculated in LB medium containing 2% maltose overnight. The cells were centrifuged at 3,020 x *g* for 10 min and resuspended in 2.5 mL of 10 mM $MgSO_4$. The λ lysate that was obtained in a freeze-dried form was resuspended in 0.5 mL of λ dilution buffer (10 mM Tris-HCl + 10 mM $MgSO_4$ pH 7.5) (Davis, *et al.*, 1980). Dilutions (10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of the λ, were made in λ dilution buffer. MM294A (0.05 mL) and each λ dilution (0.05 mL) were incubated at 37°C for 15 min. 2.5 mL of top agar was added and spread on λ plates and incubated face up overnight at 30°C.

Day 2

Single plaques were picked with a sterile Pasteur pipette and placed in two drops of λ dilution buffer and vortexed well. Two drops of overnight MM294A cells in LB medium was added and incubated at 37°C for 15 min. Top agar was added and

overlayed on λ medium plates. The plates were incubated face up for 6 h at 37°C, overlayed with 5 mL cold λ dilution buffer and kept in the cold room overnight.

2.2.2 Determining the phage titer

Day 3

The overlay solution was removed and two drops of chloroform was added and centrifuged at 12,000 x *g* for 10 min to remove the bacterial debris. The phage was grown on MM294A to determine the phage titer. Dilutions of the λ lysate (10^{-2}, 10^{-4}, 10^{-6},10^{-8}) were made in λ dilution buffer. Three independent plaques were used. MM294A (0.05 mL) and each λ dilution (0.05 mL) were incubated for 15 min at 37°C. 2.5 mL top agar was added and spread on λ plates. The plates were incubated face up for 6-8 h at 37°C, and then 5 mL of λ dilution buffer was added to the undiluted plate. Dilution plates were incubated at 37°C overnight for determining phage titers.

2.2.3 Adsorption of the phage on HG163 (*pps*⁻).

Day 4

The overlay solution from the undiluted plate was removed, two drops of chloroform were added and top agar containing the lysate was centrifuged at 12,000 x *g* for 10 min to remove the bacterial debris. Dilutions of the phage (10^{-1}, 10^{-2}, 10^{-3}) were made in λ dilution buffer. Dilutions of λ (0.3 mL) and HG163 cells (0.3 mL) were incubated in a 37°C water bath for 15 min. Two drops from each tube were

plated on kanamycin plates. Also, two drops from each tube were added to 2.5 mL of top agar and overlayed on λ agar plates to determine the reversion frequency of the phage.

2.2.4 Mutagenesis

Day 5

A 10^{-1} dilution of the phage, which produced an optimum frequency of Kan^R cells, was used for the mutagenesis experiment. A 5mL overnight culture of HG163 was centrifuged and resuspended in 2.5 mL of 10 mM $MgSO_4$. Five tubes were used for the mutagenesis experiment to avoid siblings and to obtain independent mutants.

Five tubes each containing HG163 (0.25 mL) and 10^{-1} dilution of λNK1316 (0.25 mL) was incubated at 37°C for 15 min. One mL of LB was added to each tube and then incubated in a 37°C water bath for one hour. The samples were centrifuged at 3020 x *g* for 15 min. The pellet was resuspended and washed twice in 1x Medium A (without glucose) and resuspended in 1x Medium A containing glucose and grown overnight at 37°C.

2.2.5 Penicillin Selection

Day 6

Overnight cultures in Medium A plus glucose were centrifuged and resuspended twice in 1x Medium A to remove glucose. The cells were resuspended in 25 mL Medium A plus succinate. Five 250 mL side-arm flasks were inoculated

with the appropriate washed cells to obtain a density of ~10 Klett units using a Klett Summerson colourimeter (for 100 Klett units, OD_{600} = 0.2 and corresponds to a density of 6.4 x 10^8 viable cells/mL (Goldie, Ph.D thesis, 1979)). The flasks were aerated for 4-6 h at 37°C. PenicillinG potassium was added at a concentration of 4000 U/mL. The flasks were further aerated for 2-3 h until cell debris was seen. Penicillin will lyse cells growing in succinate as a carbon source, whereas cells not growing on succinate are not lysed. The culture was centrifuged at 7710 x *g* for 15 min. The pellet was washed twice with Medium A to remove penicillinG. The cells were resuspended in Medium A plus glucose. The tubes were incubated at 37°C overnight. At this point, the cells that were dormant in the succinate medium will grow. Three rounds of penicillin selection were done. The cells were finally washed in 1 x Medium A and resuspended in Medium A + glucose and grown overnight at 37°C. Suc^- mutants from independent tubes A, B, C, D and E were diluted to 10^{-1} through 10^{-4} and spread on LB plates containing kanamycin and incubated at 37°C overnight. The plates were refrigerated for at least 4 h (this makes the Suc^- penotype more stringent) and small colonies were picked on glucose and succinate plates. Mutants with low Pck activity were further characterised for growth and Pck levels.

2.3 Pck enzyme assay

2.3.1 Processing the cell cultures

A 2 cm streak of mutant growth from a Medium A plus glucose plate was inoculated in 100 mL of LB in a 250 mL Erlenmeyer flask (The experiment was tried with a 1% inoculum and it produced similar results). Growth was monitored from 7.5 h to 17.5 h. Growth was measured in terms of Klett Units using a Klett Summerson colourimeter. Samples (5 mL) were withdrawn every 1.5 h and kept on ice. At the end of 17.5 h, the cells were treated with 500 µL of 10x CTAB buffer (0.1% CTAB, 3mM $MnCl_2$, 0.2M imidazole, pH 7.5) and vortexed vigorously. The samples were then centrifuged at 2910 x *g* for 15 min and the pellet was resuspended in 250 µL 1x CTAB buffer.

2.3.2 Pck assay

Pck was assayed by the measurement of ATP-dependent exchange of ^{14}C between $NaH^{14}CO_3$ and oxaloacetate (Utter and Kurahashi, 1954). This procedure is a modification of that of Wright and Sanwal (1969). The total volume of the reaction was 500 µL and contained 20 mM oxaloacetate, 20 mM $NaH^{14}Co_3$ (0.55 µCi), 1 mM ATP, 10 mM $MgCl_2$, 0.1 M Tris-Cl buffer (pH7.5) and 50 µL of CTAB-treated cell extract. Oxaloacetate was titrated to pH 7.0 using NaOH after first dissolving in 0.01N NaOH. The reaction mixture without oxaloacetate was dispensed in triplicate in one-dram vials and incubated at 30°C for 10 min. Reactions were started by

adding 50 µL of oxaloacetate for 10 min at 30°C and stopped by adding 500 µL of 0.1N H_2SO_4. One drop of octanol was added to each of the vials and compressed air was bubbled vigorously for 10 min to remove $^{14}CO_2$. The vials were filled with PCS scintillation fluid (Amersham Canada Ltd. Oakville, ON) and counted in a liquid scintillation counter. HG163 was used as the wild type control. Protein was assayed using the micro Lowry method (Geiger and Bessman, 1972) with 1 mg/mL BSA as a standard. This procedure was adapted from Lowry *et al.* (1951).

2.4 P1 Transduction

The procedure of P1 transduction used in this work is described by Miller (1972).

2.4.1 Preparation of P1 lysate:

One drop of the overnight culture of W3350 was inoculated in 10 mL LB containing 5 mM $CaCl_2$ and 0.1% glucose. The cells were aerated at 37°C until a slight biofringence was seen. One mL of exponentially growing cells was dispensed into 5 mL disposable culture tubes. Two drops of high titer P1 lysate was added, mixed and incubated in a 37°C water bath for 20 min without shaking. R top agar (4.5 mL kept at 45°C) was added to the tubes after incubation and this was immediately plated on a freshly made R plate and incubated face up overnight at 32°C or 37°C for 8 h. Top agar was scraped into 30 mL centrifuge tubes and the plates

were washed with 1 mL LB plus 5 mM $CaCl_2$ and 1% glucose. The top agar and the wash were collected into the same centrifuge tube. Five drops of chloroform were added and the tubes were vortexed vigorously for 30 seconds and kept at room temperature for 10 min. Cell debris was removed by centrifugation at 12,000 x *g* for 10 min and the supernatant transferred into sterile, screw-capped tubes. A few drops of chloroform were added to prevent bacterial growth in the lysates.

2.4.2 P1 transduction

Recipient bacteria were grown overnight in LB plus 5 mM $CaCl_2$ and 0.1% glucose, and centrifuged at 3,020 x *g* for 10 min. The cells were resuspended in MC buffer (0.1 M Mg SO_4, 5 mM $CaCl_2$) (Miller, 1972) and aerated for 15 min at 37°C. Dilutions of the P1 lysate (10^{-1} and 10^{-2}) were made in LB containing 5 mM $CaCl_2$ and 0.1% glucose.

Two drops of recipient bacteria in MC buffer were dispensed in eleven sterile 5 mL disposable culture tubes. Two drops of the undiluted phage were then added to the first four tubes, two drops of 10^{-1} dilution was added to the next three tubes, and two drops of 10^{-2} dilution was added to the following three tubes. The tubes were then incubated in a 37°C water bath for 20 min without shaking. At the end of the 20 min incubation, four drops of trisodium citrate was added to the tubes to stop phage adsorption and killing (Miller, 1972). Transductants were plated on appropriate selective medium plates. Plates were incubated for 24 h for LB plus antibiotics or 20-40 h for minimal medium plates plus carbon source.

2.5 ATP synthase enzyme activity measurement

2.5.1 Preparation of inside out vesicles

2 liters of Medium A containing 12.5 mL of 50% glucose and 25 mL of 20% casamino acids

↓

Inoculate with 1% overnight culture and grow overnight at 37°C under shaking conditions

↓

Centrifuge at 3020 x *g* for 15 min at 4°C

↓

supernatant (discard)

Pellet on ice

↓

Resuspend in 50 mM Tris, pH 7.8 + 5 mM $MgSO_4$ and centrifuge at 3020 x *g*/15 min at 4°C

↓

Pellet on ice

Supernatant (discard)

↓

Wash twice with ice cold 0.8% NaCl and centrifuge 3020 x *g*/15 min

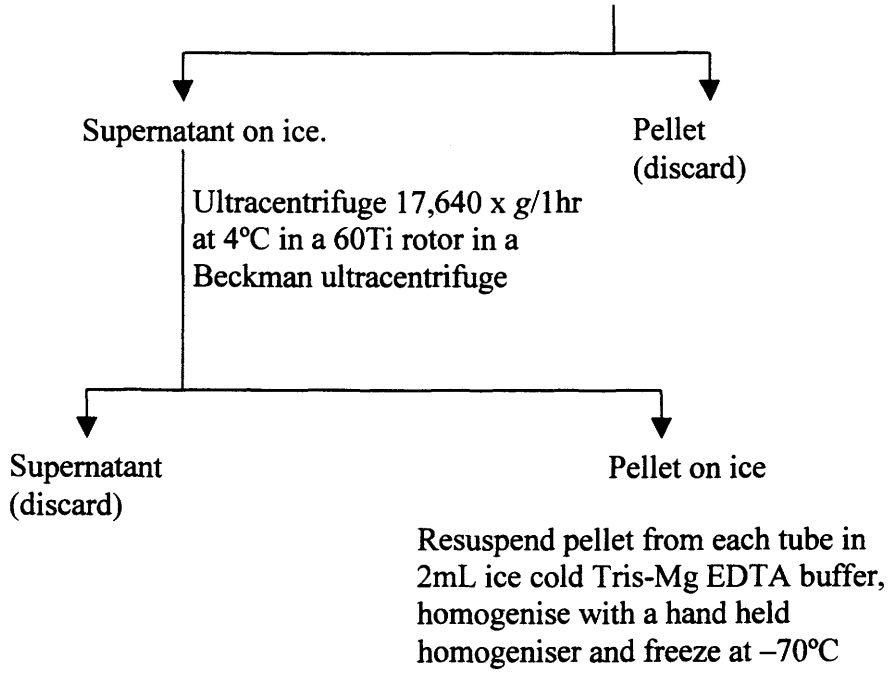
↓

Supernatant (discard)

Pellet resuspend in ice cold Tris-Mg EDTA buffer containing 1mM EDTA (1 g/10mL)

↓

French press at 1000 psi and centrifuge 12,000 x *g*/15 min



2.5.2 ATP synthase assays

The ATP synthase reaction was measured as ATP hydrolysis to yield inorganic phosphate (Pi). Pi was measured by the reaction of phosphate ions with ammonium molybdate to produce ammonium phosphomolybdate. Phosphomolybdate complexes were measured by converting them to molybdenum using ANSA reagent (Fiske and Subbarow, 1925).

Phosphate free (acid washed or disposable) tubes were used for the reactions. Three sets of reactions were carried out: Set A, with Mg^{+2}; set B, without Mg^{+2}; and set C, with Mg^{2+} and the inhibitor DCCD. Set A: Reaction tube contained 0.78 mL 50 mM Tris-sulphate buffer, pH 7.8 containing 1 mM EDTA, 75 µL 0.04 M $MgCl_2$ and 0.1 mL membranes (1:100 dilution). There was also an experimental control tube

with all the above ingredients. The tubes were incubated at 37°C for 2 min. The reaction was initiated by adding 50 µL of 0.1 M ATP and incubated for 10 min. The reaction was stopped after 10 min with 10% TCA. To the control tube 1 mL of 10% TCA was added followed by the 50 µL ATP. The samples were placed on ice. Set B, the experiment was repeated as Set A but omitting the $MgCl_2$ and adding 75 µL water instead. The control for the experiment was similar to the set A control, but without $MgCl_2$. Set C contained 0.79 mL 50 mM Tris-sulphate buffer, pH 7.8; 50 µL 0.04M $MgCl_2$; 0.1 mL membranes (1:100) and DCCD (10 µL of 10 mM in methanol). A control experiment with 10µl methanol instead of DCCD was also carried out. The tubes were incubated at 37°C for 5 min and the reactions were initiated by adding 50 µL of 0.1 M ATP. At the end of 10 min, the reactions were stopped by adding 1 mL of 10% TCA. The tubes were centrifuged at 2910 x *g* for 10 min in a Beckman high speed centrifuge.

2.5.2.1 Phosphate Estimation

The following tubes were set up for generating phosphate standards

	Additions (mL)					
Pi Standard (1µmole/mL)	0	0.2	0.4	0.6	0.8	1.0
Acid-Molybdate	1.0	1.0	1.0	1.0	1.0	1.0
H_2O	3.95	3.75	3.55	3.35	3.15	2.95

The tubes were mixed, 50 µL ANSA reagent was added and incubated for 10 min at room temperature. Absorbance was read at 660 nm and a graph was plotted of A_{660} *vs.* µmoles of Pi.

To determine Pi in each of the reaction tubes, sets of tubes were made containing 1 mL of 2.5% acid molybdate in 5 N H_2SO_4 and 3.45 mL of H_2O. Supernatant (500 µL) from tubes of set A, set B and set C and their controls were added to the appropriate tubes and mixed well. ANSA reagent (50 µL) was added to each tube and the absorbance was measured at 660 nm after 10 min.

Using the standard graph of A_{660} *vs* µmoles of Pi, the amount of Pi generated in each experimental tube was interpolated.

2.6 Molecular biology methods

2.6.1 Chromosomal DNA isolation

Overnight cultures were used for chromosomal DNA isolation as per the instruction manual in QIAamp Tissue kit (QIAgen, Valencia, CA). The DNA was quantitated by measuring the absorbance at 260 nm and used in PCR reactions.

2.6.2 PCR amplification

The mutant chromosomal DNA was amplified using primers specific to various regions of the *atp* operon (**Table 1**). The expected size of the fragment was *c.a.* 1.5 Kbp using primer pairs oHG112 and oHG113, oHG114 and oHG115,

oHG116 and oHG117, oHG118 and oHG119, oHG120 and oHG121. The PCR mixture consisted of 10 μL of PCR buffer (Amersham. Inc.), 8 μL of 2.5mM deoxynucleotides, 15 ng of DNA, 50 pmoles of primer, 1 μL of Taq polymerase and sterile water to a total volume of 100 μL. The PCR incubation cycle was as follows: step 1, denaturation at 94°C for 1 min; step 2, annealing at 55°C for 1 min; step 3, extension reaction at 72°C for 1 min. The cycle was repeated 35 times, followed by; step 4, 72°C for 5 min; step 5, hold at 4°C. Whole cell PCR was also carried out. Bacterial cells were taken from the plate with a sterile toothpick and added to the PCR reaction mixture. In that case, before adding Taq polymerase, denaturation was carried out at 95°C for 10 min and after one min at room temperature, Taq polymerase was added and the reaction was carried out as with using purified DNA.

PCR amplification of the site of insertion of mini Tn*10*-ATS in the mutants was carried out by a modified PCR method adapted from Lan *et al.* (1996) using a 25 nt primer specific to the IS10R end in the Tn*10*-ATS and REP1 or REP2 primers **(Table 1)**. The REP (Repetitive Extragenic Palindrome) element has been predicted to comprise up to 1% of the genome of *E. coli.* (Subramanian *et al.*, 1992). A ~35 bp REP palindrome sequence has been identified in the intercistronic regions. Chromosomal DNA at a concentration of 1.3 μg/μL was used for the PCR. To avoid background due to the random primer, three cycles of PCR were first done with the IS10R primer alone (Lan *et al.*, 1996). The second primer was then added and the PCR reaction was then carried out for 25 cycles (1 min each at 94°C, 50°C and 72°C).

Table 1. Oligonucleotide primers used in this study

Primer	Sequence[a]	Length	Amplified regions
oHG25	CTGATGAATCCCCTAATGATTTTGG	25 nt	IS10R
oHG26	TGGATACACATCTTGTCATATGATC	25 nt	IS10R
oHG27	CCAAAATCATTAGGGGATTCATCAG	25 nt	IS10R (bottom strand)
oHG28	GATCATATGACAAGATGTGTATCCA	25 nt	IS10R (bottom strand)
oHG29	CGATCAGCGAATCGCCGGTATCGAC	25 nt	*hybF*
oHG30	CCTGGCCTGGAACGCCAATACACAT	25 nt	*hybF* (bottom strand)
oHG31	CCGTCCGTTTTAGTTTTGAAATTGT	25 nt	*hybF*
oHG32	ATATCGTCTATAAACCCGCCCAGGC	25 nt	*hybF* (bottom strand)
oHG112	TGCTGTGCGCGAACATGCGC	20 nt	*atp I*
oHG113	GCCATGTACAGCAGATCCAT	20 nt	*atp E* (bottom strand)
oHG114	ATGGATCTGCTGTACATGGC	20 nt	*atp B-E* interface
oHG115	GGGAGATCATTTCACCCTGC	20 nt	*atpA* (bottom strand)
oHG116	GCAGGGTGAAATGATCTCCC	20 nt	*atp H-A* interface
oHG117	TGGAAGCGGCGACCATCTCC	20 nt	*atp G* (bottom strand)
oHG118	GGAGATGGTCGCCGCTTCCA	20 nt	*atp A-G* interface
oHG119	CCGGTCAGAGCAACGCGCAG	20 nt	*atp D* (bottom strand)
oHG120	CTGCGCGTTGCTCTGACCGG	20 nt	*atp D*
oHG121	GATTATCCCCGCTGACGAAA	20 nt	3' end of *atp* operon (bottom strand)
oHG138	CAGCGACATCGTGGATAAAC	20 nt	*atp F*
oHG139	GGTCGTCTTAACGCGCTCCC	20 nt	*atp H* (bottom strand)
oHG140	GGGATAACCCTTCCCTGTCC	20 nt	*atp G*
oHG141	CCGCCCGTATGGTGGCGATG	20 nt	*atp G* (bottom strand)
oHG142	GCAGCAGCTCGGCGGCGGTA	20 nt	*atp G*

(Over)

oHG143	TAAAGTTGGTCTGTTCGGTG	20 nt	*atp G* (bottom strand)
oHG144	TCTACCCGGCCGTTGACCCG	20 nt	*atp D*
oHG145	TCATGGAAGGCGAATACGAT	20 nt	*atp D* (bottom strand)
oHG146	CTATCTGTCTGGCGGCATTC	20 nt	*atp C*
oHG147	CTGAATAGCGTTCACATAGA	20 nt	*atpC*
oHG148	TCTTAAATCCTCTACGAAAT	20 nt	*atp G* (bottom strand)
oHG149	CGCCTTTCATGTCGACCGGT	20 nt	*atp G* (bottom strand)
REP1	CGCGGATCCIIIICGICGICATCIGGC	27 nt	REP sequences
REP2	CGCGGATCCICGICTTATCIGGCCTAC	27 nt	REP sequences

[a] the oligonucleotide sequences are listed 5' → 3'

The DNA was electrophoresed on an agarose gel and the DNA fragments were isolated from the gel as described below, and sent for sequencing at the DNA services, Plant Biotechnology Institute (PBI), National Research Council, Saskatoon, SK.

2.6.3 DNA electrophoresis

DNA samples mixed with agarose gel loading buffer (36% urea, 0.5% bromophenol blue, 0.5% xylene cyanol green) were electrophoresed on 1.7% agarose gels with 0.1 μg/mL ethidium bromide. Plasmid pBR322 digested with *HinfI* was used as the DNA molecular weight standard. The DNA fragments were separated at a constant voltage of 75 volts in 1x TBE (0.09 M Tris, 0.5 mM EDTA (pH 8.0), 0.09 M H_3BO_3) buffer, pH 8.3 containing 0.1 μg/mL of ethidium bromide. DNA fragments were visualised with a transilluminator with 310 nm ultraviolet light and photographed using Polaroid type 55 film (Polaroid Corporation, Cambridge, MA) and a Kodak No.1 Wratten filter.

2.6.4 Isolation of DNA fragments from agarose gels

The QIAquick spin (QIAgen, Valencia, CA) method was used for isolating DNA fragments from agarose gels. This involved electrophoresing the PCR samples on 1% agarose gels. DNA bands were cut from the agarose gel using a sharp razor blade. The DNA was extracted from the gel band in a microfuge, using the protocol described in the QIAgen QIAquick spin handbook.

2.6.5 DNA quantitation and sequencing

The extracted DNA was electrophoresed on 1% agarose mini gels using DNA quantitation standards (Life Technologies, Grand Island, NY). Six µL of the sample DNA was mixed with 2 µL of the loading buffer and was electrophoresed with standards to quantitate the amount of DNA to be sent for sequencing. Sequencing was performed at PBI using the Big dye terminator sequencing reaction method using a 373 DNA sequencer (Applied Biosystems, FosterCity, CA).

2.6.6 Plasmid DNA isolation

2.6.6.1 Mini-Preps

The plasmid amplification and isolation method was carried out as described in Maniatis *et al.* (1982). Cells were inoculated in 5 mL LB containing ampicillin and grown overnight at 37°C. The cells were centrifuged at 16,200 x *g* for 1 min and the pellet was resuspended in 350 µL of Bacterial Plasmid Miniprep solution (8% sucrose, 0.5% TritonX-100, 50 mM EDTA pH8.0, 10 mM Tris-HCl pH 8.0) and vortexed at top speed for 10 seconds. After adding 20 µL of lysozyme (10 mg/mL in 10 mM Tris-HCl pH 8.0, Sigma, St. Louis, MO), the tube was held in a boiling water bath for 50 seconds and centrifuged at 16,200 x *g* for 10 min. The bacterial pellets were discarded and 15 µL of 5 M NaCl and 800 µL of 95% ethanol was added and incubated at –20°C for at least 30 min to precipitate the DNA. The tubes were centrifuged at 16,200 x *g* for 15 min and then the pellet was air dried and resuspended

in 30 μL of distilled water. Plasmid mini preps were also done using the QIAprep spin mini prep kit (QIAgen, Valencia, CA).

2.6.6.2 Maxi-Prep

Five mL LB containing 100 μg/mL ampicillin was inoculated with one colony and grown overnight. This was used for inoculating 300 mL LB containing ticarcillin (100 μg/mL) and incubated overnight. The cells were centrifuged in a Beckman GSA rotor at 3020 x *g* for 10 min and the pellet was used for plasmid isolation as per the QIAgen Maxi protocol as described in the QIAfilter Plasmid Maxi kit. The various steps that were employed were alkaline lysis, filteration and ion exchange. Plasmids were air dried and resuspended in 200 μL of TE buffer.

