GENETIC MANIPULATION OF METABOLIC FLUXES IN *Escherichia coli*

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To...

the DIVINE Cause ...
of
the fine...
defined Course ...
of
effects —
the Fine Effects.....!

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I wish to pay special tribute to my wife, Airen for her remarkable understanding of the challenges and circumstances of the programme and for the dignity with which she coped with asperity by association...

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ABBREVIATIONS, SYMBOLS AND DEFINITIONS

Abbreviations, symbols and units of measurement have been used in this thesis according to the recommendations of *The Biochemical Journal. vol. 265, no.1 (1990)* except for the following :

-amp ampicillin

Amino acids The standard 3-letter abbreviations are used in most instances

BCIG 5-*bromo*-4-*chloro*-3-*indolyl*-β-galactoside

bp nucleotide base pair

BSA Bovine Serum Albumin (F)

ß-IAA ß-indolylacetic Acid

cfu colony-forming bacterial units

cft colony-forming transformants (bacteria)

Dry wt. Dry Weight of Bacterial Biomass

ED Entner-Doudoroff (Pathway)

EMP Embden-Meyerhof-Parnas pathway

FBP/FDP Fructose-1,6-*bis*-phosphate

G-3-P Glyceraldehyde-3-phosphate

G-6-P Glucose-6-phosphate

6-PG 6-phosphogluconate

HPLC High Performance (Pressure) Liquid Chromatography

kb/kbp 1000 nucleotide base pairs

KDPG 2-Keto-3-deoxy-6-phosphogluconate

λ Bacteriophage-Lambda

λ_{nm} Wavelength in nanometres

MBN Mung Bean Nuclease

MGT Mean generation time (min.)

n.d. not determined

n.d.a no detectable (enzyme) activity

NTPs nucleotide triphosphates

OAA oxaloacetate

OD_n apparent optical density at λ_n

OGA 2-*oxo*glutarate

PEPc phospho*enol*pyruvate carboxylase

PEPs phospho*enol*pyruvate synthase

PG phosphoglycerate

ppm parts per million (concentration)

prep sample preparation

psi pound per square inch (pressure)

PTS Phospho*enol*pyruvate: Sugar Phosphotransferase System

pyk pyruvate kinase

pyr pyruvate

REact Custom-made reaction buffers for restriction enzymes (source: BRL)

soln solution

TBE Tris Borate EDTA Buffer

TCA Tricarboxylic Acid (cycle)

TNC Too numerous to Count (esp. bacterial colonies on plates)

TOC Total organic carbon

TOCA Total organic carbon analyzer

TP Triose phosphates

μ growth rate (per hour)

μ flux (mmol. $g.^{-1}. h.^{-1}$)

v/v sample volume per volume of solution

w/v sample weight per volume of solution

w/w sample weight per weight of solution

Enzyme Commission Numbers for Enzymes Used or Cited

EC numbers for enzymes are used according to the recommendation of the Nomenclature Committee of the International Union of Biochemistry on the nomenclature and classification of enzyme-catalysed reactions (1984).

Acetate kinase	EC 2.7.2.1
Adenylate cyclase	EC 4.6.1.1
Aspartase	EC 4.3.1.1
Citrate synthase	EC 4.1.3.7
Dihydrolipoamide dh	EC 1.8.1.4
DNA Polymerase	EC 2.7.7.7.
DNA Ligase	EC 6.5.1.1-2
Enolase	EC 4.2.1.11
Fructose *bis*phosphatase	EC 3.1.3.11
FBP-aldolase	EC 4.1.2.13
Fructose-PTS	EC 2.7.1.98
Fumarate reductase	EC 1.3.99.1
Glucose-6-P isomerase	EC 5.3.1.9
ß-D-Glucuronidase	EC 3.2.1.31
Glyceraldehyde-3-P dh	EC 1.2.1.12
sn-Glycerol-3-P dh ($NADP^+$)	EC 1.1.1.94
Glycerol kinase	EC 2.7.1.30
Hexokinase	EC 2.7.1.1
*Iso*citrate dehydrogenase	EC 1.1.1.42
*Iso*citrate lyase	EC 4.1.3.1
2-KDPG aldolase	EC 4.1.2.14
Lipoamide dh (NADH)	EC 1.6.4.3
Lipoate:oxidoreductase (Pdh)	EC 1.2.4.1
Malate dehydrogenase	EC 1.1.1.37
Malate synthase	EC 4.1.3.2
Nitrate reductase	EC 1.7.99.4
*Oxo*glutarate dh (lipoamide)	EC 1.2.4.2
PEP carboxylase	EC 4.1.1.31
PEP-protein PTS	EC 2.7.3.9
PEP synthase	EC 2.7.9.2
Phospho-2-dehydro-3-deoxyheptonate aldolase	EC 4.1.2.16
6-phosphofructokinase	EC 2.7.1.11
6-phosphoglucono lactone	EC 3.1.1.31
6-PG dehydratase	EC 4.2.1.12
Phosphogluconate dh	EC 1.1.1.44
Phosphoglycerate kinase	EC 2.7.2.3
PTS enzyme I	EC 2.7.3.9
PTS enzyme II	EC 2.7.1.69
Pyruvate dehydrogenase (cyt.)	EC 1.2.2.2
Pdh (lipoamide)	EC 1.2.4.1
Pyruvate kinase	EC 2.7.1.40
RNAse A	EC 3.1.27.5
Succinate dh (fumarase)	EC 1.3.9.1
Triose phosphate isomerase	EC 5.3.1.