2.6.7 Electroporation

2.6.7.1 Preparation of competent cells for electroporation

One liter of LB was inoculated with 1% overnight inoculum and grown to mid-log phase with shaking at 37°C. The *E. coli* cells for electroporation were prepared as suggested in the BioRad *E. coli* Pulser manual. The culture was centrifuged at 3020 x *g* for 15 min and the pellet was resuspended gently in 1 liter of ice cold 10% glycerol and centrifuged again for 5 min at 3020 x *g*. The pellet was washed four times in 10% glycerol and each time the volume of glycerol was reduced by half. The last pellet was resuspended in 4 mL of 10% glycerol. Aliquots of

competent cells (25 µL) were dispensed into sterile microfuge tubes, frozen immediately in a –70°C methanol bath and stored at –70°C.

2.6.7.2 Electroporation and Plating

Competent cells were thawed on ice for 10 min. Two µL of the appropriate plasmid was added to the 25 µL of competent cells and kept on ice for 1 min. The mixture was transferred into a chilled sterile electroporation cuvette (Bio-Rad laboratories, Inc. Hercules, CA), and the cells were exposed to a voltage pulse of 1.8 kV using the *E. coli* Pulser (Bio-Rad laboratories, Inc. Hercules, CA). After electroporation, 400 µL of SOC medium (American Biorganics, Inc. Niagara falls, NY) was added immediately to the cuvette, transferred into sterile 1.5 mL microfuge tubes and placed on a shaker at 37°C for one hour. Following the incubation, 100 µL of the mixture was plated on appropriate antibiotic containing plates and incubated at 37°C for 24 h.

2.7 Bacterial strains and plasmids used in this study

2.7.1 Bacterial strains

Table 2. Bacterial strains used in this study

Strain	Genotype	Phenotype	Reference
CAG18431	λ⁻, *rph-1*, *ilvD500*::Tn*10*	Ilv⁻, Tet^R	Nichols *et al.* (1998)
CAG18491	λ⁻, *rph-1*, *metE-3079*::Tn*10*	Met⁻, Tet^R	Nichols *et al.* (1998)
CAG18492	λ⁻, *rph-1*, *Zic-4901*::Tn*10*	Tet^R	Singer *et al.* (1998)
CAG18495	λ⁻, *rph-1*, *Zih-35*::Tn*10*	Tet^R	Singer *et al.* (1998)
CAG18496	λ⁻, *rph-1*, *fad-751*::Tn*10*	Tet^R	Nichols *et al.* (1998)
CAG18499	λ⁻, *rph-1*, *Zid-501*::Tn*10*	Tet^R	Singer *et al.* (1998)
CAG18501	λ⁻, *rph-1*, *rbsD296*::Tn*10*	Rbs⁻, Tet^R	Singer *et al.* (1998)
DK8	*ilv*::Tn*10*, Δ*atpB-C*	Tet^R, Ilv⁻	Klionsky *et al.* (1984)
GC122	*rpoS*::Tn*10*	Tet^R	Schellhorn *et al.* (1992)
HG29	HfrH, *pps, leu, arg, rpsL, asd-1*	Pyr⁻, Leu⁻, Arg⁻, Asd⁻	Goldie, Ph.D. Thesis
HG87	*recA56*, *srl*::Tn*10*, *pps*, *his*, *pyr*, *tyr*, *argG*::Tn*5*,	Tet^R, Pyr⁻, His⁻, Tyr⁻, Arg⁻	Goldie, unpublished

(Over)

HG137	*pck13*::*mud1*, *pps*, Δ*lacU*169	Amp^R, Pyr^-	Goldie, 1984
HG163	*pps*	Lac^-, Pyr^-, Su^o	Chan, Y., M.Sc. Thesis
HG193	*pckA*::Tn*10*, *pps*	Suc^-, Pyr^-, Tet^R, Su^o	Goldie, unpublished
HG194	*pckA*::Tn*10*, *pps*	Suc^-, Pyr^-, Tet^R, Su^o	Goldie, unpublished
MM294A	*pro*, *thi*-1, *endA1*, *hsdR17*, *SupE*44	Pro^-	Backman *et al.* (1976)
W3350	*lac*	Lac^-, Su^o	Adhya *et al.* (1968)
HG201	Kan^R, *pps*,	Kan^R, Pyr^-, Suc^-, Su^o	This work
1B17	Kan^R, *pps*,	Kan^R, Pyr^-, Suc^-, Su^o	This work
HG202	Kan^R, *pps*,	Kan^R, Pyr^-, Suc^-, Su^o	This work
HG203	Kan^R, *pps*, *atpG*,	Kan^R, Pyr^-, Suc^-, Su^o	This work
HG204	Kan^R, *pps*	Kan^R, Pyr^-, Suc^-, Su^o	This work
HG205	Kan^R, *pps*, *atpG*,	Kan^R, Pyr^-, Suc^-, Su^o	This work
HG206	Kan^R, *pps*, *atpG*	Kan^R, Pyr^-, Suc^-, Su^o	This work

2.7.2 Plasmids

Table 3. Plasmids used in this study

Plasmid	Drug Resistance	Region expressed	Reference
pACWU1.2 (R376A)	Amp^R	*atpA*	Kuo *et al.* (1998)
pACWU1.2 (K155Q)	Amp^R	*atpD*	Kuo *et al.* (1998)
pACYC177	Kan^R, Amp^R	Vector	Chang and Cohen, (1978)
pACYC184	Cam^R, Tet^R	Vector	Chang and Cohen, (1978)
pAN45	Cam^R, Tet^R	*atp B, E, A, G, D, C*	Downie *et al.* (1980)
pBWG15	Tet^R	*atpG*	Shin *et al.* (1992)
pBWU1.2	Amp^R	*atpB-C*	N/A
pBWU1.3	Amp^R	*atpB-C*	Moriyama *et al.* (1991)
pDJK20	Tet^R	*atpB*, *atpE*, *atpF*	N/A
pDJK35	Cam^R	*atpH, A, G, D, C*	Klionsky and Simoni, (1985)
pDM8	Amp^R	*atpF*	Mclachlin and Dunn, (1997)

(Over)

pJC.1	Amp^R	*atpH*	Dunn and Chandler, (1998)
pRPG51	Cam^R	*atpA*, *atpH*	Gunsalus *et al.* (1992)
pSD13	Amp^R	*atpD*, *C*	N/A
pSD14	Amp^R	*atpE*, *F*, *D*, *C*	N/A
SBV11	Cam^R	*atpB*	N/A

CHAPTER THREE

3. Results

3.1 Generation of Mutants by mini Tn*10*-ATS transposition

Mini Tn*10*-ATS (Kan^R) was obtained on a λ NK1316 (P_{am} 80λ::miniTn*10*) vector. Due to the P_{am} mutation, λNK1316 can only grow in a strain having an amber suppressor. A phage titer of $5x10^{11}$ PFU/ml was obtained using *E. coli* strain MM294A (*glnV44* amber suppressor). The reversion frequency and the transposition frequency were determined using HG163, which is *pps*$^-$ and Su^o (has no amber suppressor); therefore, only P^+ revertants of λNK1316 can form plaques on this strain. The λ infected HG163 cells were plated on LB containing kanamycin and the transposition frequency was calculated as follows:

Transposition Frequency = No. Kan^R Transpositions/No. PFU's on MM294A

$= 5.2x10^{-3}$

Reversion Frequency = No Revertants (plaques in HG163)/No. PFU's on MM294A

$= 5.8x10^{-9}$

3.1.1 Transposon mutagenesis of Su° *pps* strain HG163

The dilution (10^{-1}) of phage λNK1316 that produced the maximum number of transpositions was used for mutagenesis of HG163 cells. Kanamycin plates were plated with 0.1 mL of appropriate dilution of the cells after infection and used for further mutant screening.

3.1.2 Penicillin selection

The cells that were infected with λNK1316 were grown on glucose in five independent tubes overnight. The cells were resuspended in Medium A + succinate and were subjected to penicillin selection. Penicillin will lyse all the cells growing in succinate as carbon source whereas the cells that do not grow on succinate will not be lysed. Suc$^-$ cells that survived after the penicillin selection may be *sdh*$^-$, *fum*$^-$, *dct*$^-$ or *pck*$^-$ (lacking succinate dehydrogenase, fumarase or dicarboxylate transport or Pck activity) or the Suc$^-$ could be due to other mutations. About 10% of the Suc$^-$ cells are Pck$^-$ (Goldie and Sanwal, 1980a). Some Penicillin^R cells could have been also selected during mutagenesis.

3.1.3 Screening of the mutants

A total of 2,500 Kan^R colonies from five independent mutagenesis tubes were isolated on LB containing kanamycin and screened on medium A plates containing succinate, glucose or pyruvate as carbon source. *pck*$^-$ *pps*$^-$ mutants will not grow on succinate (Suc$^-$ phenotype) or pyruvate (Pyr$^-$ phenotype) (Goldie and Sanwal, 1980a).

Seventy-two Suc$^-$, Pyr$^-$, Kan^R isolates were obtained. These were further purified and rechecked. Pck assays of Suc$^-$ isolates grown to stationary phase were carried out to identify the ones with decreased Pck activity (**Table 4**). The percentage Pck specific activity in the Suc$^-$ mutants was calculated, setting the value for HG163 (*pck*$^+$, *pps*$^-$) at 100%. Mutants with decreased specific activity were chosen for further studies (**Table 4**). Mutants HG201, HG202, HG203, HG204, HG205, HG206 and HG207 were chosen as potential isolates for studying regulation of Pck. The basis of choosing the mutants was as follows: there were five mutants in mutagenesis tubes C and D and all of them were used for further studies. Two mutants with low and intermediate Pck specific activities from mutagenesis tube B were also short listed.

Table 4. Screening of the mutants for Pck enzyme activity

Mutant number Suc⁻ Kan^R	**% Specific activity**	**Mutant number Suc⁻ Kan^R**	**% Specific activity**	**Mutant number Suc⁻ Kan^R**	**% Specific activity**
1B1	41	1B22	25	1B45	27
1B2	38	1B23	32	1B46	26
1B3	14	1B25	22	1B47	28
1B4	33	1B27	36	1B48	28
1B5	31	1B28	27	1B49	25
1B6	34	1B29	27	1B50	28
1B7	33	1B30	33	1B51	81
1B8	35	1B31	28	1B52	13
1B9	31	1B32	33	1B53	43
1B10	41	1B33	13	1B54	29
1B11	48	1B34	15	1B57	24
1B12	44	1B35	12	1B58	30
HG201 (1B13)	**30**	1B36	21	1B59	26
1B14	20	1B37	15	1B61	27
1B15	1	1B38	11	1B62	25
1B16	41	1B39	16	1B63	25
1B17	16	1B40	18	**HG203 (1C68)**	**7**
1B18	20	1B41	26	**HG204 (1C69)**	**4**
HG202 (1B19)	**19**	1B42	26	**HG205 (1D70)**	**5**
1B20	31	1B43	25	**HG206 (1D71)**	**8**
1B21	27	1B44	27	**HG207 (1D72)**	**17**

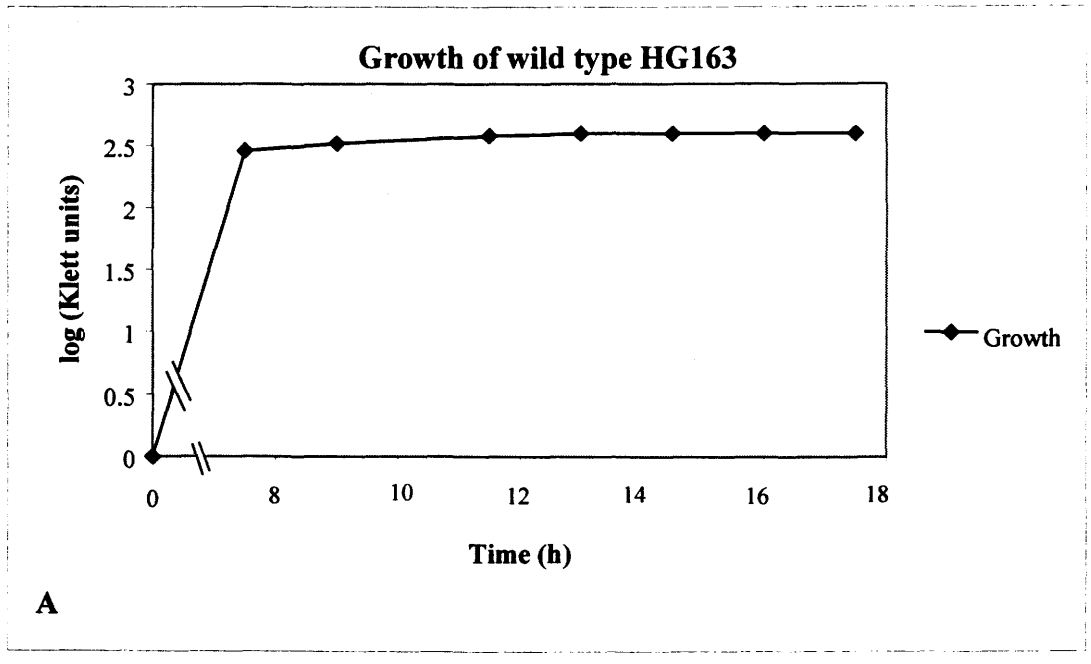
The percent specific activities of the Suc$^-$ mutants are calculated keeping the wild type HG163 Pck specific activity at 100%.

Samples were CTAB treated at early stationary phase and assayed for Pck. The activity was normalised for protein. The mutants used for further studies are indicated in bold letters. Mutants with different letters (B, C, D) are independent isolates.

3.1.4 Growth and Pck specific activity of the mutants

On LB medium, growth (log Klett units) and specific activity were plotted as a function of time for the mutants, with HG163 as the wild type control (**Figure 5).** Maximum Pck specific activity was observed at around 13 h (early stationary phase). After 14.5 h, the growth (Klett units) was constant. In subsequent work, all observations involving growth and specific activity were taken between 7.5-17.5 h after inoculation.

Figure 6A shows the growth of the wild type and the Suc^- mutants, HG203, HG204, HG205, HG206 and HG207 in LB medium as a function of time. The growth yield of mutants was generally lower than the wild type, but one mutant, HG206 showed growth comparable to the wild type. **Figure 6B** shows the comparison of the Pck specific activity as a function of time in the mutants and the wild type. The Pck specific activities of the mutants HG203, HG204, HG205, HG206 and HG207 were only about 10% that of the wild type.



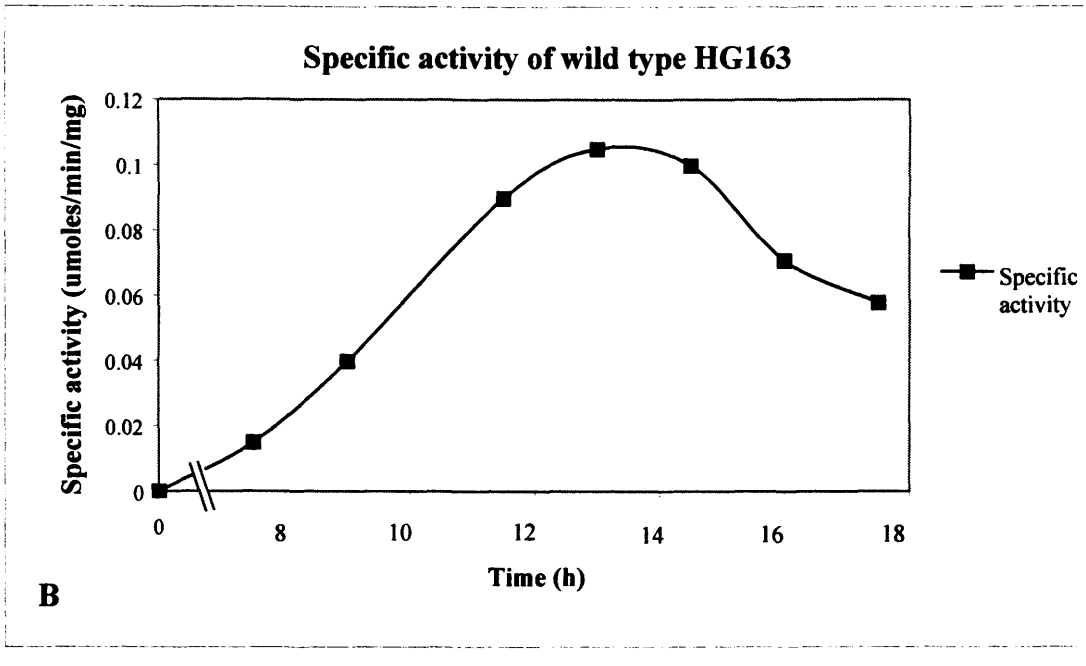
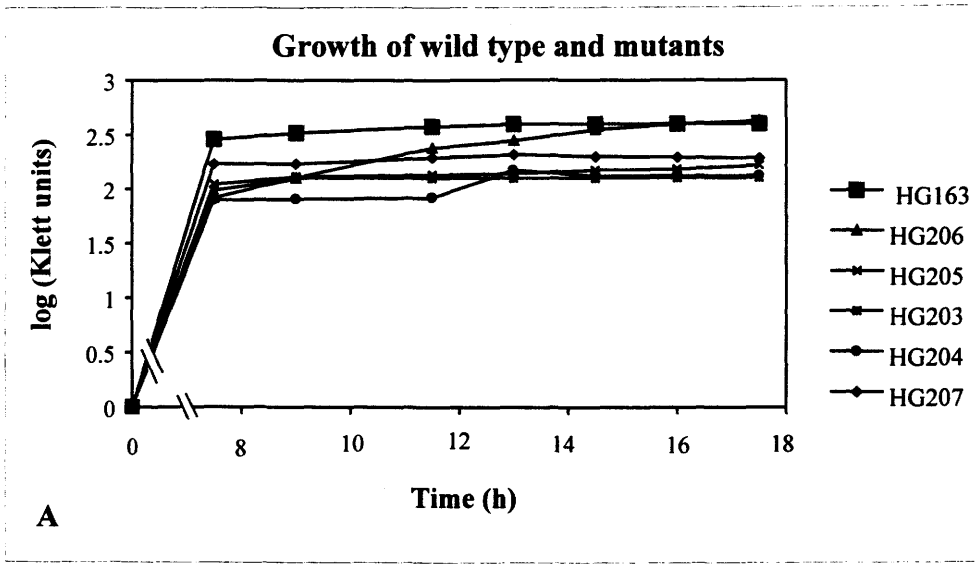


Figure 5. Growth and Pck specific activity of wild type HG163.

A. The growth and Pck specific activity of HG163 were monitored from 7.5 to 17.5 h after inoculation. **B.** Pck assays were done on CTAB treated cells (section 2.3.1). The experiment was repeated three times and the figure shows representative data.



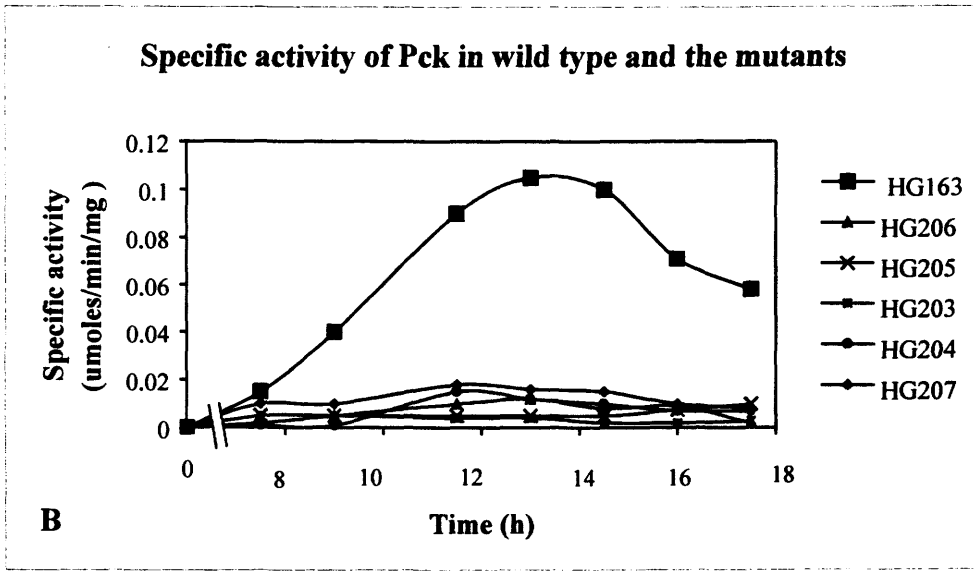


Figure 6. Growth and specific activity of Pck in wild type and in mutants HG206, HG205, HG203, HG204 and HG207.

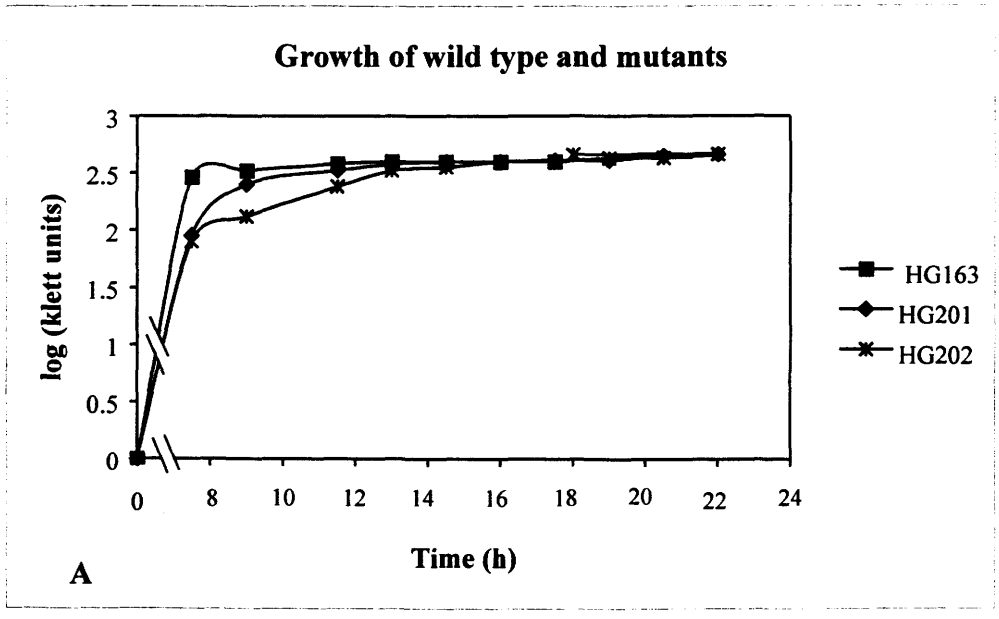
A. Growth yield of the wild type and the mutants from 7.5 to 17.5 h. **B.** Pck specific activity of the wild type and the mutants (CTAB treated cells).

Figure 7A shows the growth yield of the wild type and mutants HG201 and HG202. The growth is comparable to the wild type in these mutants. **Figure 7B** shows the Pck specific activity of mutant HG201 and HG202 and wild type. In these mutants, the specific activity increased up to 20 h and then started declining. These mutants, HG201 and HG202 seemed to differ in phenotype from the other mutants HG203, HG204, HG205, HG206 and HG207. In mutants HG201 and HG202 there seems to be delayed Pck expression versus decreased Pck expression seen in mutants HG203, HG204, HG205, HG206 and HG207. Two other mutants 1B15 and 1B17 had results similar to HG201.

3.1.5 Testing genetic linkage of the presumptive transposon insertion mutations

The next aspect to be addressed was to determine the genetic linkage of Kan^R to *pckA*. The intent was to obtain mutants with mutations not in *pckA*, but in unlinked genes, which may regulate the activity of *pckA*.

The mutants were mapped with respect to the *asd* locus by P1 transduction. *pckA* is linked 29-30% with *asd* (Goldie and Sanwal, 1980a). Transduction was done using HG49 (pck^+ asd^-) as the donor and the mutants, HG201, HG202, HG203, HG204, HG205, HG206 and HG207, as recipients. The transductants were selected on minimal medium containing succinate as a carbon source and the ASD mixture (0.01% each of threonine, methionine, lysine and diamino pimelic acid (DAP)) to isolate Suc^+ transductants. The asd^- cells will not grow in the absence of the ASD mixture. The transductants were further purified on minimal medium containing



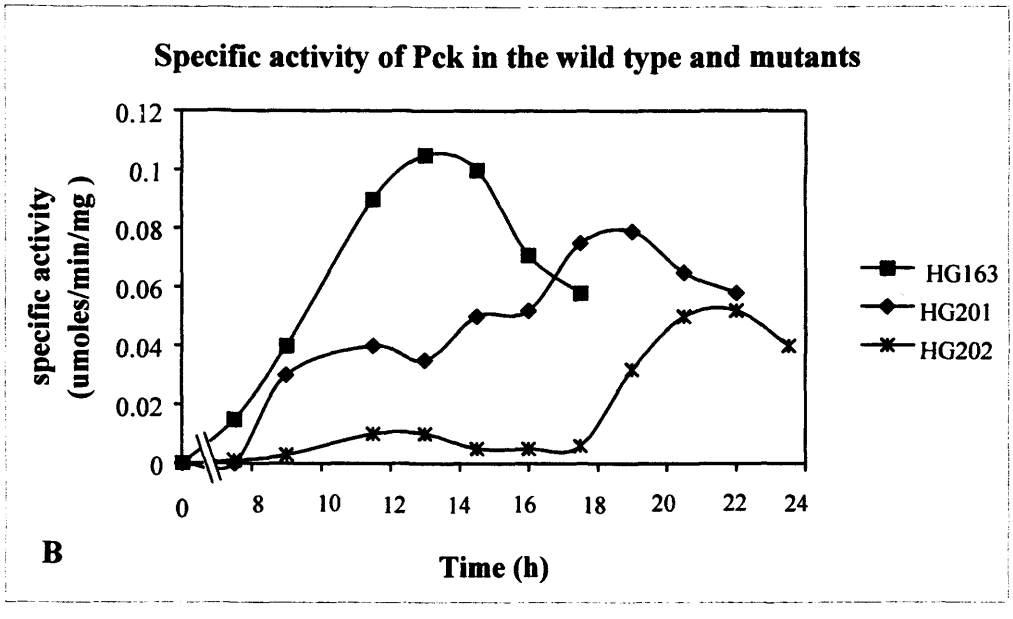


Figure 7. Growth and specific activity of Pck in wild type HG163 and in mutants HG201 and HG202.

A. Growth yield of the wild type and the mutants from 7.5 to 22 h. Growth was measured in log Klett units. **B.** Pck assays were done on CTAB treated cells. The experiment was repeated three times and the figure shows representative data.

Succinate + ASD to avoid possible abortive transductants. The transductants were screened on minimal medium plates containing (i) succinate + ASD, (ii) glucose + ASD, (iii) glucose, (iv) pyruvate + ASD, and (v) LB plates containing kanamycin. **(Table 5)**

In mutants HG203, HG204, HG205, HG206, all the transductants were Suc^+ and kanamycin sensitive, which means that they had lost the Kan^R mutation. This indicates that in these mutants, the Kan^R is linked to the Suc^- phenotype. These mutations are not linked to *asd*, since the transductants are *asd*$^+$. None of the transductants were Kan^R or *asd* $^-$. In transduction using mutants HG201, HG202 and HG207 as recipients, all Kan^R, transductants were Suc^+ *asd*$^+$, which indicates that the Kan^R in these mutants is not linked to the Suc^- phenotype. Mutants HG206 and HG207 are from the same mutagenesis tube D. In transduction using mutant HG206 as recipient, all the Kan^R transductants were linked to Suc^- and not linked to *asd*, since only Suc^+ Kan^S *asd*$^+$ transductants were obtained. In mutant HG207 as P1 recipient, Suc^- was not linked to Kan^R since only Suc^+ Kan^R *asd*$^+$ transductants were obtained. The Kan^R mutations in strains HG201 and HG205 represent independent isolates of mutations, which appear to affect *pckA* expression. From transductions using strains HG201, HG202 and HG207 as P1 recipients, there was no evidence of linkage of Kan^R and Suc^-. Since all the transductants are Kan^R and Suc^+, there may be an additional mutation in the mutants that is independent of a transposition event. Therefore the mutants HG201, HG202 and HG207, were not studied further.

Table 5. Determination of linkage of Kan^R to *pckA*

Mutant Number	Number of Suc^+ selected	Kan^R *asd*$^+$	Kan^R *asd*$^-$	Kan^S *asd*$^+$	Kan^S *asd*$^-$	% linkage of Kan^R to Suc^-	% linkage of Suc^+ to *asd*$^-$
HG201	200	200	0	0	0	< 0.5%	NA
HG202	200	200	0	0	0	< 0.5%	NA
HG203	258	0	0	258	0	100	< 0.5%
HG204	240	0	0	240	0	100	< 0.5%
HG205	221	0	0	221	0	100	< 0.5%
HG206	258	0	0	258	0	100	< 0.5%
HG207	300	300	0	0	0	< 0.5%	NA

NA = Not Applicable

The donor HG49 is Kan^S Suc^+ *asd*$^-$. Transductants were selected on Succinate and DAP containing minimal medium plates. Linkage of Kan^R with Suc^- and Suc^+ with *asd* is indicated. *pckA* is linked 30% to *asd*.

Linkage experiments resulted in four mutants HG203, HG204, HG205 and HG206 where the Kan^R was not linked to *asd* and therefore not linked to *pckA*. The transduction experiments show that in these mutants the Kan^R was 100% linked to Suc^-. These mutations possibly affect expression of *pckA*, but do not map at the *pckA* locus and therefore could be regulatory mutants of *pckA*.

P1 transduction was done to transduce the Kan^R Suc^- from the mutants HG203, HG204, HG205 and HG206 into HG163 (wild type). After repeated efforts, Kan^R Suc^- transductants were obtained at very low frequency. The Kan^R Suc^- transductants from HG206 mutant exhibited poor growth. By contrast, mutant HG206 exhibited growth yield comparable to the wild type, which could indicate the presence of a second mutation in the mutant, which suppresses the Kan^R linked poor growth phenotype seen in mutant HG206.

3.1.6 Effect of slow growth on Pck activity

The growth experiments with mutants showed that some mutants had low growth yield and low Pck specific activity. In some cases, the Pck specific activity was only 10% that of the wild type. Is slow growth of the mutants responsible for low Pck specific activity or is the low specific activity responsible for slow growth? To address these questions, growth and Pck specific activity were investigated in HG193 and HG194 (both *pckA*::Tn*10* insertions) that were available in the lab. In the insertion mutant, if low Pck activity was the result of slow growth, the growth of the mutant should be low in comparison to the wild type since the Pck specific activity is

undetectable. **Figure 8A** shows the comparison of growth between wild type HG163, HG193 (*pckA*::Tn*10*) and HG194 (*pckA*::Tn*10*). There is no difference in growth yield between the wild type and the *pckA*::Tn*10* mutants. **Figure 8B** shows the difference in Pck specific activity between HG163 and *pckA*::Tn*10* insertion strains HG193 and HG194. The Pck specific activity in the insertion mutants are undetectable but this did not affect the growth of the *pckA*::Tn*10* insertion mutants.

To confirm if the low Pck activity was responsible for slow growth or vice versa, growth and Pck specific activity were determined in HG87, a *recA* mutant available in the lab. The *recA* mutants have slow growth and lower growth yield, probably due to a defect in recombination and DNA repair pathway (Michel *et al.*, 1997), which could affect Pck specific activity.

Figure 9A shows the comparison of growth between the wild type and *recA*$^-$ strain. The growth yield of the *recA*$^-$ strain is lower than the wild type. **Figure 9B** shows the comparison of Pck specific activities between the wild type and the *recA* mutant. On comparing the growth of the wild type and the mutants with the growth of the wild type and *recA* strain, the *recA* strain grew slightly slower than the mutants. But on comparing the Pck specific activity between the *recA* strain and our mutants, the *recA* strain had much higher Pck specific activity than the mutant although the growth of the *recA* strain was slower than the mutants. This indicates that in a *recA* strain though the growth yield is lower than the wild type, the Pck specific activity is not significantly affected, which is an indication that Pck specific activity is not

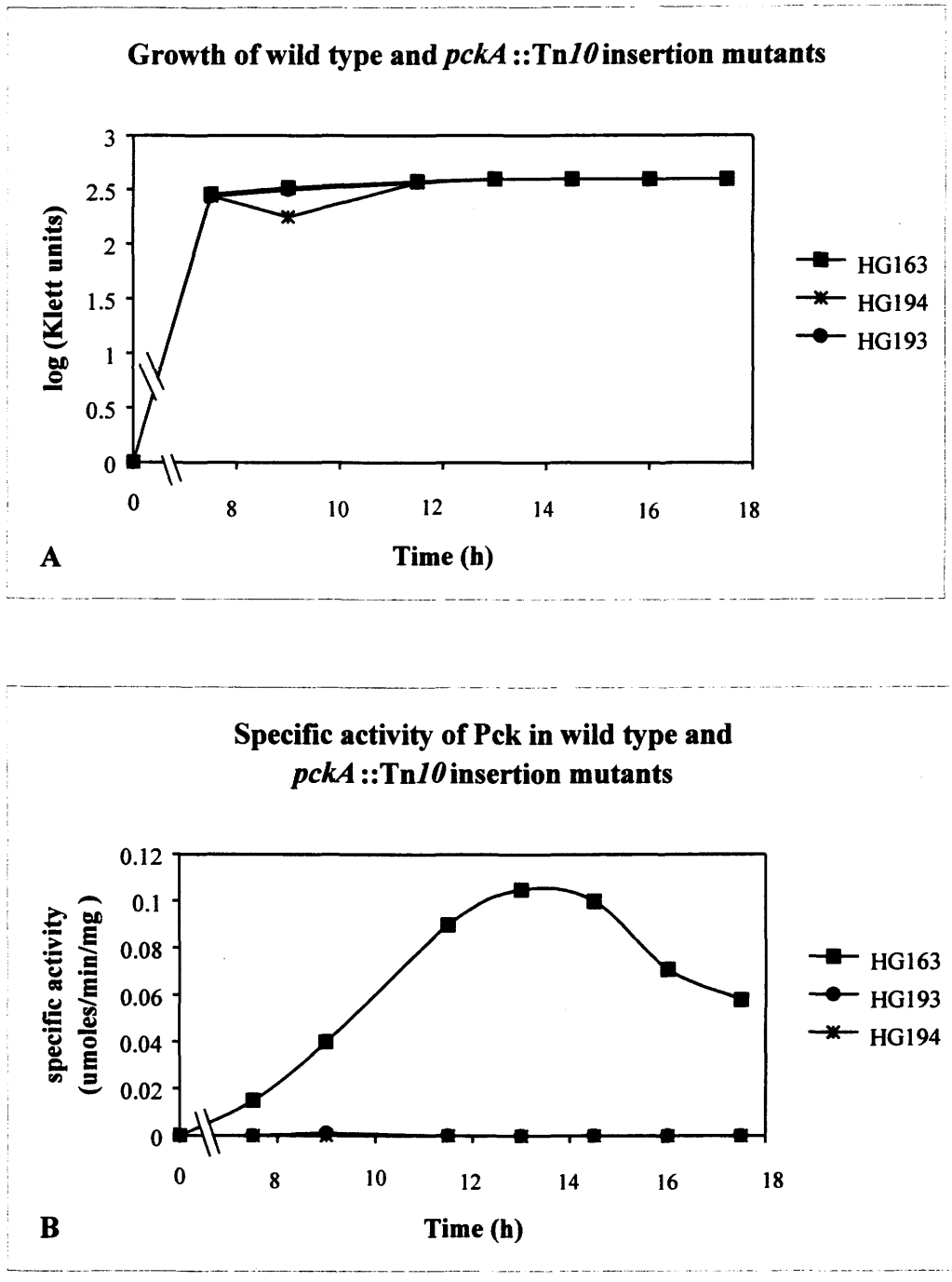
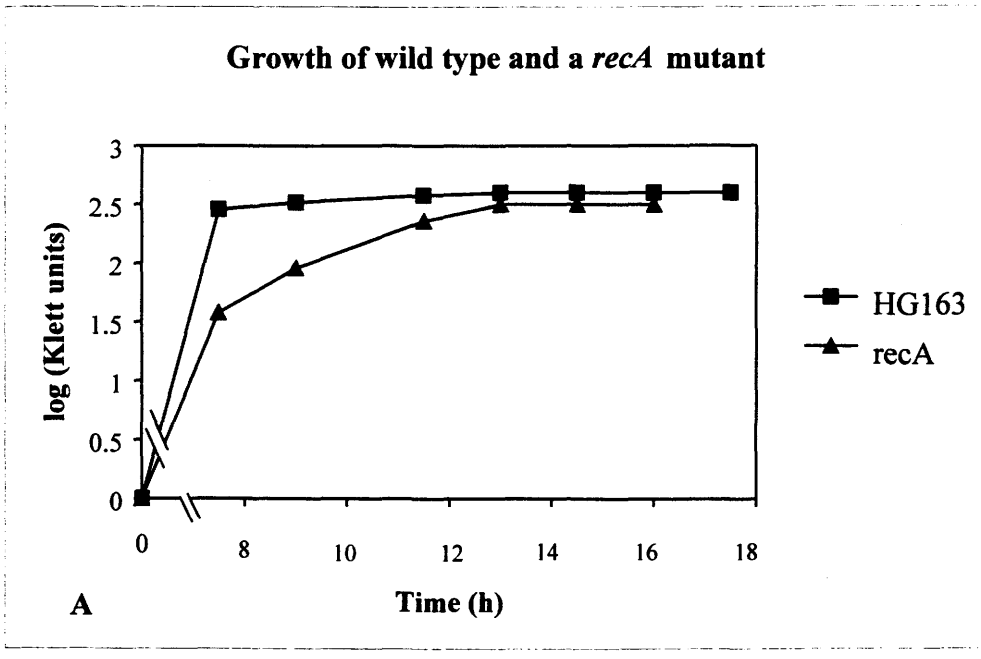


Figure 8. Growth and Pck specific activity of HG163 and HG193 (*pckA*::Tn*10*) and HG194 (*pckA*::Tn*10*)
A. Growth yield of the wild type HG163, HG193 (*pckA*::Tn*10*) and HG194 (*pckA*::Tn*10*). **B.** Pck assays were done on CTAB treated cells.



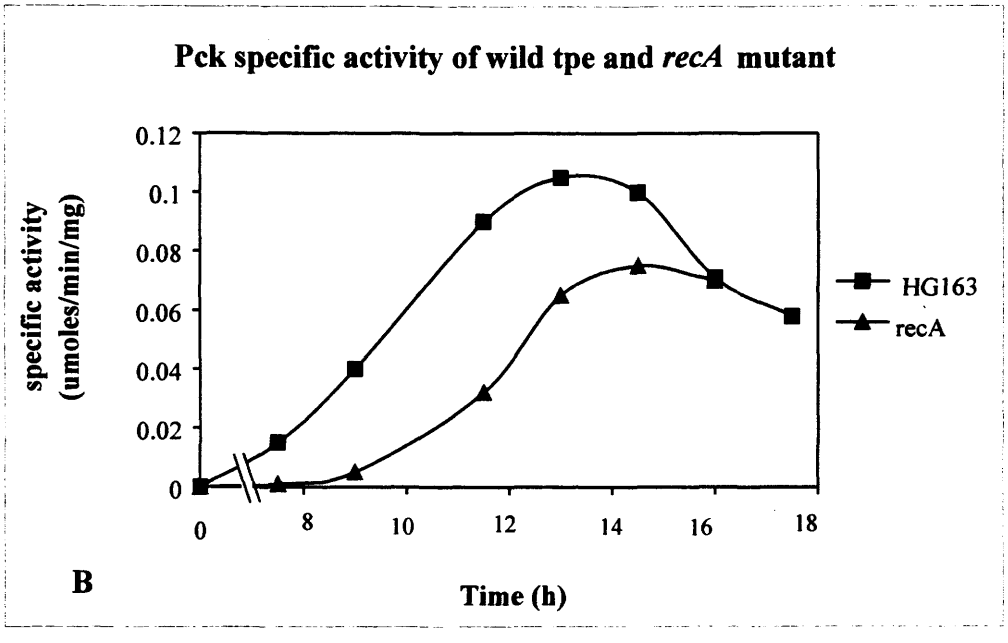


Figure 9. Growth and Pck specific activity of the wild type HG163 and a *recA* mutant.

A. Growth yield of the wild type and the *recA* mutant from 7.5 to 17.5 h. **B.** Pck assays were done on CTAB treated cells.

growth yield dependent. Therefore, the Pck$^-$ phenotypes of the mutants cannot simply be explained by their growth phenotypes. The mutations in mutants HG203, HG204, HG205 and HG206 affect the expression of *pckA* and in turn may have a pleiotropic effect.

3.1.7 Mutations are not in *cya* or *crp*

Transcription of *pckA* is subject to catabolite repression when cells are grown on media containing carbohydrates. Also, cAMP and cAMP receptor protein (Crp) are required for expression of Pck and for the transcription of *pck-lacZ* fusions (Goldie and Sanwal, 1980a, Goldie, 1984). The *pckA* gene has been sequenced and the transcriptional start site mapped (Medina *et al.*, 1990). Gel shift assays indicate the possibility of three Crp binding sites near the *pckA* promoter. Two footprints have been obtained for Crp near the *pckA* promoter (Goldie, unpublished results).

From all the known facts about catabolite repression in stationary phase and experiments with the *pck-lacZ* fusions regulated by cAMP and Crp, there was a possibility that the isolates could have mutations in *cya* or *crp*. To test this possibility, the mutants were picked on to MacConkey's plates containing maltose and arabinose. **Table 6** shows the results of the experiment. *crp* or *cya* mutants do not ferment maltose or arabinose. When checked on MacConkey's plates containing maltose or arabinose, the *cya* mutants were seen as white colonies. The mutants HG203, HG204, HG205 and HG206 tested positive for fermentation on maltose and

Table 6. Growth on different carbon sources and fermentation of sugars on MacConkey's plates

Strain	Glucose	Pyruvate	Succinate	MacConkey's	
				Maltose	Arabinose
HG203	+++	-	-	+++	+++
HG204	+++	-	-	+++	+++
HG205	+++	-	-	+++	+++
HG206	+++	-	-	+++	+++
HG163	+++	-	+	+++	+++
CA8306	+++	++	-	-	-
Δ *cya*					

\+ fermentation (Red colonies) on MacConkey's
\- non fermentation (White colonies) on MacConkey's

arabinose (red coloured colonies). It is, therefore, unlikely that mutants HG203, HG204, HG205 and HG206 had *cya* or *crp* mutations.

3.1.8 Mapping of the mini Tn*10*-ATS insertions by PCR

It was shown from earlier experiments that the Suc$^-$ Kan^R mutants are affected in Pck specific activity (Pck$^-$ phenotype). The presumptive mini Tn*10*-ATS insertions were not linked to *pckA* as concluded from P1 transduction experiments involving determination of linkage to *asd*. The next step was to attempt to identify the location of the mini Tn*10*-ATS in the four mutant HG203, HG204, HG205 and HG206 by PCR of DNA flanking the insertion points. PCR was done using the chromosomal DNA of each mutant, with a Tn*10* specific primer and a REP primer (REP primers were homologous to REP1 and REP2 repetitive sequences of *E. coli*) (Subramanian *et al.*, 1994). PCR products of 1.6kb and 1.2kb size were purified and sequenced at the Plant Biotechnology Institute (PBI), National Research Council, Saskatoon, SK, Canada. A BLAST search on the sequence showed 97% homology with *hybF*, a potential regulatory gene in the *hyb* operon (Menon *et al.*, 1994). It was hypothesised at this stage that the mini Tn*10*-ATS was inserted in *hybF* in these strains and that *hybF* somehow regulates *pckA*. If this was true, then transforming the mutants with *hybF* plasmid should restore the Suc$^+$ phenotype in the mutants.

3.2 Determination that the insertion mutations were not in *hybF*

To test the hypothesis that the insertion was in *hybF*, the mutants HG203, HG204, HG205 and HG206 were transformed with plasmid pHyb33 containing the complete *hyb* operon *hyb*(*A-G*) (Menon *et al.*, 1991). Growth and Pck specific activity was determined for HG203, HG204, HG205 and HG206 mutants transformed with the *hyb*(*A-G*) plasmid, and compared to the mutant control. Pck specific activity was not restored in the mutants transformed with the *hybA-G* plasmid (data not shown). This indicated that the mutations in the mutants were not in *hybF*. PCR was carried out using two primers oHG95 and oHG98, flanking the hypothetical insertions in *hybF* for mutants HG203, HG204, HG205 and HG206 and the wild type HG163 (results not shown). Only one single amplified band (of *c.a.* 500bp size) was seen in the wild type control and the mutants. If there were a mini Tn*10* insertion in that region, the amplified fragment would have been of larger molecular weight than the band seen in HG163. This indicated that there were no mini Tn*10*-ATS insertions in *hyb*. This was a curious finding, considering that Kan^R was tightly linked to Suc^- in the mutants by P1 transduction.

3.3 Effect of *pps*$^+$ background on the Suc$^-$ phenotype

Since the mutation was not in *hyb*, an attempt was made to further characterise the mutation. The Suc$^-$ phenotype of mutations in *pckA* depends on the presence of a *pps*$^-$ mutation, since *pckA pps*$^+$ strains are Suc$^+$. Experiments were done to change the background of the mutants to *pps*$^+$. If the mutations had a simple effect on expression of *pckA*, then *pps*$^+$ transductants should be Suc$^+$.

P1 transduction was carried out using W3350 (*pps*$^+$) as a donor and HG203, HG204, HG205 and HG206 as recipients. The *pps*$^+$ transductants were selected on minimal medium plates containing pyruvate as a sole carbon source. Fifty transductants were then tested on minimal medium containing succinate, glucose and pyruvate as carbon source and also LB plates containing kanamycin (**Table 7).**

Mutants HG203, HG204, HG205 and HG206 are Suc$^-$, Pyr$^-$ (*pps*$^-$), Kan^R. When *pps*$^+$ was transduced into the mutants, the mutants remained Suc$^-$. If the mutation had a simple effect on expression of *pckA*, then the transductants would have been Suc$^+$ since a *pckA*$^-$*pps*$^+$ will grow on media with succinate as a carbon source. This suggested that the mutations were in a gene or genes where the Suc$^-$ phenotype is independent of expression of the *pckA* gene.

Table 7. Effect of *pps*$^+$ on the Suc$^-$ phenotype of the mutants

Mutant	Number of Pyr$^+$ colonies tested	Suc$^+$	Suc$^-$	Kan^R	Kan^S
HG203	50	0	50	50	0
HG204	50	0	50	50	0
HG205	50	0	50	50	0
HG206	46	0	46	46	0

The transductants were screened on minimal medium containing glucose, succinate and pyruvate as carbon source and LB containing kanamycin.

3.4 Relationship of spontaneous Kan^R resistance and the Suc$^-$ phenotype to *atp* mutations

Earlier experiments involving PCR amplification of the mini Tn*10*-ATS insertion showed the absence of a mini Tn*10* in the mutants HG203, HG204, HG205 and HG206 (Kan^R and Suc$^-$). The Kan^R in these strains could be due to spontaneous mutations. It has been found by earlier investigators that mutations in *atp* can confer upon the cells resistance to aminoglycosides, and some *atp* mutations are also known to be Kan^R (Thorbjarnardottir *et al.*, 1978). Kan^R is also conferred by *kanA* or *ecfB* (Thorbjarnardottir *et al.*, 1978). It is also known that *atp* mutants do not grow in the presence of succinate as a sole carbon source, possibly due to defects in transport for succinate (Boogerd *et al.*, 1998) and also due to defects in oxidative phosphorylation. Therefore, it was hypothesised that these spontaneous Kan^R mutations that confer a Suc$^-$ phenotype are in one of the genes of the *atp* operon. Previously, an *atpA*::Tn*10* mutant was isolated in the lab that has 10% of wild type Pck specific activity and is Suc$^-$ (Goldie, unpublished results).

3.5 Mapping the Kan^R Suc$^-$ isolates using mutations linked to the *atp* operon

Since it was hypothesised that the spontaneous Kan^R mutation could be in *atp* genes, it was decided to test them for genetic linkage to mutations linked to the *atp* operon. The *atp* is at 84.6' on the *E. coli* chromosome. HG203, HG205 and HG206 mutants (Suc$^-$, Kan^R) were checked for linkage of Kan^R and Suc$^-$ by transducing mutations close to the *atp* operon (obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT). The expected linkages are shown in **Figure 10.** P1 transduction studies were done using Tn*10* (Tet^R) insertion mutations. If the strains have mutations in *atp* genes, then transducing the Tn*10* markers close to the *atp* should restore the Suc$^+$ phenotype in some of the transductants. This would be an indication that the mutation lies in the region of 83.2-86.7 min. According to the linkage map, the expected theoretical linkage of *atp* to *rbs* is 53% and to *ilvD* is 2% (Miller, 1992). No linkage is expected with the other markers farther away from *atp*. However, unusually high cotransduction frequencies have been observed in this region (Von-Meyenberg *et al.*, 1979) and the genetic map does not correlate well with the physical map.