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SYNOPSIS

The excretion of acetate by *E. coli* ATCC15224 is a form of metabolite leakage from the central metabolic pathways and a symptom of disproportionate partition of *input* carbon to metabolites required as precursors for cellular biosynthesis. Preliminary analysis of carbon flux through the central pathways suggested that throughputs of carbon to biosynthetic precursors from the tricarboxylic acid (TCA) cycle may be sub-optimal and limiting for the net synthesis of new cell material and that partition of flux at phospho*enol*pyruvate (PEP) was central to the throughputs of carbon to intermediate metabolites for the synthesis of about 70% of amino acids, 50% of nucleotides and eventually to the output of acetate. Carboxylation of PEP during flux through phospho*enol*pyruvate carboxylase (PEPc) played a primary role in throughput of carbon to the TCA cycle and two biosynthetic precursors—oxaloacetate and 2-*oxo*glutarate. Using a combination of flux and genetic manipulations, the activity of PEPc and throughput of PEP to oxaloacetate was amplified. Recombinant plasmid pJOE4 (9.36 kb) which contained the *ppc* gene encoding PEPc, was isolated from a comprehensive bank of *Escherichia coli* genomic DNA (in the form of a population of synthetic hybrid plasmids of *Sau*3A-generated fragments of *E. coli* chromosomal DNA interposing the unique *Bam*HI recognition sequence of plasmid vector pBR322) by complementing the PEPc lesion in transformants of *E. coli* PA342. The intrinsic PEPc activity of 24.4*nmol. mg.* protein.$^{-1}$. min.$^{-1}$ in *E. coli* ATCC15224, was overexpressed 75-fold in *E. coli* ATCC15224-pJOE4 recombinant strain. Frameshift mutation of plasmid pJOE4 by limited deletion of bases at a unique *Sac*II site on the insert,

produced recombinant plasmid pJOE12. No PEPc activity was detected in ultrasonic extracts of recombinant strain—*E. coli* PA342-pJOE12. Comparison of growth and substrate utilization by *E. coli* ATCC15224 and ATCC15224-pJOE4 showed that overexpression of PEPc activity diminished fluxes to acetate excretion from 5.12*mmol. g.* dry weight.$^{-1}$. hour.$^{-1}$, to 2.04*mmol. g.*$^{-1}$. h^{-1}, during aerobic metabolism of glucose. This was equivalent to 60% reduction in acetate excretion. The recombinant strain also excreted 34% less acetate during aerobic growth on pyruvate and 20-25% less acetate on D-gluconate, D-glucuronate respectively. Both strains did not operate fluxes to acetate excretion during fructose and glycerol metabolism. In addition, carbon conversion coefficients by *E. coli* ATCC15224-pJOE4 were enhanced to a greater or lesser extent, *vis-a-vis* the parent growing on the various substrates: $Y_{GLUCOSE}$ increased from 90.5*g.* dry weight. *mol.*$^{-1}$. to 114.4*g. mol.*$^{-1}$, which represented 27% improvement in growth yield. $Y_{FRUCTOSE}$ also increased by 9.4%, $Y_{PYRUVATE}$ by 6.7%, $Y_{GLUCURONATE}$ and $Y_{GLUCONATE}$ by 4.7 and 3.0 respectively. These effects were also demonstrated qualitatively in a recombinant strain of *E. coli* K10 transformed with plasmid pJOE4. Since growth on fructose did not result in acetate excretion, overexpression of PEPc activity probably increased growth yield by diminishing flux around a possible futile cycle between fructose-6-phosphate and fructose-1,6-*bis*phosphate. The complete abolition of this cycle was also a strong possibility. Overexpression of PEPc activity had no detectable effects on glycerol metabolism where the most important determinants of carbon flux partition were independent of PEP. A significant observation from the analysis of carbon flux in *E. coli* ATCC15224-pJOE4, was the strong likelihood of a zero flux through pyruvate kinase, as a