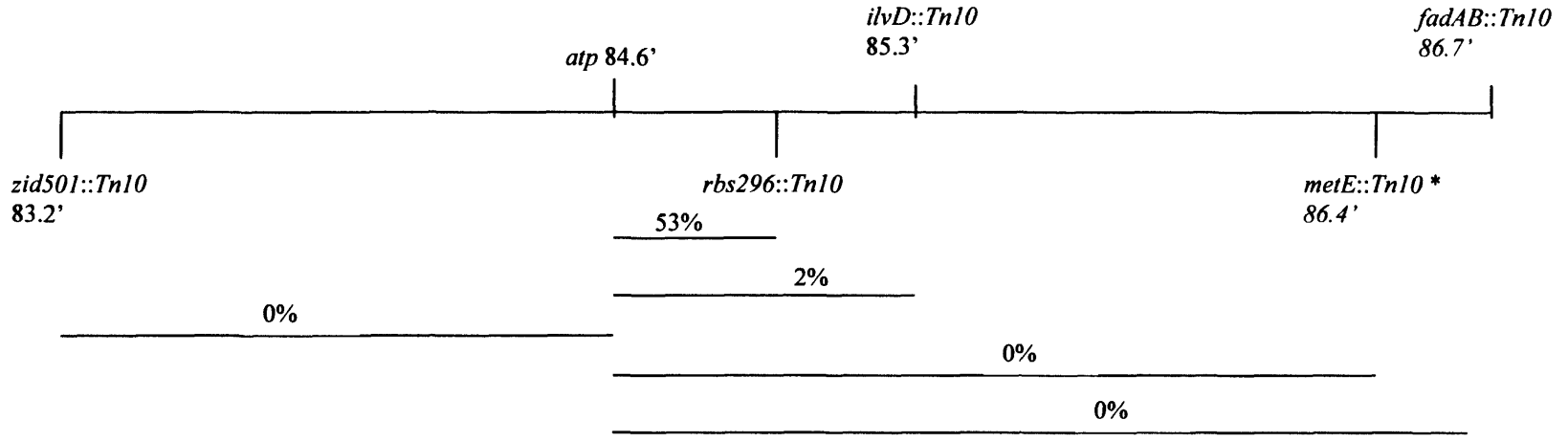


Figure 10. Positions of the markers used for transduction experiments with respect to the *atp* operon.

The markers were obtained from the *E coli* genetic stock center. The expected linkage of the markers with *atp* (Tet^R to Suc^+) based on the map distances, are indicated; however, linkages higher than expected are observed in this region, which is close to the origin of DNA replication.

3.5.1 Mutant HG205

The transduction results of the mutants are shown in **Table 8.** 100% linkage was observed with both *ilvD*::Tn*10* (85.3') and with *rbs296*::Tn*10* (85') markers, where only a 2% linkage of Suc^+ and *ilvD* and a 53% linkage of Suc^+ and *rbs* are expected. One possibility for the high linkage might be due to a deletion in that region of the chromosome. Transduction was done with markers further away from the *atp* (84.6'). The markers that were tested were *metE*::Tn*10* (86.4'), *fadAB*::Tn*10* (86.7') and *zid501*::Tn*10* (83.2'). Linkage of Suc^+ and Tet^R of 7.6% was observed after transducing with *metE*::Tn*10*, and no linkage was obtained with the other mutations. A linkage map of HG205 mutant with the tested markers is shown in **Figure 11.** No linkage of Tet^R and Suc^+ was observed with the markers further from *atp*. The high cotransduction frequencies observed with markers *ilvD*::Tn*10* and *rbs296*::Tn*10* , however are consistent with the observations of other workers for genes in this region (Rosen, 1973) probably due to the closeness to the origin of DNA replication. The possibility of a small deletion in this region has not been ruled out. This can be tested by a reciprocal cross using the Kan^R Suc^- mutations as P1 donors.

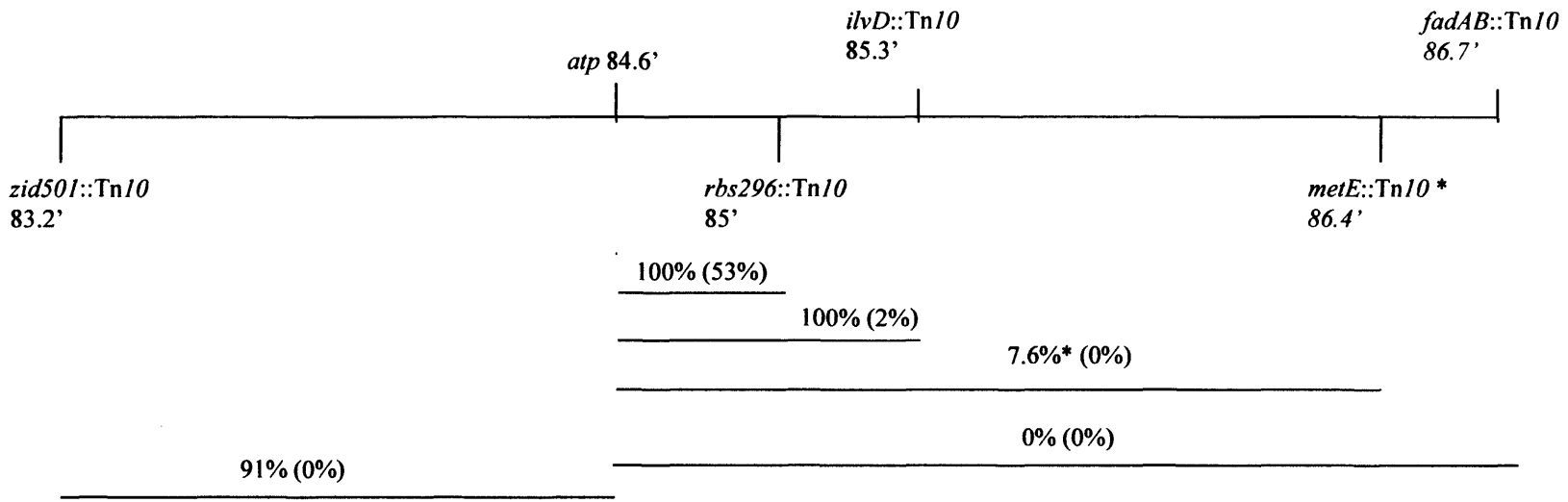
Table 8. Linkage of Tn*10* insertions to Suc^+ and Kan^S in mutant HG205

Strain used as donor for transduction	Selected mutation	Map position of selected mutation	No. of Tet^R selected	Ilv^-	Met^-	Kan^S Suc^+	Kan^S Suc^-	Kan^R Suc^-	Kan^R Suc^+	Percentage Linkage of Suc^+ and Kan^S to Tn*10*
CAG18431	*ilvD*::Tn*10*	85.3'	180	180	-[a]	180	0	0	0	100
CAG18501	*rbs296*::Tn*10*	85'	176	-	-	176	0	0	0	100
CAG18491	*metE*::Tn*0*	86.4'	100	-	13[b]	1	12	0	0	7.6[b]
CAG18496	*fadAB*::Tn*10*	86.7'	100	-	-	0	100	0	0	< 0.5%
CAG18499	*zid501*::Tn*10*	83.2'	100	-	-	91	9	0	0	91

The *atp* operon is at 84.6' on the *E coli* chromosome. Linkage of Suc^+ and Kan^S to Tet^R is determined in the mutant. Markers in the region of 83.2-86.7' in the *E coli* chromosome were tested for linkage.

[a] Not applicable

[b] *metE* data are probably not reliable, since only 13 of 100 Tet^R transductants were Met^-. The Tn*10* insertion in this strain may have transposed to one or more secondary sites.



*Met⁻ was linked only 13% with Tet^R in this donor strain (*metE*::Tn*10*). There may be two or more Tn*10*s in this strain.

Figure 11. Linkage of the Tn*10* insertions to Kan^S and Suc^+ in mutant HG205.

The markers were obtained from the *E coli* genetic stock center.
The percentage linkage of *atp* to Suc^- was tested by determined by linkage of Suc^+ and Kan^S to Tet^R. The numbers in brackets indicate the expected linkage of Suc^+ to Tet^R based on the physical map of the genome. However, linkages higher than expected are observed in this region, probably due to the proximity of the origin of DNA replication.

3.5.2 Mutant HG203

The transduction results of the mutant HG203 are shown in **Table 9**. Linkage of Suc^+ and Tet^R of 84% was observed on transducing the *ilvD*::Tn*10* (85.3') and 91% linkage of Suc^+ and Tet^R on transducing the *rbs296*::Tn*10* (85'), where the expected linkages are 2% and 53% respectively. This led to the possibility of a deletion between 84.6 and 85.3' in the mutant HG203 chromosome. Transduction with markers further away from *atp* showed linkage data as expected. **Figure 12** shows the linkage map of *atp* with the markers tested in the HG203 mutant. Linkages observed with *ilvD*::Tn*10* and *rbs296*::Tn*10* were higher than predicted by the genetic map, yet are consistent with other cotransduction data for genes in this region (Rosen, 1973). The kanamycin resistant phenotype is very erratic. The Kan^R Suc^+ phenotype that is seen (**Table 9**) could be due to spontaneous kan^R mutations and the Kan^R Suc^- colonies that are seen in the transduction experiments may be nontransductants (recipient from the transduction plate).

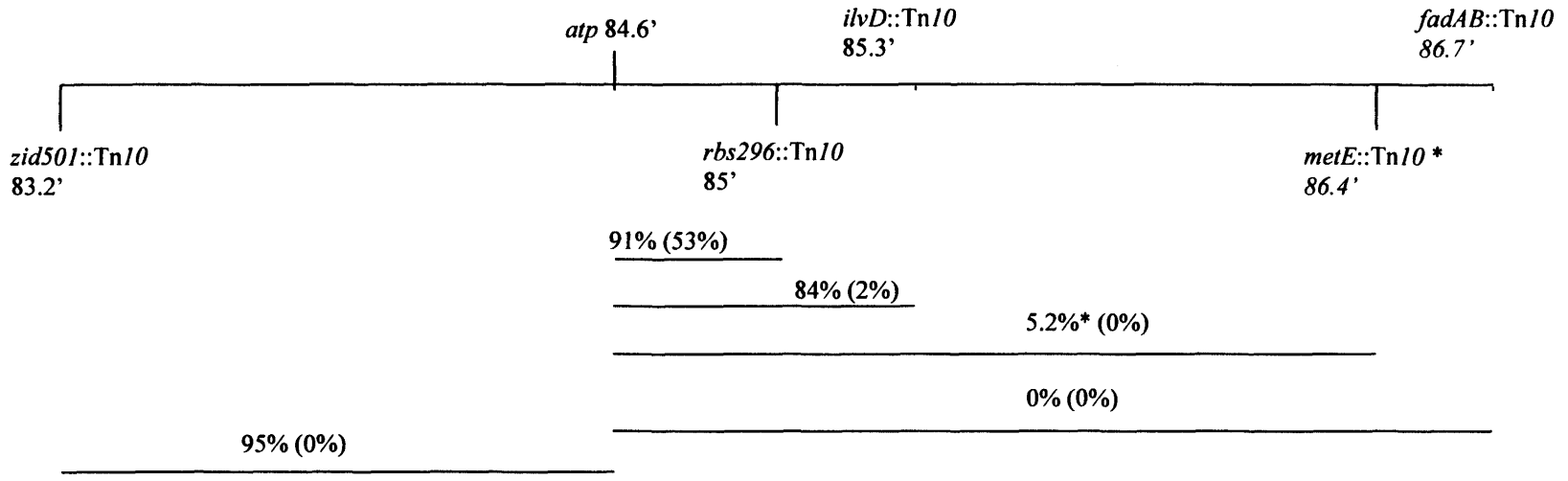
Table 9. Linkage of Tn*10* insertions to Suc^+ and Kan^S in mutant HG203

Strain used as donor for transduction	**Selected mutation**	**Map position of the selected mutation**	**No. of Tet^R selected**	**Ilv^-**	**Met^-**	**Kan^S Suc^+**	**Kan^S Suc^-**	**Kan^R Suc^-**	**Kan^R Suc^+**	**Percent linkage Suc^+ and Kan^S to Tn*10***
CAG18431	*ilvD*::Tn*10*	85.3'	100	100	-	84	3	2	11	84
CAG18501	*rbs296*::Tn*10*	85'	90	-	-[a]	82	3	1	4	91
CAG18491	*metE*::Tn*0*	86.4'	100	-	19[b]	1	18	0	0	5.2[b]
CAG18496	*fadAB*::Tn*10*	86.7'	100	-	-	0	100	0	0	0
CAG18499	*zid501*::Tn*10*	83.2'	99	-	-	7	2	2	88	95

The *atp* operon is at 84.6' on the *E coli* chromosome. Linkage of Suc^+ and Kan^S to Tet^R is determined in the mutant. Markers in the region of 83.2-86.7' in the *E coli* chromosome were tested for linkage.

[a] Not applicable

[b] *metE* data are probably not reliable, since only 19 of 100 Tet^R transductants were Met^-. The Tn*10* insertion is this strain may have transposed to one or more secondary sites.



*Met⁻ was linked only 13% with Tet^R in this donor strain (*metE*::Tn*10*). There may be two or moreTn*10*s in this strain.

Figure 12. Linkage of the Tn*10* insertions to Kan^S and Suc^+ in mutant HG203.

The markers were obtained from the *E coli* genetic stock center.
The percentage linkage of *atp* to Suc^- was tested by determining the linkage of Suc^+ and Kan^S to Tet^R. The numbers in brackets indicate the expected linkage of Suc^+ to Tet^R based on the physical map of the genome. However, linkages higher than expected are observed in this region, probably due to the proximity of the origin of DNA replication.

3.5.3 Mutant HG206

Linkage of Suc$^-$ in mutant HG206 to markers on either side of *atp* is shown in **Table 10**. Linkage of 87% of Suc$^+$ and Kan^S to *ilvD*::Tn*10* and 92% to *rbs296*::Tn*10* were observed. There could be a deletion in the region between 84.6' to 85.3' in the mutant HG206 chromosome, causing the higher linkages. Linkages of Suc$^+$ Kan^S to *metE*::Tn*10* (86.4'), *fadAB*::Tn*10* (86.7') and *zid501*::Tn*10* were determined. **Figure 13** shows the linkage of Tn*10* to Suc$^+$ and Kan^S in mutant HG206

In all four mutants, the *ilvD*::Tn*10* and *rbs296*::Tn*10* was tightly linked with Suc$^+$ and Kan^S. Linkage expected from the map positions was much lower in comparison to what was observed in all three mutants HG203, HG205 and HG206. Earlier experiments by other researchers, trying to transduce the *atp* operon, was always done using the cotranduction with *ilvD*::Tn*10* where they observed a 60% linkage of *atp* to *ilvD* (Klionsky *et al.*, 1984), consistent with the cotransduction data for genes in this region.

In the HG204 mutant there was no linkage of the Kan^R to the Suc$^-$ phenotype. There could be some other mutation in this strain that confers the Kan^R such as *kanA* or *ecfB*, (Thorbjarnardottir *et al.*, 1978) which may be sources of spontaneous Kan^R in *E coli* K12. This mutant was not used for further mapping studies.

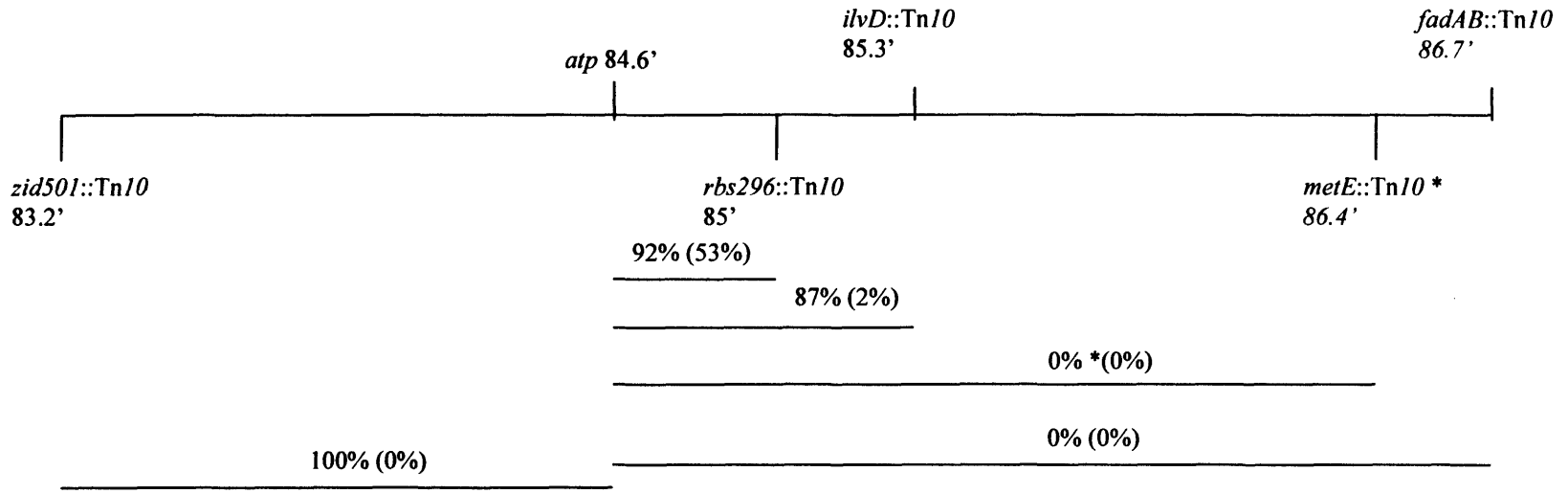
Table 10. Linkage of Tn*10* insertions to Suc^+ and Kan^S in mutant HG206

Strain used as donor for transduction	Selected mutation	Map position of selected mutation	No. of Tet^R selected	Ilv^-	Met^-	Kan^S Suc^+	Kan^S Suc^-	Kan^R Suc^-	Kan^R Suc^+	Percent linkage Suc^+ to Tn*10*
CAG18431	*ilvD*::Tn*10*	85.3'	100	100	-[a]	87	10	2	1	87
CAG18501	*rbs296*::Tn*10*	85'	100	-	-	92	8	0	0	92
CAG18491	*metE*::Tn*0*	86.4'	100	-	21[b]	0	21	0	0	0[b]
CAG18496	*fadAB*::Tn*10*	86.7'	100	-	-	0	100	0	0	0
CAG18499	*zid501*::Tn*10*	83.2'	100	-	-	100	0	0	0	100

The *atp* operon is at 84.6' on the *E coli* chromosome. Linkage of Suc^+ and Kan^S to Tet^R is determined in the mutant. Markers in the region of 83.2-86.7' in the *E coli* chromosome were tested for linkage.

[a] Not applicable

[b] *metE* data are probably not reliable, since only 21 of 100 Tet^R transductants were Met^-. The Tn*10* insertion is this strain may have transposed to one or more secondary sites.



*Met$^-$ was linked only 13% with Tet^R in this donor strain (*metE*::Tn*10*). There may be two or more Tn*10*s in this strain.

Figure 13. Linkage of the Tn*10* insertions to Kan^S and Suc$^+$ in mutant HG206.

The markers were obtained from the *E coli* genetic stock center.
The percentage linkage of *atp* to Suc$^-$ was tested by determined by linkage of Suc$^+$ and Kan^S to Tet^R. The numbers in brackets indicate the expected linkage of Suc$^+$ to Tet^R based on the physical map of the genome. However, linkages higher than expected are observed in this region, probably due to the proximity of the origin of DNA replication.

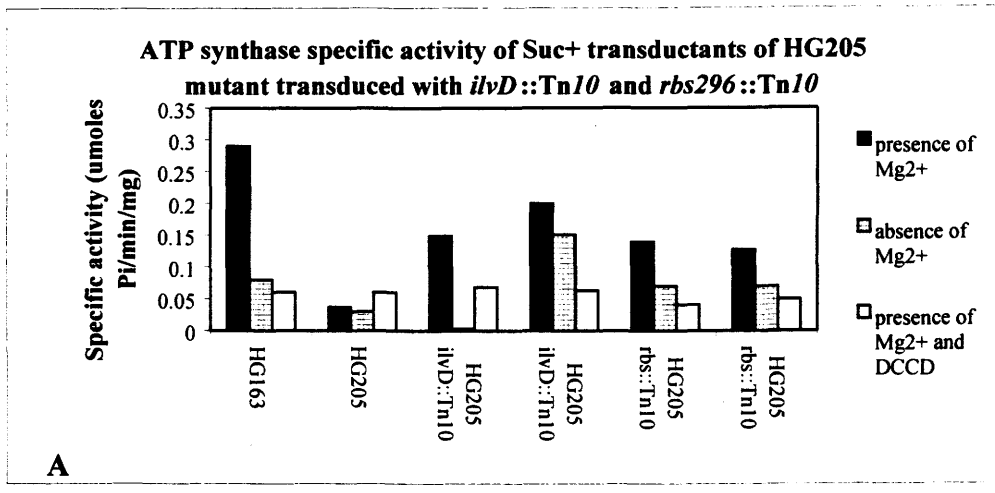
3.5.4 Restoration of ATP synthase activity in the transductants

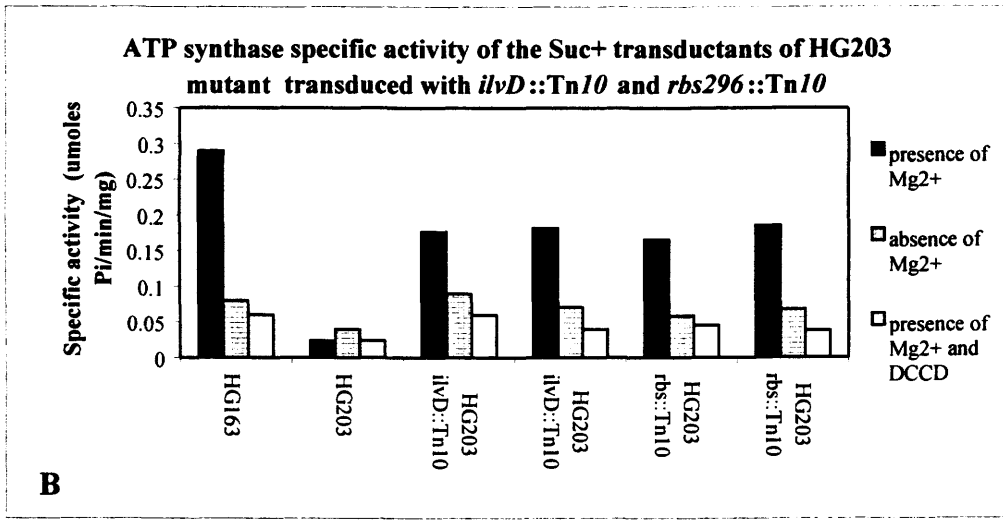
Transduction experiments with *ilvD*::Tn*10* and *rbs296*::Tn*10* show tight linkage of the Suc^+ and Tet^R, which would be consistent with the mutation lying in the *atp* operon. If the mutation is in *atp* as hypothesised, then ATP synthase activity should be reduced or eliminated in the mutants and transducing the Tet^R from *ilvD*::Tn*10* and *rbs296*::Tn*10* that resulted in a Suc^+ phenotype should restore the ATP synthase activity in the Suc^+ transductants. Inside out vesicles were made of the respective mutants and the *ilvD*::Tn*10* and *rbs296*::Tn*10* transduced mutants HG203, HG205 and HG206. The transductants were Suc^+ Tet^R Kan^S. ATP synthase activity in the mutants HG203, HG205 and HG206 were greatly reduced if not completely eliminated. ATP synthase specific activity of the transductants was compared to the mutant controls and the HG163 wild type control. An ATP synthase assay was done in the presence or absence of Mg^{2+}, and in the presence or absence of DCCD, a specific inhibitor of the F_0F_1 ATP synthase (West and Mitchell, 1974). Lower ATP synthase specific activity is expected in the absence of Mg^{2+}, given the fact that Mg^{2+} is a cofactor for the enzyme. The transductants were assayed in duplicate. **Figure 14A** shows the ATP synthase specific activity of the inside-out vesicles of the HG205 Suc^+ transductants in the presence of Mg^{2+}. ATP synthase specific activity of both the transductants was about three fold higher than the specific activity of the HG205 mutant control. In **Figure 14A**, it is seen that, in the presence of DCCD and Mg^{2+} or in the absence of Mg^{2+}, the specific activity was lower indicating that the F_0F_1 ATP synthase is probably the major activity that is present in the inside-out vesicles.