result of the increased demand for PEP by amplified PEPc activity. This was corroborated by experiments in *E. coli* HW1387 (*pykA, F*) which carried lesions for both pyruvate kinases A and F and *E. coli* HW0760, its parent strain. $Y_{GLUCOSE}$ in *E. coli* HW0760 was $72 g.\ mol.^{-1}$, at $\mu = 0.47 h.^{-1}$. Overexpression of PEPc activity in the pyruvate kinase-negative strain brought both growth rate and growth yield to the level obtained in the parent strain. However, these observation were complicated both by slow growth rates and non-excretion of acetate by *E. coli* HW0760 and *E. coli* HW1387-pJOE4. It is proposed that the contrasting effects of amplified PEPc activity on acetate excretion and growth yield, was possible through a combination of competitive reduction of flux from PEP to pyruvate via pyruvate kinase on the one hand, and increased oxidation of acetyl-CoA through increased flux of PEP to oxaloacetate, the coreactant/carrier-molecule of acetyl-CoA, on the other. The results demonstrate how a studied manipulation of metabolic fluxes can improve microbial productivity by enhancing conversion of input-carbon to desired outputs and conservation of feed-stock by arresting effluxes to undesirable outputs.

CHAPTER 1

GENERAL INTRODUCTION

1.1 METABOLISM : CONCEPT AND SCOPE

Cellular metabolism is a dynamic event involving an interplay of a myriad of biophysical and biochemical processes for producing, reproducing, sustaining and maintaining living systems (Kliger, 1916; Krebs, 1937; 1981; Gunsalus, 1948; Entner and Doudoroff, 1952; Dawes, 1986; Miles and Guest, 1987; Ratledge, 1987; Goodridge, 1990). Its scope includes nutrition (Snell, 1949; Owens and Legan, 1987; Linton, 1990) and growth (Monod, 1949; Morita, 1988; Wanner and Egli, 1990), while its course involves defined chemical reactions (Kornberg, 1965; Morowitz, 1978) whose complexity is often concealed by the arrows and chemical equations by which convention represents them (Davis, 1961; Sinclair, 1987).

Classical metabolism consists of degradative (*catabolic*) and biosynthetic (*anabolic*) reactions (Sanwal, 1970; Ratledge, 1987; Mulder *et al.*, 1988) which are unidirectional and unifunctional (Sanwal, Kapoor and Duckworth, 1971; Roels, 1987). The two processes are often opposite but complementary and may be aerobic or anaerobic (Griffiths and Cole, 1987; Iuchi, Cameron and Lin, 1989; Namdari and Cabelli, 1990). Nearly half of the intermediate products of catabolism eventually initiate or feed into anabolic routes, giving rise to bidirectional, bifunctional *amphibolic* pathways (Davis, 1961).

Most of the underlying mechanisms of metabolism have been studied and are now well known (Davis, 1961; Moat and Foster, 1988); the unknown remain the subject of extensive contemporary research. Only a minority of metabolic reactions are spontaneous because the energy barrier associated with the majority precludes their occurrence under normal physiological conditions in living systems (Dawes and Large, 1982; Schlegel, 1988). However, it is common knowledge that these reactions not only occur, but are tolerated *in vivo* largely due to biocatalysis by reaction-specific enzymes (Cohen, 1965; Beeckmans and Kanarek, 1987; Acerenza and Kacser, 1990). Enzymes are vital in general metabolism and are major determinants of the nutritional capabilities of living systems (Chance, 1961; Richmond, 1968; Guest, 1981). Enzyme-catalyzed pathways also determine the character of metabolism (Crabtree and Newsholme, 1987). The catabolic pathways for instance, are exergonic, yielding energy and carbon skeleton from nutrient sources (Dawes and Large, 1982; Mulder *et*

al., 1988). The carbon units are eventually transformed into cellular structures by endergonic, anabolic pathways at the expense of metabolic energy (Harrison, 1978; Schlegel, 1988). The coupling of both catabolic and anabolic pathways is the key to thermodynamic efficiency in cellular metabolism (Stücki, 1980; Roels,1987). In effect, any exergonic process could become an energy source, provided the energy output is of anabolic value (Kelly, 1990).