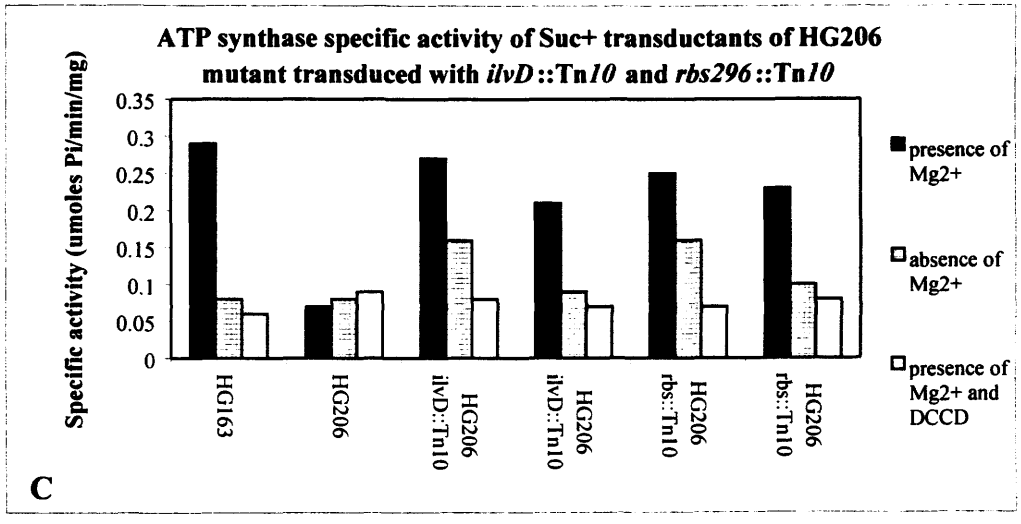
Figure 14B shows the ATP synthase specific activity of the Suc^+ transductants of mutant HG203, in presence and absence of Mg^{2+}, and in the presence and absence of DCCD. It also shows the difference in specific activity between wild type HG163 and mutant HG203. In the presence of Mg^{2+}, both Suc^+ Kan^S *ilvD*::Tn*10* and *rbs296*::Tn*10* transductants show ATP synthase specific activity that is about three fold higher than the mutant. There was a two-fold decrease in ATP synthase specific activity in the absence of Mg^{2+}. Specific activity decreased about three fold in the presence of DCCD in the transductants. The ATP synthase specific activity of the mutant HG203 was considerably lower than the wild type control and the Suc^+ Kan^S transductants. **Figure 14C** shows the ATP synthase activity of Suc^+ Kan^S transductants of mutant HG206. ATP synthase specific activity in the presence of Mg^{2+} was comparable to the specific activity of the wild type control and was about six fold higher than the HG206 mutant.

Figure 14. ATP synthase activity of mutants HG205, HG203, HG206 transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10*.

ATP synthase specific activity in inside-out vesicles of mutants HG205, HG203 and HG206 transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10* in comparison to the respective mutant controls and the wild type control. Transductants were assayed in duplicate. Wild type HG163 was used as the control. **A.** HG205 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10* the presence of Mg^{2+}, absence of Mg^{2+} and in the presence of DCCD. **B.** HG203 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10* in the presence and absence of Mg^{2+} and DCCD. **C.** HG206 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10* in the presence and absence of Mg^{+2} and DCCD


ATP synthase specific activity of Suc+ transductants of HG205 mutant transduced with ilvD::Tn10 and rbs296::Tn10
Specific activity (umoles Pi/min/mg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HG163
HG205
HG205 ilvD::Tn10
HG205 ilvD::Tn10
HG205 rbs::Tn10
HG205 rbs::Tn10
presence of Mg2+
absence of Mg2+
presence of Mg2+ and DCCD
A


ATP synthase specific activity of the Suc+ transductants of HG203 mutant transduced with ilvD::Tn10 and rbs296::Tn10
Specific activity (umoles Pi/min/mg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HG163
HG203
HG203 ilvD::Tn10
HG203 ilvD::Tn10
HG203 rbs::Tn10
HG203 rbs::Tn10
presence of Mg2+
absence of Mg2+
presence of Mg2+ and DCCD
B


ATP synthase specific activity of Suc+ transductants of HG206 mutant transduced with ilvD::Tn10 and rbs296::Tn10
Specific activity (umoles Pi/min/mg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HG163
HG206
HG206 ilvD::Tn10
HG206 ilvD::Tn10
HG206 rbs::Tn10
HG206 rbs::Tn10
presence of Mg2+
absence of Mg2+
presence of Mg2+ and DCCD
C

3.5.5 Restoration of Pck activity in transductants

The Suc^+ Kan^S contransduced with the *ilvD*::Tn*10* and *rbs296*::Tn*10* in mutants HG203, HG205 and HG206. The ATP synthase specific activity was lower in the mutants and restored in the Suc^+ Kan^S transductants. Therefore, the hypothesis that the spontaneous Kan^R mutation is in *atp* is probably correct. The next question that was addressed was how did transduction to Suc^+ and Kan^S affect the specific activity of Pck in the mutants?

The *ilvD*::Tn*10* and *rbs296*::Tn*10* transductants, HG163 wild type control, and the mutant controls were grown to stationary phase (13 h). This time point was chosen because the wild type showed maximum Pck specific activity at this time point. **Figure 15A** shows the comparison of Pck specific activity of Suc^+ Kan^S transductants of HG205 mutant. The Pck specific activity in the transductants is approximately seven fold higher in comparison to the HG205. This indicates that the Pck^+ phenotype cotransduced with the Suc^+ and the Kan^S phenotypes.

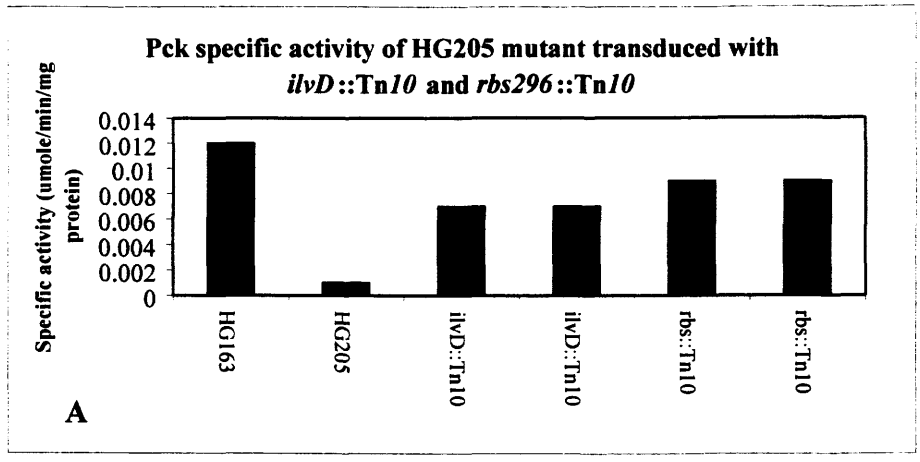
Figure 15B shows the comparison of Pck specific activity between HG203 mutant transduced with *ilvD*::Tn*10*, *rbs296*::Tn*10* and the controls (wild type HG163 and the HG203 mutant). The Pck specific activity of both the transductants was about six fold higher than the HG203 mutant control and was comparable to the wild type. These experimental results provided further support for the presence of the Kan^R spontaneous mutation in the region of 84.6-85.3 min in the HG203 mutant chromosome. This also indicated that the Pck^+ phenotype cotransduced with the Suc^+ and the Kan^S phenotypes.

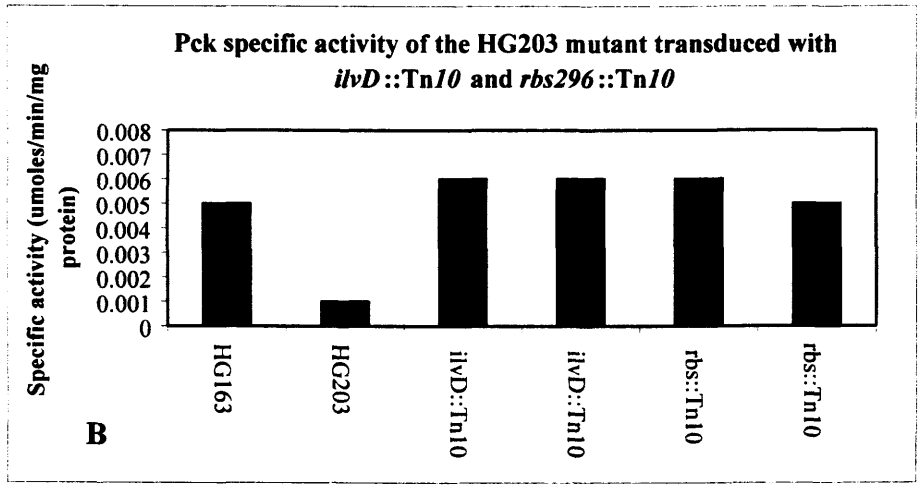
Pck specific activity of Suc^+ Kan^S transductants of HG206 was compared with mutant HG206 and HG163 wild type (**Figure 15C**). The results obtained were similar to those obtained for mutants HG203 and HG205.

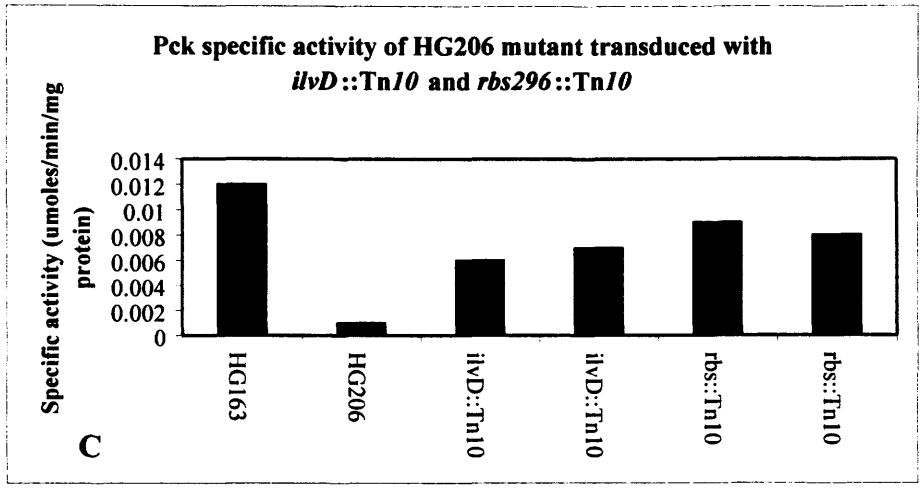
Figure 15. Pck specific activity of mutants HG205, HG203 and HG206 transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10*.

The wild type, mutant and the *ilvD*::Tn*10* and *rbs296*::Tn*10* transduced mutants were assayed for Pck at 13 h. Transductants have been assayed in duplicate. **A.** HG205 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10*. **B.** HG203 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10*. **C.** HG206 mutant transduced with *ilvD*::Tn*10* and *rbs296*::Tn*10*.

Pck specific activity of Suc^+ Kan^S transductants of HG206 was compared with mutant HG206 and HG163 wild type (**Figure 15C**). The results obtained were similar to those obtained for mutants HG203 and HG205.


Pck specific activity of HG205 mutant transduced with
ilvD::Tn10 and rbs296::Tn10
Specific activity (umole/min/mg protein)
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
HG163
HG205
ilvD::Tn10
ilvD::Tn10
rbs::Tn10
rbs::Tn10
A


Pck specific activity of the HG203 mutant transduced with
ilvD::Tn10 and rbs296::Tn10
Specific activity (umoles/min/mg protein)
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
HG163
HG203
ilvD::Tn10
ilvD::Tn10
rbs::Tn10
rbs::Tn10
B


Pck specific activity of HG206 mutant transduced with
ilvD::Tn10 and rbs296::Tn10
Specific activity (umoles/min/mg protein)
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
HG163
HG206
ilvD::Tn10
ilvD::Tn10
rbs::Tn10
rbs::Tn10
C

3.6 Identification of the mutation

Transduction experiments with markers close to the *atp* locus led to the restoration of the Suc^+ phenotype in the mutants. Linkage data from the P1 transduction experiments suggest the absence of any large deletions in the mutants, HG203, HG205 and HG206. Determination of ATP synthase specific activity in inside-out vesicles shows that the original mutant HG203, HG205 and HG206 had lowered ATP synthase activities and the ATP synthase specific activity was increased in the *ilvD*::Tn*10* and *rbs296*::Tn*10* transductants and was comparable to the wild type HG163. Following this, Pck specific activity was also assessed in the transductants to check if the spontaneous mutations have an effect on Pck specific activity. It was found that the Pck specific activity was much higher in the transductants in comparison to the mutant controls and specific activity was comparable to the wild type. These results suggest that single mutations mapping near the *atp* operon, may have produced the Suc^-, Kan^R and Pck^- phenotypes in mutants HG203, HG205 and HG206.

Since it is known that mutations in the *atp* operon confer resistance to aminoglycoside (Thorbjarnardottir *et al.*, 1978), and leads to loss of growth on succinate containing medium, (Boogerd, 1998) and the data presented here show that the mutants HG203, HG205 and HG206 have low ATP synthase activity, mutations in *atp* were the most probable candidates for the mutations in strains HG203, HG205 and HG206.

3.6.1 Complementation of the Suc$^-$ phenotype

Plasmid pBWU1.3 containing the intact *atp* operon (Moriyama *et al.*, 1991) was transformed into mutant HG205, HG203 and HG206. This plasmid is a pBR322 derivative containing the *atp* operon under the control of the *atp* promoter. Transformations were also performed using pBWU1.2 (Kuo *et al.*, 1998) and pAN45 (Downie *et al.*, 1980), which contain the *atp* operon and are derivatives of pACYC177 and pACYC184, respectively. These are low copy number plasmids, whereas pBR322 is a medium to high copy number plasmid. The Amp^R transformants were screened on minimal medium plates containing succinate, glucose and pyruvate as carbon source (**Table 11**). Ten transformants were screened for each of the three mutants transformed with pBWU1.3, pBWU1.2 and pAN45. The ten transformants screened for each plasmid had the same phenotypes and one example of each is characterised in Table 11. The mutants were also transformed with pBR322, pACYC184 and pACYC177 vectors as controls. Plasmids pBWU1.3, pBWU1.2 and pAN45 each complemented the Suc$^-$ and Kan^R phenotypes of each mutant (**Table 11**). Controls of mutants transformed with plasmid vector alone did not grow on succinate and retained the Kan^R phenotype. Complementation of the Suc$^-$ and Kan^R by intact *atp* plasmid was the first direct evidence that the mutations were in the *atp* operon in mutants HG205, HG203 and HG206.

Figure 27. DNA sequence of the F_1 region of ATP synthase in mutants HG205, HG203 and HG206.

Nucleotides 5503-5588 of the Walker sequence is shown. All the three mutants have a deletion in the *atpG* coding for the γ subunit of F_0F_1 ATP synthase. The complete F_1 region was sequenced in all the three mutants. The deletion is indicated by * in the mutants. The mutated nucleotides are indicated in bold letters in the wild type.

+++ Growth observed after incubation at 37°C for 24 h. Succinate and pyruvate plates were incubated for 48 h.
- No growth observed after incubation at 37°C for 24 h on kanamycin plates or after 48 h on succinate and pyruvate plates.
NA Not applicable

Mutants HG205, HG203 and HG206 were transformed with plasmids pBWU1.3, pBWU1.2 and pAN45 containing complete *atp* operon. The control experiment of the mutants transformed with the vector is also shown. pBWU1.3, is a derivative of pBR322, pBWU1.2, a derivative of pACYC177 and pAN45, a derivative of pACYC184.

3.6.2 Restoration of Pck activity in the transformed mutants

Since the Suc^+ phenotype was restored in the mutants upon transforming with *atp* plasmids, the effect of pBWU1.3 on Pck specific activity was examined. Growth and Pck specific activities were done with HG205, HG203 and HG206 mutants transformed with intact *atp* operon plasmid pBWU1.3 and compared with the wild type HG163 and the mutant controls.

Growth and specific activities of the wild type HG163 and mutants were compared as a function of time (**Figure 16**). This was done as the control for the experiments with pBWU1.3 (intact *atp* operon plasmid) transformed mutants. **Figure 16A** shows the growth of the mutants in comparison with the wild type. The wild type growth yield was about 50 Klett units higher than all the mutants. **Figure 16B** shows the Pck specific activities of the wild type and the mutants. The mutants have lower Pck specific activities in comparison with the wild type. Pck specific activity was approximately 20% of the wild type in the three mutants tested.

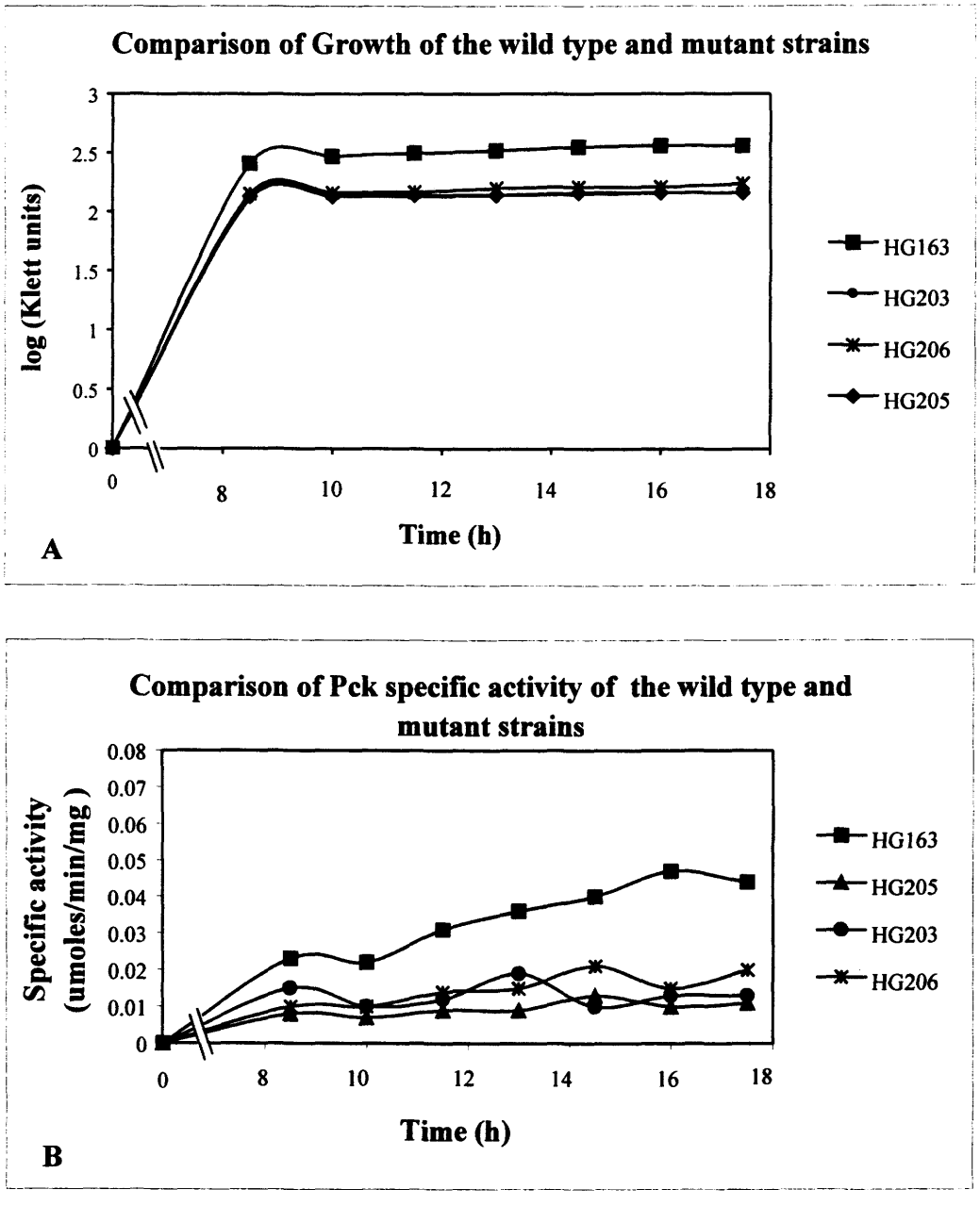


Figure 16. Growth and Pck specific activity of the wild type and mutants HG205, HG203 and HG206.

Comparison of growth dependent Pck specific activity between the wild type HG163 and the mutants HG205, HG203 and HG206. **A.** Growth yield of the wild type and the mutants from 8.5 to 17.5 h. Growth was measured in terms of log Klett units. **B.** Pck specific activity of the wild type and the mutants. Samples were taken from 8.5 to 17.5 h and were CTAB-treated and used for the Pck assay.

Growth and Pck specific activities were determined in mutants transformed with plasmid pBWU1.3, which expresses the complete *atp* operon. **Figure 17A** shows the comparison of growth between the wild type and the pBWU1.3 transformed mutants. Growth yield of the HG205 and HG206 transformed with pBWU1.3 was essentially identical to the wild type though HG203 transformed with pBWU1.3 had a growth yield lower than the wild type. **Figure 17B** shows the Pck specific activities of the pBWU1.3 transformed mutants and the wild type control. Mutant HG205 transformed with pBWU1.3 had a specific activity about six fold higher than the HG205 mutant control. The HG203 transformed with pBWU1.3 had a two fold increase in specific activity as compared to the HG203 control (**Figure 17B**). In case of the HG206 mutant transformed with pBWU1.3, the Pck specific activity was about four fold higher than the HG206 mutant (**Figure 17B**). The Pck specific activities of mutants HG205 and HG206 transformed with pBWU1.3 were higher than the wild type. In all the three mutants, transformation with plasmid pBWU1.3 led to an increase in Pck specific activity.

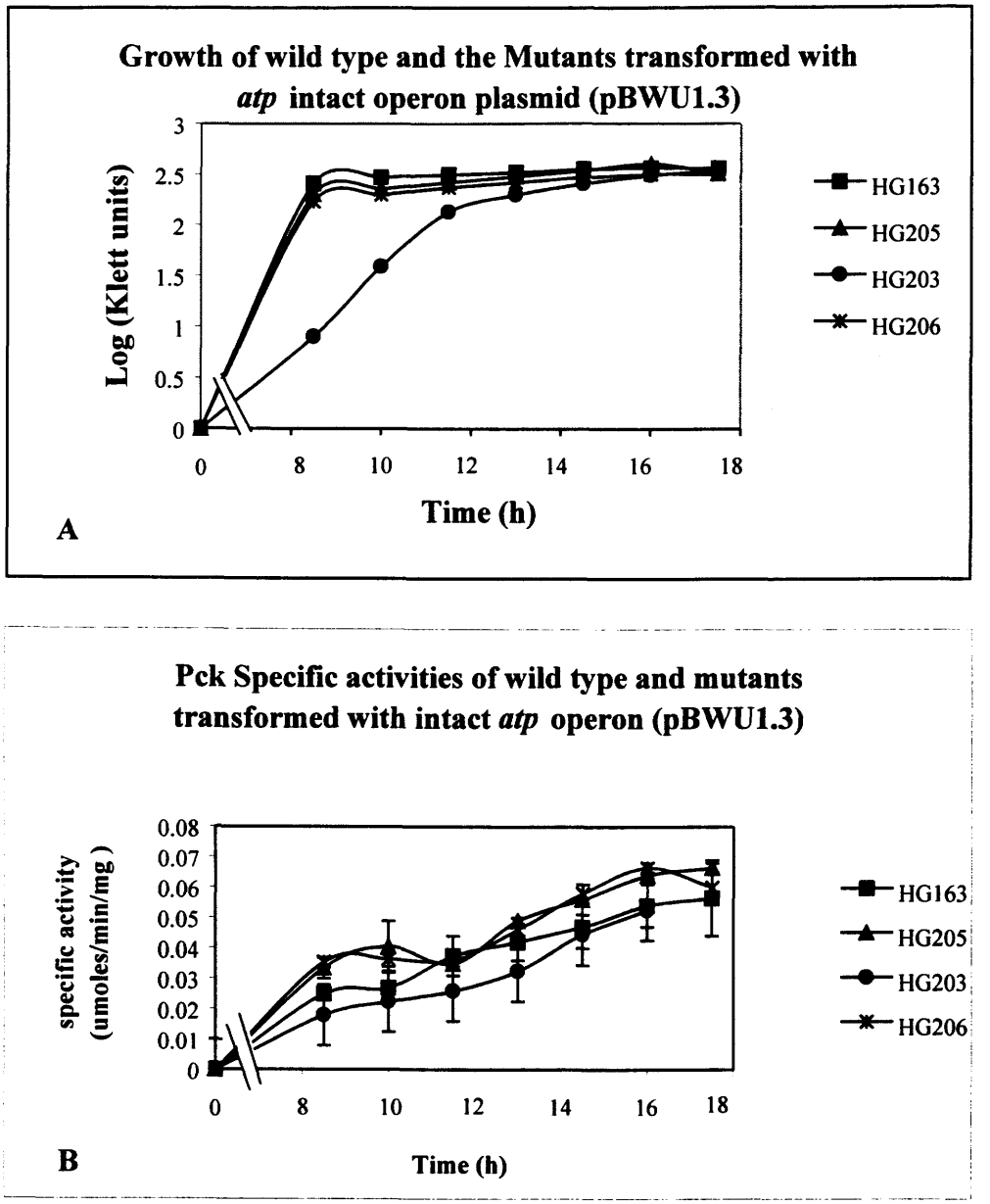
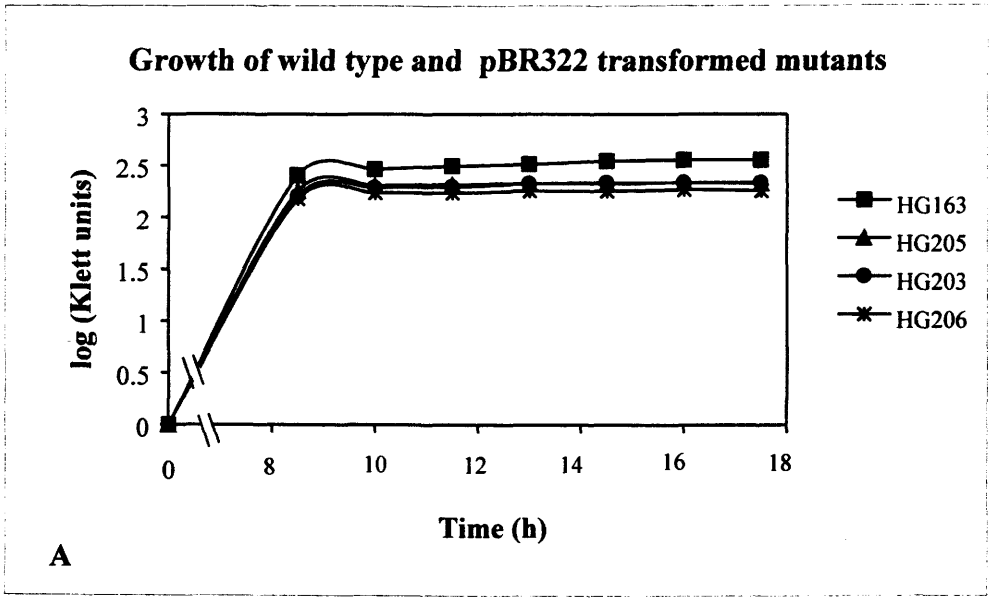


Figure 17. Effect of transformation with complete *atp* operon on growth and Pck specific activity in mutants HG205, HG203 and HG206.

Wild type HG163 was used as the control. **A.** Growth of wild type and the pBWU1.3 transformed mutants. **B.** Pck specific activities of the wild type and the transformed mutants. Sample was withdrawn at regular intervals of time from 8.5 to 17.5 h and Pck assays were done on CTAB treated cells. The experiment was repeated three times and the figure shows representative data.

A control experiment was done with mutants transformed with pBR322 (pBWU1.3 is a derivative of pBR322) to determine the effect of the vector on growth and Pck specific activities in the mutants. Mutants transformed with pBR322 were monitored from 8.5 to 17.5 h (**Figure 18A**). The growth of all three mutants transformed with pBR322 plasmid was comparable to the mutant controls and was lower than the wild type (**Figure 18A**). Pck specific activities of pBR322 transformed mutant was determined as a function of growth as shown in **Figure 18B**. In mutant HG205 and HG206 transformed with pBR322, Pck activity was higher than the respective mutant control. Mutant HG203 transformed with pBR322 showed Pck specific activity comparable to the HG203 mutant control. The mutant controls are shown in Figure 15B. This is not totally unexpected for pBR322 because pBR322 can cause increased respiratory and ATP synthase activities in *E. coli* (Eisenbraun *et al.*, 1993). Increase in Pck specific activity in pBR322 transformed mutants lacking ATP synthase activity suggests that Pck expression may respond to respiratory changes. This has not been investigated further.

Comparison of the growth and Pck specific activities of pBWU1.3 transformed mutants with the mutant controls showed that Pck specific activities in mutants transformed with the complete *atp* operon were higher. Since the plasmid carrying an intact *atp* operon complemented the Suc$^-$ phenotype of the mutants and it caused increased Pck specific activity, the next question addressed was does it lead to a restoration of ATP synthase activity in the mutants?



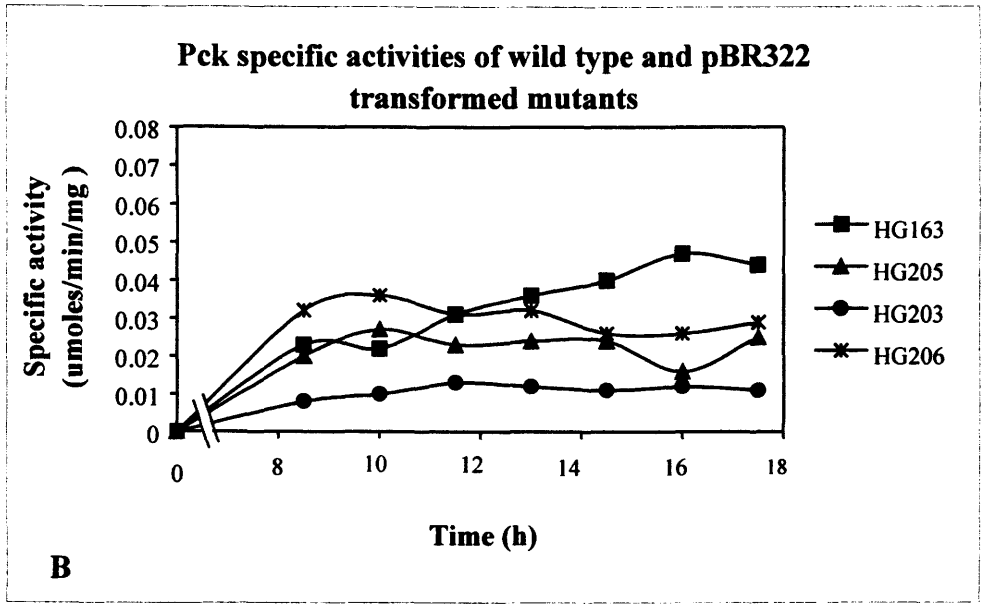


Figure 18. Effect of transformation with empty vector pBR322, on growth and Pck specific activities in mutants HG205, HG203 and HG206.

A. Growth of the wild type and pBR322 transformed mutants. **B**. Pck specific activities of the wild type and the pBR322 transformed mutants.

3.6.3 Restoration of ATP synthase activity in the transformed mutants

ATP synthase specific activities were determined for inside out vesicles of HG205, HG203 and HG206 mutants transformed with pBWU1.3, and compared with the wild type and the mutant controls. ATP synthase activity was determined in the presence or absence of Mg^{2+} and in the presence of DCCD. **Figure 19A** shows the ATP synthase specific activity of the HG205 mutant transformed with pBWU1.3. The ATP synthase specific activity of the pBWU.13 transformed mutant was comparable to the wild type. The specific activity was significantly higher than for the HG205 control. In the absence of Mg^{2+}, and in the presence of Mg^{2+} and DCCD, the specific activities were low as expected for the F_0F_1 ATP synthase.

The HG203 mutant transformed with pBWU1.3 was tested for ATP synthase specific activity (**Figure 19B**). ATP synthase specific activity of the HG203 mutant transformed with pBWU1.3 in the presence of Mg^{2+} was comparable to the wild type control and was higher than the mutant control. The specific activity was lower in both the pBWU1.3 transformed HG203 and in HG163 control in the absence of Mg^{2+} than in the presence of Mg^{2+}. DCCD inhibited the ATP synthase specific activity in HG203 mutant transformed with pBWU1.3 and HG163 wild type control. HG203 mutant control showed no difference in specific activity either in the presence of Mg^{2+}, absence of Mg^{2+}or in the presence of DCCD.

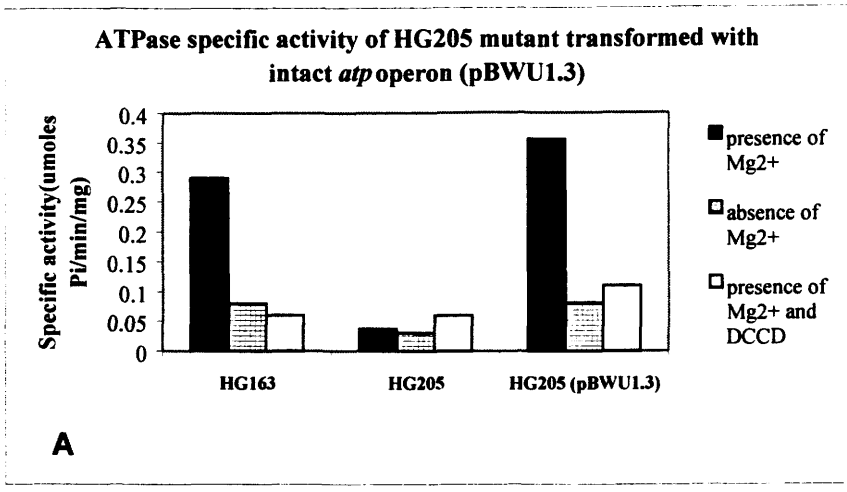
ATP synthase specific activity of mutant HG206 transformed with pBWU1.3 was compared with HG163 wild type and HG206 mutant control (**Figure 19C**). Specific activity of the pBWU1.3 transformed HG206 was comparable to the wild

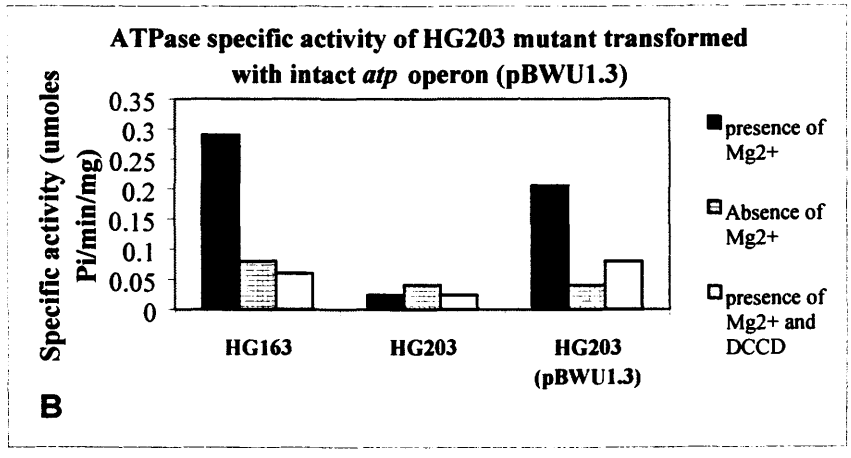
type and substantially higher than the mutant control. In both the wild type and HG206 transformed with pBWU1.3, the ATP synthase specific activity was lower in the absence of Mg^{2+}. DCCD mediated inhibition was observed in both the wild type and the pBWU1.3 transformed HG206.

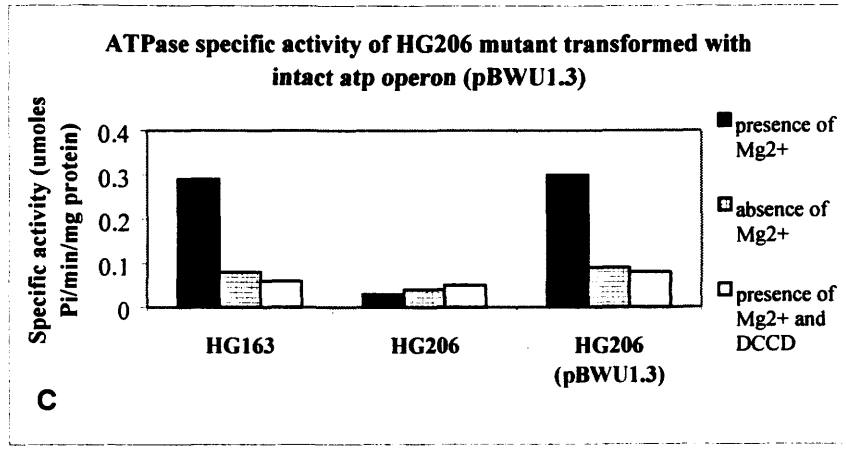
Figure 19. ATP synthase specific activities of mutants transformed with the complete *atp* operon (pBWU1.3).

Inside out vesicles were made of the mutants HG205, HG203 and HG206 transformed with pBWU1.3; wild type HG163 and the mutants.

A. ATP synthase specific activity of the HG205 mutant transformed with pBWU1.3 compared to wild type HG163 and mutant HG205 controls in the presence of Mg^{2+}, absence of Mg^{2+} and in the presence of DCCD. **B.** ATP synthase specific activity of HG203 mutant transformed with pBWU1.3. **C.** ATP synthase specific activity of HG206 mutant transformed with pBWU1.3.


ATPase specific activity of HG205 mutant transformed with intact *atp* operon (pBWU1.3)
Specific activity(umoles Pi/min/mg)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HG163
HG205
HG205 (pBWU1.3)
presence of Mg2+
absence of Mg2+
presence of Mg2+ and DCCD
A


ATPase specific activity of HG203 mutant transformed with intact *atp* operon (pBWU1.3)
Specific activity (umoles Pi/min/mg)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
HG163
HG203
HG203 (pBWU1.3)
presence of Mg2+
Absence of Mg2+
presence of Mg2+ and DCCD
B


ATPase specific activity of HG206 mutant transformed with intact atp operon (pBWU1.3)
Specific activity (umoles Pi/min/mg protein)
0.4
0.3
0.2
0.1
0
HG163
HG206
HG206 (pBWU1.3)
presence of Mg2+
absence of Mg2+
presence of Mg2+ and DCCD
C

3.7 Identifying the mutations in the F_1 region of ATP synthase

ATP synthase enzyme consists of two regions, F_0 and F_1. F_0 is an integral membrane protein responsible for proton translocation, whereas the F_1 region is a peripheral membrane protein, which possesses the ATP synthesis and hydrolyzing functions. F_0 is comprised of subunits *a*, *c* and *b* encoded by *atpB, atpE* and *atpF* respectively and F_1 is comprised of subunits, δ, α, γ, β and ε, encoded by *atpH, atpA, atpG, atpD* and *atpC* respectively. The F_1 subunit harbours the catalytic portion of ATP synthase $\alpha_3 \beta_3 \gamma$.

Transformation with the complete *atp* operon complemented the Suc^- and Kan^R phenotypes and led to an increase in the Pck and ATP synthase specific activities in mutants HG205, HG203 and HG206. To identify which cistron in the *atp* operon was complementing, the mutants were transformed with various genes of the *atp* operon, alone or in groups.

3.7.1 Transformation of the mutants with plasmids expressing various regions of the *atp* operon

Mutants HG205, HG203 and HG206 were transformed with the plasmids expressing various genes of the *atp* operon. The transformants were selected on appropriate antibiotic plates and ten transformants from each transformation were checked on minimal medium plates containing succinate, pyruvate or glucose as carbon source and also LB plates containing the respective antibiotics. Since all ten

colonies tested for each of the plasmids showed the same result, and only one example has been described in **Table 12.**

In all three mutants HG205, HG203 and HG206, plasmid pDJK35 (expressing the F_1 portion of ATP synthase) (Klionsky and Simoni, 1985) complemented the Suc^- phenotype and resulted in kanamycin sensitivity, suggesting the possibility of the occurrence of the spontaneous Kan^R in the F_1 region of the ATP synthase in the mutants. The results of the complementation experiments suggested that the mutations could be in any one of the five genes for the F_1 region of ATP synthase (*atpH*, *atpA*, *atpG*, *atpD*, *atpC*).

In earlier experiments, among the three mutants HG205, HG203 and HG206, HG205 showed the highest increase in Pck specific activity when transformed with the plasmid pBWU1.3, which expresses the complete *atp* operon. Therefore, HG205 was used for further complementation studies.

Table 12. Complementation of the Suc$^-$ phenotype on transformation with plasmids expressing different subunits of ATP synthase

	Plasmid	Subunits expressed	Glucose	Succinate	Pyruvate	Amp^R	Cam^R	Kan^R	LB
HG205	pACWU1.2	α mutation	+++	-	-	+++	NA	+++	+++
	pACWU1.2	β mutation	+++	-	-	+++	NA	+++	+++
	pDM8	b	+++	-	-	+++	NA	+++	+++
	SBV11	a	+++	-	-	NA	+++	+++	+++
	pDJK20	a, c, b	+++	-	-	NA	+++	+++	+++
	pRPG51	b, δ	+++	-	-	+++	NA	+++	+++
	pDJK35	**δ, α, γ, β, ε**	**+++**	**++**	-	NA	**+++**	-	**+++**
HG203	pACWU1.2	α mutation	+++	-	-	+++	NA	+++	+++
	pACWU1.2	β mutation	+++	-	-	+++	NA	+++	+++
	pDM8	b	+++	-	-	+++	NA	+++	+++
	SBV11	a	+++	-	-	NA	+++	+++	+++
	pDJK20	a, c, b	+++	-	-	NA	+++	+++	+++
	pRPG51	b, δ	+++	-	-	+++	NA	+++	+++
	pDJK35	**δ, α, γ, β, ε**	**+++**	**++**	-	NA	**+++**	-	**+++**
HG206	pACWU1.2	α mutation	+++	-	-	+++	NA	+++	+++
	pACWU1.2	β mutation	+++	-	-	+++	NA	+++	+++
	pDM8	b	+++	-	-	+++	NA	+++	+++
	SBV11	a	+++	-	-	NA	+++	+++	+++
	pDJK20	a, c, b	+++	-	-	NA	+++	+++	+++
	pRPG51	b, δ	+++	-	-	+++	NA	+++	+++
	pDJK35	**δ, α, γ, β, ε**	**+++**	**++**	-	NA	**+++**	-	**+++**

+++ Growth observed after incubation at 37°C for 24 h. Succinate and pyruvate plates were incubated for 48 h.
- No growth observed after incubation at 37°C for 24 h on kanamycin, ampicillin chloramphenicol or LB plates or after 48 h on succinate and pyruvate plates
NA Not applicable

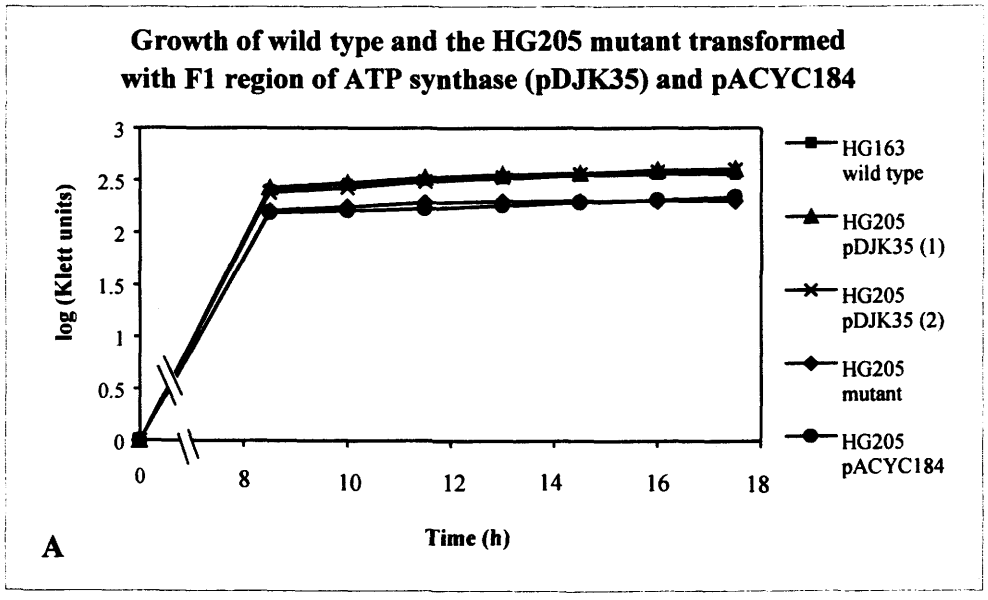
Ten separate transformants were screened for each plasmid for all the three mutants. The same results obtained for all the ten transformants. The plasmid that complemented the Suc$^-$ phenotype is indicated in bold.

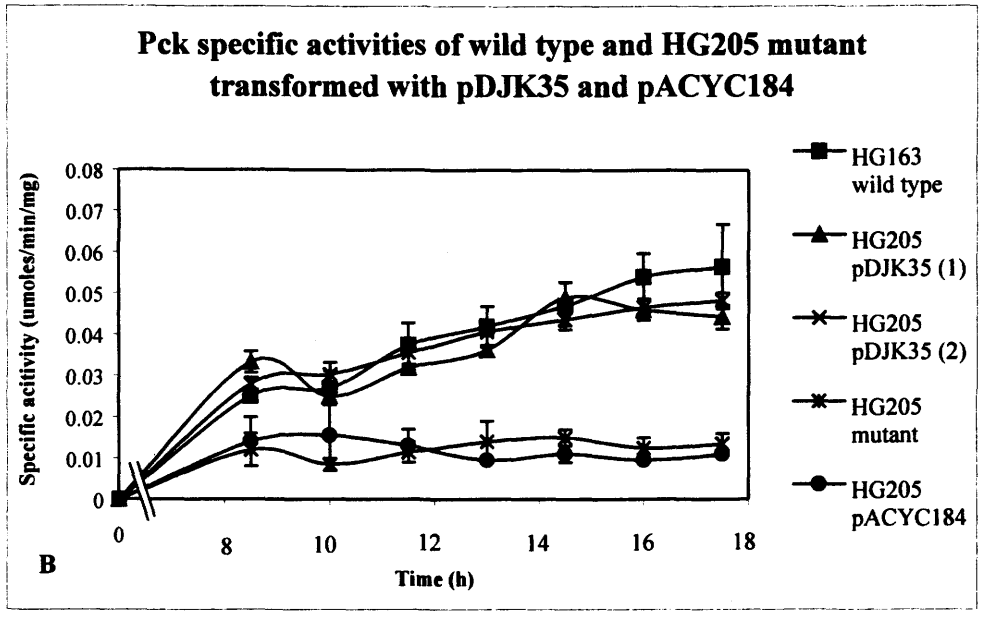
3.7.2 Complementation of the Pck$^-$ phenotype of mutant HG205 by an *atpH, A, G, D, C* plasmid

Growth and Pck specific activity was determined in HG205 transformed with plasmid pDJK35 expressing the F_1 region of the ATP synthase, (δ, α, γ, β and ε subunits). Plasmid pDJK35 is a derivative of pACYC184 and is therefore a low copy number plasmid. **Figure 20A** shows the growth yield of the HG205 mutant transformed with pDJK35. The assay was done in duplicate for two separate transformants (numbered as 1 and 2). Growth of pDJK35 transformed HG205 was comparable to the wild type. HG205 mutant control and, HG205 transformed with vector pACYC184 (control) had growth yields lower than the pDJK35 transformed HG205. **Figure 20B** shows that the Pck specific activity of HG205 transformed with pDJK35 was about five fold higher than the HG205 control. HG205 transformed with the vector had Pck specific activity slightly higher than the HG205 mutant but was significantly lower than the HG205 mutant transformed with pDJK35. The Pck specific activity of the pDJK35 transformed HG205 was comparable to the wild type. This indicates that the Pck$^-$ phenotype of mutant HG205 was complemented by a plasmid expressing the F_1 region of ATP synthase.

Figure 20. Effect of transformation with plasmid expressing F_1 region of ATP synthase on growth and Pck specific activity in mutant HG205.

Growth and Pck specific activities of HG205 mutant transformed with pDJK35. HG205 mutant; HG205 transformed with vector pACYC84; and wild type HG163; were used as controls. Two separate colonies of the Suc^+ transformants were used for the Pck assay. They have been denoted as HG205 pDJK35 (1) and HG205 pDJK35 (2). **A.** Growth of the pDJK35 transformed mutant and the controls. **B.** Pck assays on CTAB treated cells of HG205 transformed with pDJK35 (*atpH, atpA, atpG, atpD, atpC*), wild type HG163 and HG205 controls. The experiment was repeated three times and the figure shows representative data.


Growth of wild type and the HG205 mutant transformed with F1 region of ATP synthase (pDJK35) and pACYC184
log (Klett units)
3
2.5
2
1.5
1
0.5
0
0
8
10
12
14
16
18
Time (h)
HG163 wild type
HG205 pDJK35 (1)
HG205 pDJK35 (2)
HG205 mutant
HG205 pACYC184
A


Pck specific activities of wild type and HG205 mutant transformed with pDJK35 and pACYC184
Specific acitivity (umoles/min/mg)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
8
10
12
14
16
18
Time (h)
HG163 wild type
HG205 pDJK35 (1)
HG205 pDJK35 (2)
HG205 mutant
HG205 pACYC184
B

3.7.3 Complementation of the ATP synthase$^-$ phenotype of mutant HG205

The transformation of mutant HG205 with a plasmid expressing the F_1 region of the ATP synthase (pDJK35) resulted in complementation of the Suc$^-$, Kan^R and Pck$^-$ phenotypes. The next step was to check for complementation of the ATP synthase$^-$ phenotype of the HG205 mutant. Inside-out vesicles of mutant HG205 transformed with pDJK35, HG205 mutant control and HG163 wild type control were assayed for ATP synthase. **Figure 21** shows that the ATP synthase specific activity of the HG205 mutant transformed with pDJK35 was comparable the wild type and was higher than the HG205 mutant control. In the presence of Mg^{2+}, the ATP synthase specific activity of pDJK35 transformed HG205 was much higher than the specific activity in the absence of Mg^{2+}. The specific activity was significantly lower in the presence of DCCD. This demonstrates the F_0F_1 ATP synthase was being measured since DCCD is a specific inhibitor of F_0F_1 ATP synthase.

The results of the complementation experiments with the complete *atp* operon plasmid and plasmids expressing the F_1 region of ATP synthase suggest a relationship between the Pck expression and ATP synthase activity. It will be interesting to determine how ATP synthase affects Pck expression, whether the effect is at transcriptional or translational level or some other level. The effect could also be due to the levels of ATP or could be via an effect on the pH gradient, which might affect the assembly or activity of the enzyme.

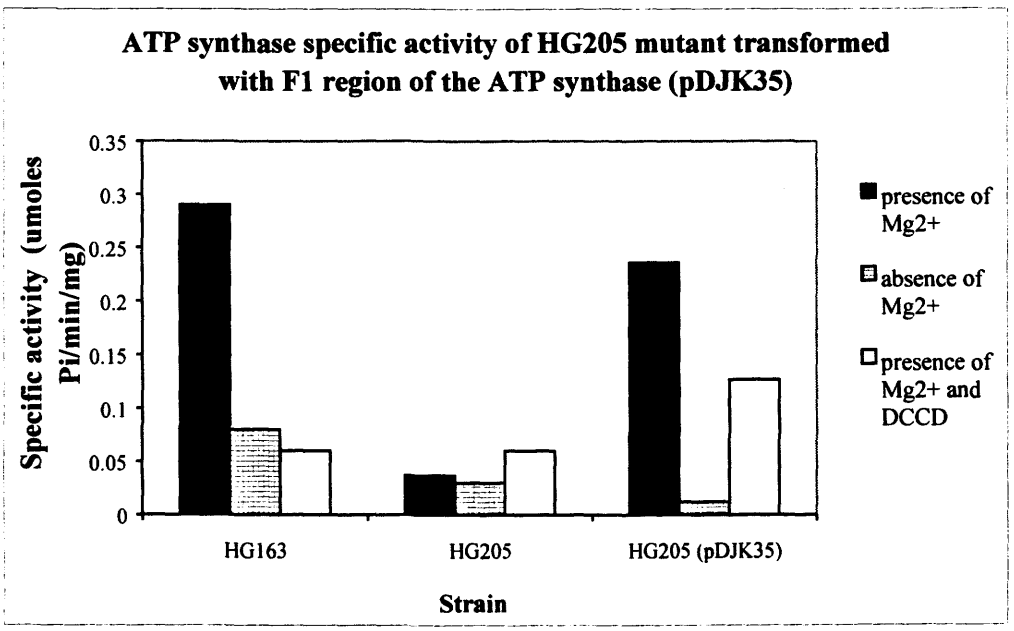


Figure 21. ATP synthase specific activity of mutant HG205 transformed with plasmid pDJK35 encoding the F_1 region of ATP synthase

ATP synthase specific activity in inside out vesicles of HG205 mutant complemented with pDJK35 expressing the F_1 region of ATP synthase. The specific activity was determined in the presence of Mg^{2+}, absence of Mg^{2+} and in the presence of inhibitor DCCD.

3.7.4 ATP synthase activity in an *atpB-C* deletion strain

An *atp* deletion strain was constructed by cotransducing an *atpB-C* deletion with *ilvD*::Tn*10* into *E coli* K12 strain 1100 to produce strain DK8 Δ (*uncB-uncC*) (Klionsky, *et al.*, 1984). ATP synthase specific activities of the wild type HG163 and DK8 were determined (**Figure 22).** The activity of ATP synthase in the deletion strain was much lower than the wild type. In the wild type, the ATP synthase activity in the absence of Mg^{2+} was significantly lower than the specific activity in the presence of Mg^{2+}.

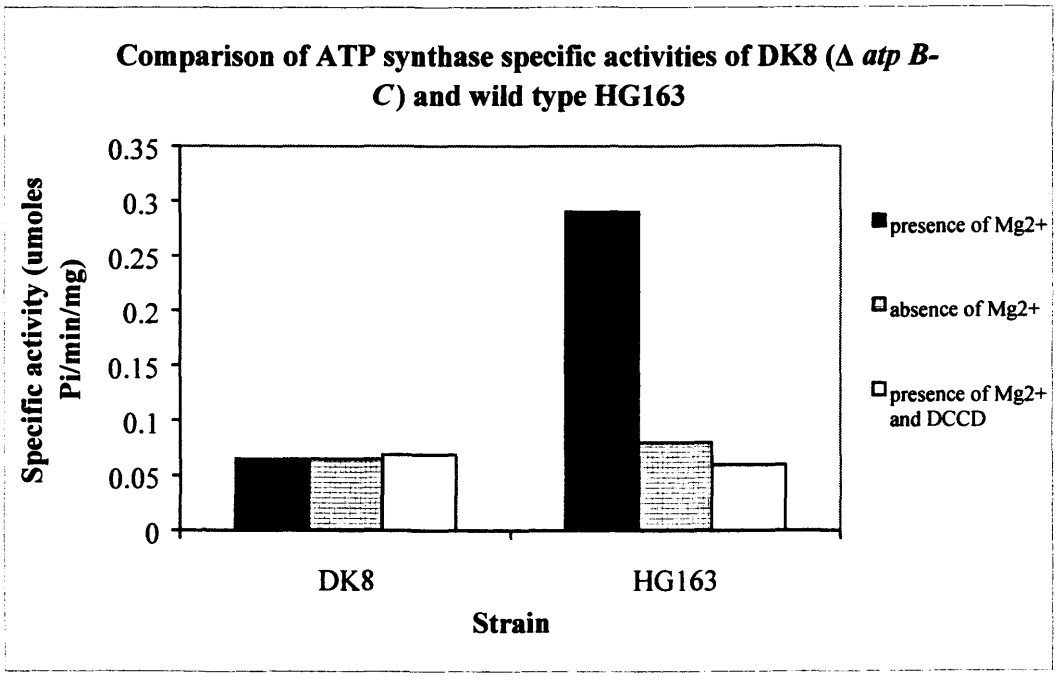


Figure 22. ATP synthase specific activity of Δ *atpB-C* strain DK8 and HG163.

ATP synthase specific activity in inside-out vesicles of wild type HG613 and *atp* deletion strain DK8, in the presence of Mg^{2+}, absence of Mg^{2+} and inhibitor DCCD.

3.8 Absence of large deletions in the *atp* operon in mutants HG205, HG203 and HG206

Since the plasmid expressing the F_1 region of ATP synthase complemented the Suc^-, Kan^R, Pck^- and the ATP $synthase^-$ phenotypes of mutants HG205, HG203 and HG206, it was inferred that there could be a mutation in the genes encoding the F_1 region of ATP synthase in the mutants. DNA from three mutants was amplified using primers homologous to the *atp* operon. The sets of primers used for amplification are indicated in **Figure 23**. Wild type HG163 was used as the control to determine if there were any large deletions of this region in the mutants. No difference in banding pattern was observed between the mutants and the wild type **(Figures 24, 25, and 26).** This indicated that there were no large deletions in the three mutants. It was hypothesised that a small deletion or a point mutation that cannot be detected by PCR is present in these mutants. It was decided to sequence the mutant DNA.

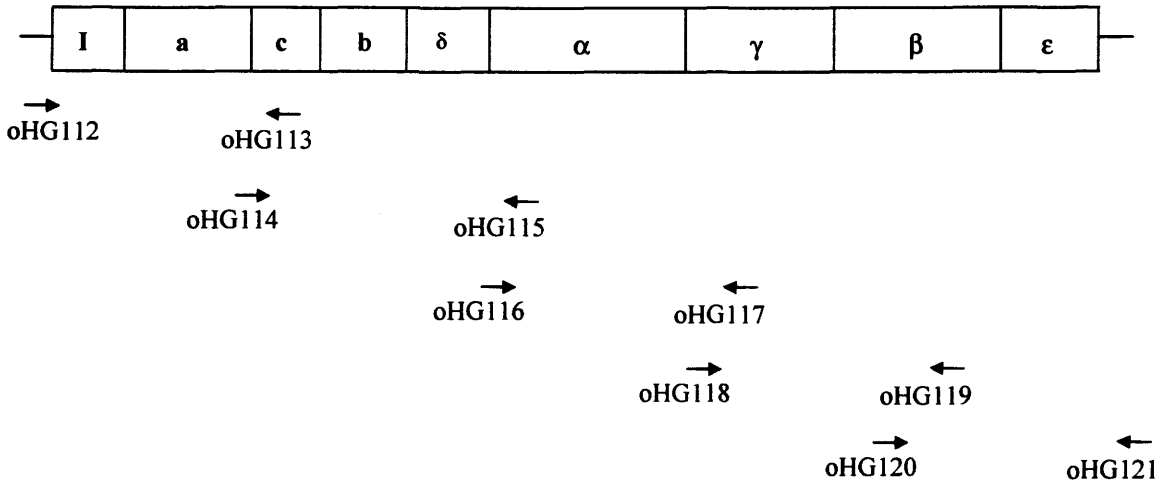


Figure 23. Sets of primers used to amplify *atp* (*I-C*) of the *atp* operon (F_0F_1 ATP synthase) in mutants HG205, HG203 and HG206.

The expected size of the amplified fragment is *c.a.* 1.5 kb with the primer sets.

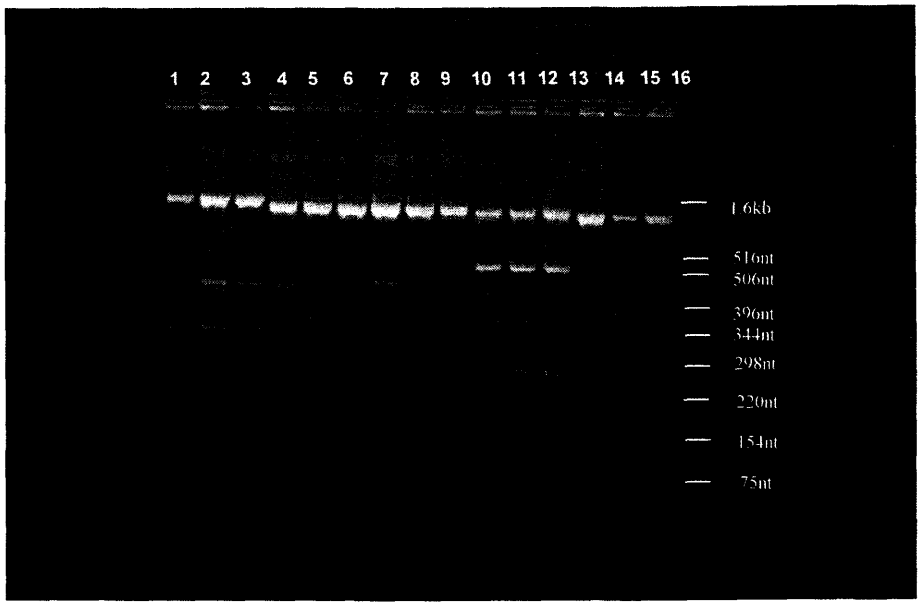


Figure 24. PCR amplification of the *atp* operon encoding F_0F_1 ATP synthase in mutant HG205.

Lane1 MM294A with oHG112 and oHG113 primers
Lane2HG205 with oHG112 and oHG113 primers
Lane3 HG163 with oHG112 and oHG113 primers
Lane4 MM294A with oHG114 and oHG115 primers
Lane5HG205 with oHG114 and oHG115 primers
Lane6 HG163 with oHG114 and oHG115 primers
Lane7 MM294A with oHG116 and oHG117 primers
Lane8 HG205 with oHG116 and oHG117 primers
Lane9 HG163 with oHG116 and oHG117 primers
Lane10 MM294A with oHG118 and oHG119 primers
Lane11HG205 with oHG118 and oHG119 primers
Lane12 HG163 with oHG118 and oHG119 primers
Lane13 MM294A with oHG120 and oHG121 primers
Lane14 HG205 with oHG120 and oHG121 primers
Lane15 HG163 with oHG120 and oHG121 primers
Lane16 marker (pBR322 digested with *HinfI*)

The PCR amplification was done using MM294A and HG163 (genotypes explained in section 2.7.1) as the controls. Primers oHG112 and oHG113 amplifies *atpI-E*, oHG114 and oHG115 amplifies *atpB-A*, oHG116 and oHG117 amplifies *atpH-G*, oHG118 and oHG119 amplifies *atpA-D* and oHG120 and oHG121 amplifies *atpD*-end of the *atp* operon.

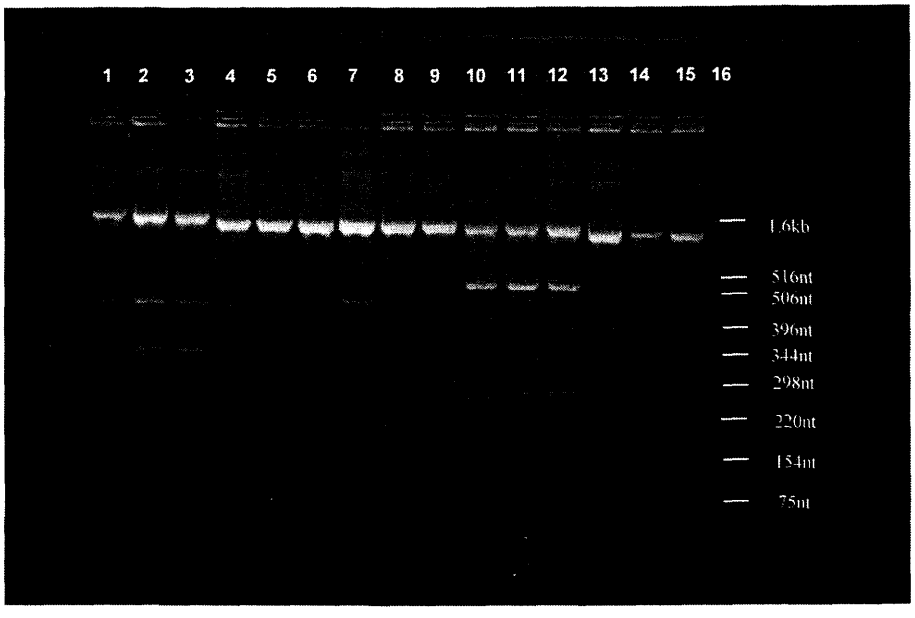


Figure 25. PCR amplification of the *atp* operon encoding F_0F_1 ATP synthase in mutant HG203.

Lane1 MM294A with oHG112 and oHG113 primers
Lane2HG203 with oHG112 and oHG113 primers
Lane3 HG163 with oHG112 and oHG113 primers
Lane4 MM294A with oHG114 and oHG115 primers
Lane5HG203 with oHG114 and oHG115 primers
Lane6 HG163 with oHG114 and oHG115 primers
Lane7 MM294A with oHG116 and oHG117 primers
Lane8 HG203 with oHG116 and oHG117 primers
Lane9 HG163 with oHG116 and oHG117 primers
Lane10 MM294A with oHG118 and oHG119 primers
Lane11HG203 with oHG118 and oHG119 primers
Lane12 HG163 with oHG118 and oHG119 primers
Lane13 MM294A with oHG120 and oHG121 primers
Lane14 HG203 with oHG120 and oHG121 primers
Lane15 HG163 with oHG120 and oHG121 primers
Lane16 marker (pBR322 digested with *HinfI*)

The PCR amplification was done using MM294A and HG163 (genotypes explained in section 2.7.1) as the controls. Primers oHG112 and oHG113 amplifies *atpI-E*, oHG114 and oHG115 amplifies *atpB-A*, oHG116 and oHG117 amplifies *atpH-G*, oHG118 and oHG119 amplifies *atpA-D* and oHG120 and oHG121 amplifies *atpD*-end of the *atp* operon.

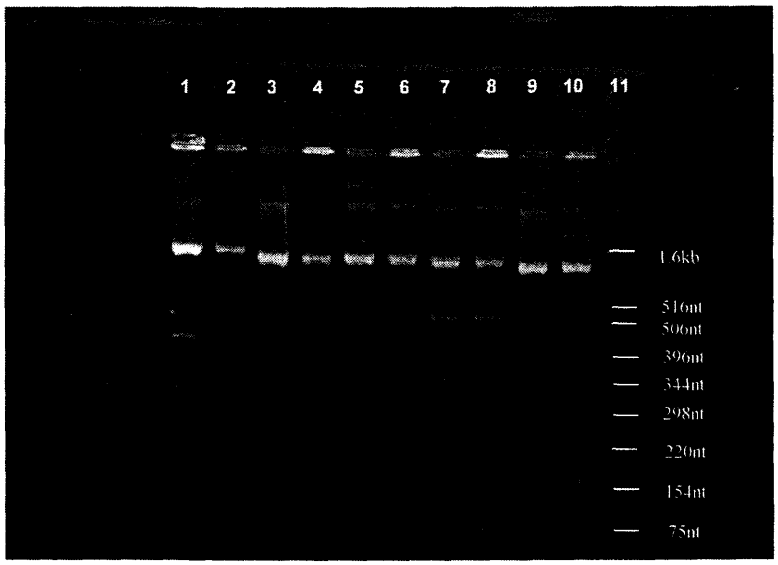


Figure 26. PCR amplification of the *atp* operon encoding F_0F_1 ATP synthase in mutant HG206.

Lane1 MM294A with oHG112 and oHG113 primers
Lane2 HG206 with oHG112 andoHG113 primers
Lane3 MM294A with oHG114 and oHG115 primers
Lane4HG206 with oHG114 and oHG115 primers
Lane5 MM294A with oHG116 and oHG117 primers
Lane6 HG206 with oHG116 and oHG117 primers
Lane7 MM294A with oHG118 and oHG119 primers
Lane8 HG206 with oHG118 and oHG119 primers
Lane9 MM294A with oHG120 and oHG121 primers
Lane10 HG206 with oHG120 and oHG121 primers
Lane11 marker (pBR322 digested with *HinfI*)

The PCR amplification was done using MM294A (genotype explained in section 2.7.1) as the control. Primers oHG112 and oHG113 amplifies *atpI-E*, oHG114 and oHG115 amplifies *atpB-A*, oHG116 and oHG117 amplifies *atpH-G*, oHG118 and oHG119 amplifies *atpA-D* and oHG120 and oHG121 amplifies *atpD*-end of the *atp* operon.

3.9 Sequencing the Suc$^-$ Kan^R Pck$^-$ mutations

The *atp* operon is at 84.6 min on the *E coli* chromosome. The size of the operon is 7.86 Kb and it consists of eight genes. Subunits *a*, *c* and *b* encoded by *atpB*, *atpE* and *atpF* comprise the F_0 region of the ATP synthase. Subunits δ, α, γ, β and ε encoded by *atpH*, *atpA*, *atpG*, *atpD* and *atpC* make up the F_1 region of ATP synthase. The *atp* operon was amplified in the mutants using six primer sets shown in Figure 23. The wild type HG163 was also amplified with these primers. The amplified regions were sequenced with the primers distinct from the primers used for PCR. Sequencing was done with 18 different primers so as to obtain overlapping sequences.

In all of the three mutants sequenced, the mutation was in *atpG*, encoding the γ subunit of ATP synthase. The mutations were confirmed by sequencing the opposite strand. Sequencing results of both strands are shown in **Figure 27.** In mutants HG205 and HG203, there is a "GC" deletion in the *atpG* gene. This leads to a frame shift and truncation of 28 amino acids at the carboxyl terminal end. The nucleotides that have been deleted are indicated in bold in the wild type sequence. The sequence was compared to wild type HG163, which is the isogenic parent. The GC deletion is at nt 5530-5531 (using the numbering of Walker *et al.*, 1984). In mutant HG206, there is a deletion of "T" in *atpG* at position 5538 in the Walker sequence. This causes a truncation of 40 amino acids at the carboxyl terminus in the HG206 mutant.

Figure 27. DNA sequence of the F_1 region of ATP synthase in mutants HG205, HG203 and HG206.

Nucleotides 5503-5588 of the Walker sequence is shown. All the three mutants have a deletion in the *atpG* coding for the γ subunit of F_0F_1 ATP synthase. The complete F_1 region was sequenced in all the three mutants. The deletion is indicated by * in the mutants. The mutated nucleotides are indicated in bold letters in the wild type.

Published V V E N L A S E D A A R M V A M K A A T D N G G S L I K
Sequence GTGGTTGAAAACCTGGCCAGCGAGCAG**GC**CGCCCG**T**ATGGTGGCGATGAAAGCCGCGACCGACAATGGCGGCAGCCTGATTAAGA
(Walker *et al.*, 1984)

HG163 V V E N L A S E D A A R M V A M K A A T D N G G S L I K
wild type GTGGTTGAAAACCTGGCCAGCGAGCAG**GC**CGCCCG**T**ATGGTGGCGATGAAAGCCGCGACCGACAATGGCGGCAGCCTGATTAAGA

HG205 V V E N L A S E D R P Y G G D E S R D R Q W R Q P D **Stop**
Mutant GTGGTTGAAAACCTGGCCAGCGAGCAG******CGCCCG**T**ATGGTGGCGATGAAAGCCGCGACCGACAATGGCGGCAGCCTGAT**TAA**GA

HG203 V V E N L A S E D R P Y G G D E S R D R Q W R Q P D **Stop**
Mutant GTGGTTGAAAACCTGGCCAGCGAGCAG******CGCCCG**T**ATGGTGGCGATGAAAGCCGCGACCGACAATGGCGGCAGCCTGAT**TAA**GA

HG206 V V E N L A S E D A A R W W R **Stop**
Mutant GTGGTTGAAAACCTGGCCAGCGAGCAG**GC**CGCCCG*****ATGGTGGCGA**TGA**AAGCCGCGACCGACAATGGCGGCAGCCTGATTAAAG

The opposite strand was sequenced and it showed the same mutation.

Published
Sequence TTAATCAGGCTGCCGCCATTGTCGGTCGCGGCTTTCATCGCCACCAT**A**CGGGCG**GC**CTGCTCGCTGGCCAGGTTTTCAACCACGCCC

HG163 TTAATCAGGCTGCCGCCATTGTCGGTCGCGGCTTTCATCGCCACCAT**A**CGGGCG**GC**CTGCTCGCTGGCCAGGTTTTCAACCACGCCC

Stop
HG205 **TTA**ATCAGGCTGCCGCCATTGTCGGTCGCGGCTTTCATCGCCACCAT**A**CGGGCG******CTGCTCGCTGGCCAGGTTTTCAACCACGCCC

Stop
HG203 **TTA**ATCAGGCTGCCGCCATTGTCGGTCGCGGCTTTCATCGCCACCAT**A**CGGGCG******CTGCTCGCTGGCCAGGTTTTCAACCACGCCC

Stop
HG206 **TTA**ATCAGGCTGCCGCCATTGTCGGTCGCGGCTTTCATCGCCACCAT*****CGGGCG**GC**CTGCTCGCTGGCCAGGTTTTCAACCACGCCC

3.10 Complementation of the Suc$^-$ phenotype in mutant HG205 with plasmid expressing γ subunit of ATP synthase

Mutant HG205 (Suc$^-$) was transformed with plasmid pBWG15 (Shin *et al*, 1992) expressing the γ subunit of ATP synthase. Plasmid pBWG15 is a pBR322 derivative and *atpG* is under the control of the β-lactamase promoter.

Transformants were selected on tetracycline plates and eight transformants were checked on minimal medium plates containing glucose, succinate, and pyruvate as carbon sources and also on LB plates containing tetracycline and kanamycin. Mutant HG205 was also transformed with pBR322 as a control (**Table 13**). Plasmid expressing the γ subunit complemented the Suc$^-$ and Kan^R phenotype in mutant HG205, which confirms that the mutation that is causing the Pck$^-$, Suc$^-$ and Kan^R phenotypes lies in the γ subunit encoded by *atpG* of the *atp* operon.

Table 13. Transformation with plasmid pBGW15 expressing the γ subunit of ATP synthase restores the Suc^+ phenotype in mutant HG205

HG205 transformed with pBWG15	Glucose	Succinate	Pyruvate	Tet^R	Kan^R
1	+++	++	-	+++	-
2	+++	++	-	+++	-
3	+++	++	-	+++	-
4	+++	++	-	+++	-
5	+++	++	-	+++	-
6	+++	++	-	+++	-
7	+++	++	-	+++	-
8	+++	++	-	+++	-

+++ Growth
- No growth
LB + tetracycline, LB + kanamycin and minimal medium + glucose plates were incubated at 37°C for 24 h. Minimal medium + succinate and Minimal medium + pyruvate plates were incubated at 37°C for 48 h.

CHAPTER FOUR

4.0 Discussion and Conclusions

Mutants of *Escherichia coli* containing genetic fusions of *lacZ* to the *pckA* locus were isolated using Mu d1 (*lacZ* Amp^R) bacteriophage (Goldie, 1984). Synthesis of β-galactosidase in these strains is regulated by cyclic AMP and by carbohydrates (catabolite repression). Synthesis of β-galactosidase by *pck-lacZ* fusions was induced in log-phase cells growing on gluconeogenic media, was repressed by glucose, and was also induced up to 100 fold at the onset of stationary phase in LB medium (Goldie, 1984). This stationary phase induction required cyclic AMP and some other unknown regulatory signal since induction occurs even in the presence of 5mM cAMP.

Stationary phase sigma factor, σ^S, encoded by *rpoS* is a very possible candidate involved in the regulation of *pckA* in stationary phase. We transduced *rpoS*::Tn*10* (Schellhorn *et al.*, 1992) into a *pck-lacZ* strain (Goldie and Medina, 1990). There was no effect of the *rpoS*::Tn*10* insertion on the transcriptional fusion as observed by the fact that all the transductants were the same blue colour on X-gal plates as the $rpoS^+$ controls. There is no difference in β-galactosidase activity in a *pck-lacZ* fusion in a $rpoS^+$ or a $rpoS^-$ background (data not shown). This indicates

that induction of *pckA* is probably not dependent on σ^S. A number of other stationary phase regulators were also ruled out (nitrogen limitation *glnG*, *relA* (Goldie and Sanwal, 1980a), phosphate limitation (*phoR*), oxygen limitation (*arcA*, *fnr*). It was decided to isolate mutants to address the question of how *pckA* is regulated in stationary phase.

4.1. Mutagenesis

Mutagenesis was carried out using mini Tn*10*-ATS (Kleckner *et al.*, 1991) for isolating regulatory mutants of *pckA*. MiniTn10-ATS inserts at random sites on the *E. coli* chromosome. Mini Tn*10*-Kan/Ptac-ATS transposase is a derivative of Tn*10*. It has altered target specificity, which makes it less target specific and also the mini Tn*10* lacks the transposase gene, causing it to generate more stable insertions and has a Kan^R marker, which is stable and can be easily selected for. We isolated 72 Suc^- mutants, assayed for Pck specific activity and seven mutants having low Pck specific activity were used for further studies. Experiments with a *recA* strain and *pckA*::Tn*10* insertion showed that the low Pck specific activity in the mutants was not due to slow growth but could be vice versa. The *recA* mutants have slow growth and lower growth yield, probably due to a defect in recombination and DNA repair pathway (Michel *et al.*, 1997). Pck specific activity in the *recA* mutant although slightly lower, was still comparable to the wild type though the growth of the *recA* mutant was significantly lower. Pck specific activity in the *pckA*::Tn*10* was undetectable, but

the growth yield was comparable to the wild type. The results from the *pckA*::Tn*10* and *recA* mutant indicate that growth yield and Pck specific activity were not related.

4.2 Characterisation of mutants

The mutants were further checked for genetic linkage to *asd*, as *pck* is linked 20-30% to *asd* (Goldie and Sanwal, 1980a). In four mutants, HG203, HG204, HG205 and HG206 the Kan^R Suc^- was not linked to *asd* and therefore not to *pckA*. Gel shift experiments have indicated the possibility of three Crp binding sites near the *pckA* promoter region and two Crp binding sites have been identified by DNA footprinting studies (Goldie, unpublished results). The main element for catabolite repression is the cAMP-Crp complex. Previous mutagenesis carried out using Mu d1 phage to isolate regulatory mutants of *pckA* yielded all Mu d1 insertions in *cya* (Chan, Y., M.Sc. Thesis). Another mutagenesis attempted using Tn10 also yielded some mutants in *cya* (Goldie, unpublished results). Considering the fact that *cya* mutations seem to be one of the important class of mutants that rise while selecting for regulatory mutants of *pckA*, it was surprising that our mutagenesis screen did not yield any insertions in *cya*. Mutants defective in *cya* or *crp* will not ferment sugars such as maltose and arabinose since cAMP-Crp complex is required for the activation of the *mal* regulon and *ara* operon. Mutants HG203, HG204, HG205 and HG206 fermented these sugars; therefore, the mutations affecting *pckA* expression were not in *crp* or *cya*.

4.2.1 Effect of *pps*$^+$ on Suc$^-$ phenotype in the mutants

PEP can be synthesised during gluconeogenesis by conversion of pyruvate to PEP by phosphoenolpyruvate synthase (Pps) or by conversion of Krebs cycle intermediates to OAA and conversion of OAA to PEP by phosphoenolpyruvate carboxykinase. Cells which are *pps*$^+$ and *pckA*$^-$ grow on succinate as a carbon source (Goldie and Sanwal, 1980a). This is because succinate is converted to malate and malate to pyruvate by NAD dependent malic enzyme and PEP synthase converts it to pyruvate. Only *pps*$^-$ *pckA*$^-$ strains have a Suc$^-$ phenotype. When the mutants HG203, HG204, HG205, HG206 were tranduced to *pps*$^+$, all the transductants were Suc$^-$ and Kan^R, which shows that the Suc$^-$ was not solely due to the down regulation of *pckA*, but probably due to a mutation in some other locus.

4.2.2 Identification of the insertion

Mini Tn10-ATS derivative 103 which we used in mutagenesis, has two ends which are perfect inverted repeats of a 70 bp segment carrying the outside end of the IS10R (Kleckner *et al.*, 1991). PCR amplification was carried out for detection of the insertion in our mutant from the ends of the transposon using an IS10 primer and the REP primer. REP (Repetitive Extragenic Palindrome) comprises about 1% of the *E. coli* genome. The ~35 bp palindromic sequence has been identified in intercistronic regions. Subramanian *et al.* (1992) have used an outward facing primer corresponding to either half of the REP palindrome and a Tn5 specific primer to amplify chromosomal DNA from a Tn*5* insertion mutant strain. PCR amplification

products contain unique sequences that lie between the transposon and adjacent REP sites as well as between adjacent REP sequence. The amplified PCR products from our mutants had a short region with 97% homology to *hybF*, a regulatory gene in the hydrogenase operon. However, transformation of the mutants with plasmids expressing the *hyb* operon products did not complement the Suc$^-$ phenotype. PCR done with hyb primers showed no difference between the wild type and the mutants, which indicated the absence of mini Tn10 insertions in the *hyb* operon in the mutants. The original PCR products obtained using an IS10 primer and REP primer were due to a 10 base pair sequence in the mini Tn10 ends which is identical to part of *hybF*.

4.3 Identification of spontaneous mutations in the *atp* operon

A spontaneous mutation probably made the mutants Suc$^-$ and Kan^R. A mutation isolated earlier (*atpA*::Tn*10*) results in 10% Pck activity and is Suc$^-$ (Goldie, unpublished results). During oxidative phosphorylation, which occurs during growth on succinate, ATP synthase synthesizes ATP utilising a pH gradient. Mutants in the *atp* operon cannot grow on nonfermentable carbon sources like succinate and also have a lower growth yield (Downie *et al.*, 1980). The Suc$^-$ phenotype of the *atp* operon mutants may be also due to a succinate transport deficiency (Boogerd *et al.*, 1998) or due to a defect in oxidative phosphorylation. Mutations in *atp* genes can also make the cells resistant to aminoglycoside antibiotics (Thorbjarnardottir *et al.*, 1978), kanamycin being an aminoglycoside antibiotic. The mutations that confer

resistance to aminoglycoside were first identified as mutants that are defective in membrane energisation mediated by ATP synthase, which were unable to maintain a normal proton gradient. Therefore the *atp* mutants are probably resistant to kanamycin due to an altered electrochemical gradient, which causes a defect in antibiotic transport into the cell.

Transduction of mutants HG203, HG205 and HG206 with Tn10 insertions mapping near the *atp* operon showed that the Tet^R was linked to Kan^R and Suc^- phenotypes. All the Suc^+ transductants were Kan^S and vice versa showing that the Suc^- and the Kan^R are tightly linked. Linkage obtained was higher than the expected values. Only a 2% linkage should have been seen between the *ilvD* (85.3') and *atp* (84.6') based on physical map distance (Miller, 1992). In earlier experiments however, 40% cotransduction of the *atp* operon with *ilvD* has been observed (Rosen, 1973). The *atp* operon is very close to the origin of replication (84 min). Frequencies of cotransduction around the origin of replication seem to be high. This could probably be due to extensive recombination and replication that occurs around that region. We obtained a 100% linkage of *ilvD* to *atp* in the mutants. The same cotransduction frequency was observed with some other proximal markers but, as we moved away from *atp*, linkage was as expected. Transduction results indicate that Kan^R is very tightly linked to the phenotypes, Suc^- and Pck^-. It is quite likely that single mutations in an *atp* gene were responsible for both the phenotypes.

The *atp* deletion mutants are Kan^R Suc^-, probably due to uncoupling and disruption of the electrochemical gradient and also due to transport defects (Boogerd

et al., 1998). The mutants, HG203, HG204, HG205 and HG206, had the same Suc^- phenotype as the deletion mutant (Klionsky *et al.*, 1984). Transformation of mutants HG203, HG205 and HG206 with plasmids expressing the complete *atp* operon changed the phenotype to Kan^S Suc^+. The pBWU1.3 transformed mutants had increased Pck specific activity and growth yield. It has been seen that *atp* mutations have no effect on the growth rate but only the growth yield (Downie *et al.*, 1980). An ATP synthase deletion mutant has high growth rate on minimal medium with glucose as a carbon source, but has lower growth yields (Klionsky *et al.*, 1984). When the mutants HG203, HG205 and HG206 were transformed with intact *atp* operon, it led to increased growth yields. In the three mutants, Pck specific activity was not restored to wild type levels on transforming with the *atp* genes. This could be because the *atpG* mutation is co-dominant.

Mutants transformed with pBR322 had slightly higher Pck specific activity than the mutant controls. Tet^C (tetracycline transport protein) in pBR322 brings about aminoglycoside uptake by lowering the membrane potential or by increasing the level of oligopeptide binding or both (Eisenbraur and Griffith, 1993). They found that when vesicles were prepared from pBR322 transformed cells, the mean rates of oxygen consumption coupled to oxidation of succinate were about 172-292% higher than for vesicles made from the isogenic wild type parent. Possibly, multi copy pBR322 somehow lowers the membrane potential, which affects Pck specific activity in our mutants. This has not been investigated further. This might be an indication that membrane potential and pH gradient are linked to expression of the *pckA* gene.

Plasmid pDJK35 expressing the F_1 region of ATP synthase (Klionsky and Simoni, 1985) complemented the Suc$^-$ phenotypes of the mutants, HG203, HG205 and HG206 and the Pck$^-$ and ATPase$^-$ of mutant HG205. Later, transformation with plasmid pBWG15 expressing the γ subunit of ATP synthase (Shin *et al.*, 1992) complemented the Suc$^-$ phenotype in mutant HG205, which indicated that the mutation is in the γ subunit of ATP synthase.

4.4 Identification of the mutations in the *atpG* gene

In mutants HG203, HG205 and HG206, there was no mini Tn10-ATS insertion. In all the three mutants, the Kan^R mutations are in *atpG*, which is surprising given the fact that there are no reported hot spots for Tn*10* insertions in that region of the *E. coli* chromosome. Probably our initial penicillin selection procedure selected some spontaneous Kan^R mutants. In mutants HG203 and HG205, there are identical "GC" deletions in *atpG*, which bring about truncation of 28 amino acids at the carboxyl terminal end of the γ subunit of ATP synthase. These isolates could be siblings of a spontaneous mutation occurring before transposon mutagenesis. In mutant HG206, there is a "T" deletion also in *atpG*, which leads to a 40 amino acid truncation at the carboxyl terminus. The γ subunit of ATP synthase is a part of the catalytic site and is required for the formation of the minimal catalytic complex $\alpha_3\beta_3\gamma$ (Dunn and Futai, 1980). The γ subunit interacts with the catalytic β subunit. Suppressor mutations in the β subunit have been isolated which suppresses the effect

of a deletion of 16 amino acids of the γ subunit. This shows the interaction of the γ and β subunits and the functional involvement of the γ-β subunit interaction at the stalk (Jeanteur-De Beukelaer *et al.*, 1995). The δ and ε subunits connect to the F_0 and the other subunits, forming a part of the proton pathway through γ, which is a gateway of proton flow coupled to ATP hydrolysis or synthesis. The γ subunit does not have ATP synthase catalytic activity (does not bind ATP), but is essential for functioning of the ATP synthase F_1 complex.

Proton transport in the F_0F_1 ATP synthase is coupled to ATP synthesis via a change in conformation. The γ subunit participates in this by interacting with the other subunits and plays an important role in coupling ATP synthesis to proton transport (Iwamoto *et al.*, 1990). The carboxyl terminus of the γ subunit is important for ATP synthase catalytic activity. The γGln-269→end mutation had no activity (Shin *et al.*, 1992). Deleting either the carboxyl terminus or the amino terminus of the γ subunit, results in the failure of F_1 to assemble. Shin *et al.*, 1992, have shown that residues in this region are important for energy coupling. Site directed mutagenesis of the carboxyl terminus conserved amino acids caused lower ATP synthase activity indicating the importance of γ in catalysis. Nakamoto and Al-Shawi (1995), showed, through mutagenesis, that a conserved terminal region in the γ subunit is responsible for coupling of ATP synthesis to proton transport. It can be deduced that if the γ subunit is mutated, then ATP synthase will only act as a proton pore, disrupting the pH gradient in the cell. Mutation in the γ subunit of ATP synthase also confers aminoglycoside resistance to the cell (Hunbert and Attendorf,

1989). Aminoglycoside uptake occurs at lower membrane potential, when the γ subunit is mutated, the ATP synthase acts as a proton pore and the membrane potential is higher. This prevents the uptake of the antibiotic. Considering all the known facts about the γ subunit, it plays a very important function in catalysis, with every 120° rotation of the enzyme complex around the γ, one ATP is hydrolysed to energise the membrane (Garcia and Capadi, 1998). It will be interesting to determine how ATP synthase affects *pckA* expression, whether the effect is at transcriptional or translational or some other level. The effect could also be due to the levels of ATP or could be via an effect on the pH gradient, which might affect the assembly or activity of the enzyme.

When cells are grown on LB medium (without glucose), oxidative phosphorylation occurs to bring about ATP synthesis via the formation of a pH gradient. If the cells have a mutation in *atpG*, they will have a disrupted pH gradient, which could be compensated by having larger electrochemical gradient for K^+ and possibly for other ions and the membrane potential will be affected (Trchounian *et al.*, 1998). There is also likelihood that the *atpA*::Tn*10* mutant (Goldie, unpublished results), defective in the α subunit could cause a polar effect on the γ subunit, which would explain why the Pck specific activity in the *atpA*::Tn*10* strain is only 10% of the wild type.

It has been observed in intact cells that ADP stimulates glycolysis. When the H^+-ATP synthase concentration is reduced by mutation in the *atp* operon, the amount of ADP formed is high and it increases glycolysis. This may in turn lead to an

increased redox state of the cell and therefore leads to an increase in respiration (Jensen and Michelsen, 1992). Gluconeogenesis and glycolysis are opposing reactions and are stringently controlled. Therefore, it might be safe to say that an increase in ADP, symptomatic of low energy levels, might be inhibitory for gluconeogenesis, which is an energy requiring process. If ATP synthase is not functional, then ATP cannot be synthesised via H^+-ATP synthase and there is an accumulation of ADP in the cells, which can lead to stimulation of glycolysis. This can account for a control on respiration. This might lead to a futile cycle, accounting for low growth yields in the mutants.

During fermentative growth of E. coli, membrane permeable weak acids are excreted resulting in an increase in intracellular pH. The weak acids concentrate down the pH gradient acidifying the cytoplasm (Bock and Sawers, 1996). *E. coli* maintains its internal pH between 7.4 and 7.8 during aerobic growth at an external pH of 5.0 to 9.0 (Lambert *et al.*, 1997). Motile bacteria are guided to oxygen concentrations that favour growth of the species by a process called aerotaxis (Bespalov *et al.*, 1996). There is evidence that suggests that bacteria can sense changes in the proton motive force (shioi *et al.*, 1988). There is tight coupling of proton translocation to electron transport. The proton motive force across the membrane is the primary source of energy for the cell (Boyer, 1977). Sensing the changes in proton motive force help bacteria to find optimal conditions for energy generation. The changes in PMF could affect the induction of various gluconegenic enzymes like Pck.

According to the chemiosmotic theory of Mitchell, the link between synthesis of ATP and other energy requiring reactions is the transmembrane electrochemical gradient of protons, $\Delta\mu_H^+$, which gives rise to the proton motive force (Kashket, 1985). Components of proton motive force (PMF, Δp or protonic potential difference in mV) consist of pH, difference in intracellular pH (pH_i) and extracellular pH (pH_o) and $\Delta\chi$ (transmembrane potential in electric potential). In most bacteria, the interior is alkaline and negative as compared to the exterior. Given these conditions, Δp drives the H^+ into the cells. All measurements of Δp are based on a few assumptions (Kashket, 1985): (i) steady state conditions do exist, (ii) the Δp probes are free in solution, (iii) there is only one cellular compartment and (iv) the measurement of H^+ electrochemical gradients is a relevant one i.e., that is the mechanism by which $\Delta\mu_H^+$ generators (e.g. the respiratory chain) and $\Delta\mu_H^+$ consumers (e.g. H^+-ATP synthase) are coupled.

4.5 Models for regulation of *pckA* by *atp* and future directions

4.5.1 Regulation via effect on pH gradient

Since the γ subunit is involved in the assembly of the minimal complex for catalysis, ATP synthase with a faulty γ subunit will be incapable of ATP synthesis. In mutants HG203, HG205 and HG206, the ATP synthase is malfunctional and the activity is negligible. The mutants show a decrease in Pck specific activity. Probably *pckA* regulation senses and is dependent on the activity of ATP synthase. The effect

on *pckA* expression could be via a proton gradient or ATP levels since gluconeogenesis is an energy consuming process or a combination of both. To determine if this hypothesis holds true only for Pck or also for other gluconeogenic enzymes, could be tested by measuring the activity of other gluconeogenic enzymes in the mutants or even by testing the activity of major enzymes in the Kreb's cycle, like malate dehydrogenase, fumarase and succinate dehydrogenase.

The γ subunit is the gate for proton flow and it links ATP synthesis to proton translocation. Mutations in the γ subunit can result in free flow of protons across the membrane, which lead to disruption of the pH gradient. The pH gradient might affect the Pck activity in the cell, which will be interesting to investigate. To check the effect of the pH gradient on Pck enzyme activity, suspensions of the wild type can be treated with ionophores like carbonyl cyanide-p-trifluoro methoxyl phenyl hydrazone (FCCP) and tetrachloro salicylanilide (TCS). Similar experiments have been done on the tetrachloroethane respiration in Dehalospirillum meltivorans (Miller *et al.*, 1996). Measuring Pck specific activity in a strain that is known to have a disrupted pH gradient can be used as the control. Pck specific activity can also be determined in other *atp* mutants which have lost the ATP synthase but still has a pH gradient (i.e. not totally depolarized). This will give an idea of the effect of membrane polarity on Pck activity. It is not easy to measure PMF (ΔP) in bacteria with microelectrodes because of the small size. Therefore, direct measurements cannot be made. Flourescence of a number of acridine related amines are quenched when the dyes are taken up by membrane vesicles or by cells with acidic interiors (Deamer *et al.*, 1972).

These compounds have advantages of rapid response and detection at small concentrations. Considered to be the most reliable, 9-aminoacridine (9-AA), has been used with chromatophores as well as with *E. coli* and with everted vesicles (Kashket, 1985).

4.5.2 Regulation via changes in intracellular pH

Intracellular pH might also affect the expression/activity of Pck. The pH in the medium can be measured in inside out vesicles by electrodes, which can detect small changes in pH in the medium (Kashket, 1985). After transition of the cells from a de-energised (anaerobic) to an energised (aerobic) state when respiraton starts, the H^+ ions are extruded and the pH_O (pH outside) is more acidic. When respiration stops, H^+ is passively transported into the cell until there is a pH equilibrium between the inside and the outside of the cell. The ΔpH can be calculated from the change in pH and the external and the internal buffering powers can be measured by titration in detergent treated cells (Kashket, 1985). In *E. coli* there are many genes like *ompF*, *polA*, SOS genes (Olsen, 1989), mar regulon (Lambert *et al.*, 1997), etc. whose expression changes in response to pH.

4.5.3 Effect on *pckA* transcription

The *atpG* mutations might be affecting Pck at the genetic level. Mutations in the γ subunit might have a regulatory effect on *pckA* transcription. Transducing the mutation into a *pck-lacZ* fusion strain and assaying β-galactosidase levels can test this

hypothesis. Northern blots could also be done to determine the levels of the *pck* mRNA in stationary phase in the mutants and compared with an *atp* operon deletion DK8 (Klionsky *et al.*, 1984) and a wild type strain.

Another possibility is that ATP synthase interacts with a protein kinase or is a protein kinase and affects the phosphorylation of a protein that activates the expression of *pckA*.

From the results of this work, the *atpG* gene seems to play an important role in the expression of Pck activity. The *atpG* genes could regulate *pckA* at the transcriptional or protein levels. The *atpG* gene may be a component of a signal system for stationary phase induction of *pckA*, since mutants transformed with an *atpG* plasmid showed *pck* expression pattern and enzyme levels similar to the wild type. This opens up a new area of investigation of the stationary phase regulation of *pckA*. It will be interesting to investigate if there are other physiologically important molecules or processes that influence phosphoenolpyruvate carobxykinase in *Escherichia coli*, which an important enzyme in the regulation of gluconeogeneis in most organisms.

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