Anabolic and catabolic pathways operate in conjunction with the bidirectional routes which form the bulk of intermediary metabolism from which all carbon for cellular biosynthesis is derived (Sanwal *et al.*, 1971; Dawes, 1986). The amphibolic pathways also mediate the distribution of centrally-generated metabolites to ancillary pathways (Dawes and Sutherland, 1976). Thus, a *'basic ground plan'* exists in which an intermediate system serves as the common origin of a variety of divergent peripheral pathways (Umbreit, 1949; Dawes and Large, 1982; Gottschalk, 1986). Up to thirty intermediate metabolites and large amounts of energy-related compounds make up the *central metabolic pathways* (Holms, 1986a).

1.2 Bacterial Metabolism

Bacterial metabolism is concerned with the growth, survival and sustenance of cells (Dawes and Sutherland, 1976), and depends on the availability of suitable sources of nutrients (Owens and Legan,1987). Bacteria show considerable versatility in their quest for carbon sources (Umbreit, 1949; Dawes, 1986; Schlegel, 1988). In natural ecosystems however, bioavailable energy is in short supply and the preponderance of bacteria exist in a state of *"starvation survival"*(Morita, 1988) as atypical physiological and morphological forms (Chesbro, 1988).

Bacteria possess specialized mechanisms for accumulating biologically-important extracellular molecules in the cell (Futai, 1978; Rosen, 1978; Roseman, 1990), which may be concentration-dependent or energy-consuming (Sato *et al.*, 1990).

The preponderance of bacteria are chemoorganotrophs which must rely on an exogenous supply of organic compounds for their carbon and energy requirements (Dawes and Sutherland, 1976; Kelly, 1990). In one sense, living systems such as bacteria can be visualized as open, energy transducing systems which continuously demand free energy for growth

and maintenance (Dawes, 1986; Roels, 1987). Such *'free'* energy must be available in biologically useful form (Stouthamer, 1976; Morita, 1988) such as *adenosine 5'-triphosphate* (ATP), the universal carrier of metabolic energy in living systems (Bauchop and.Elsden, 1960; Pirt, 1965; Morowitz, 1968; Battley,1987). When the oxidation of ATP is coupled to anabolic pathways, the energy evolved can be harnessed for biosynthesis (Kornberg,1965; Harrison, 1978; Morowitz, 1978;Ratledge, 1987).

1.3 The Theory of Metabolic Fluxes

From a thermodynamic perspective, the bacterial cell is a discrete network of at least a thousand essential chemical, kinetic reactions (Ratledge, 1987; Koch, 1988) which constitutes a chemical-energy transducing unit (Acerenza and Kacser, 1990). During metabolism, the cell exchanges chemical matter and energy with its environment (figure 1.1) and the intracellular environment varies in proportion to the overall metabolism of the exogenous nutrient (Kornberg, 1965). In a *steady state,* internal energy is in a state of dynamic equilibrium between potential energy of *inflow* and *outflow* (Roels, 1987).

The ultimate purpose of metabolizing systems is the reproduction of other viable metabolizing systems (Ratledge, 1987). The resources for this are accumulated from the inflow of nutrients (Morita, 1988), or *input* , such that products of metabolism form the outflow (Mulder *et al*, 1988), or *output.* Monod (1949) demonstrated that a given output was a function of a combination of specific input and the attendant metabolic events which transformed the latter to the former (figure 1.1). This forms a practical basis for analysing and predicting the course of input (Holms, 1986a).

Output encompasses the succeeding generations of viable metabolizing units, catabolites of input in varying degrees of complexity and non-biomass products (Sinclair, 1987; Wanner and Egli, 1990). Net input and the resultant net output are defined by the net *throughput* (Holms, 1986a). In this context, bacterial growth represents a decrease in entropy and a cumulative aggregate of specific outputs into quantifiable units (Harrison, 1978; Gottschalk, 1986; Chesbro, 1988). The rate of growth is an index of the velocity of net throughput (Monod, 1949; Andresen and von Meyenburg, 1980); although dynamic, growth remains relatively steady over a certain phase of the growing cycle (Crabtree and Newsholme, 1987; Owens and Legan, 1987). This rate can be measured as the specific growth rate (μ) and expressed relative to standard throughput as the net *flux* (Holms, 1986a).

Fluxes transform the potential chemical energy of input into metabolic energy by the hydrolysis of macromolecules into their constituent monomers (Morowitz, 1978). Although the course and fate of the monomeric outputs are multifarious, catabolic fluxes generally converge at pyruvate which may be metabolized further into products which vary with organisms and their growth environments (Krebs, 1937; 1940a; b; vanUrk *et al.*, 1990).

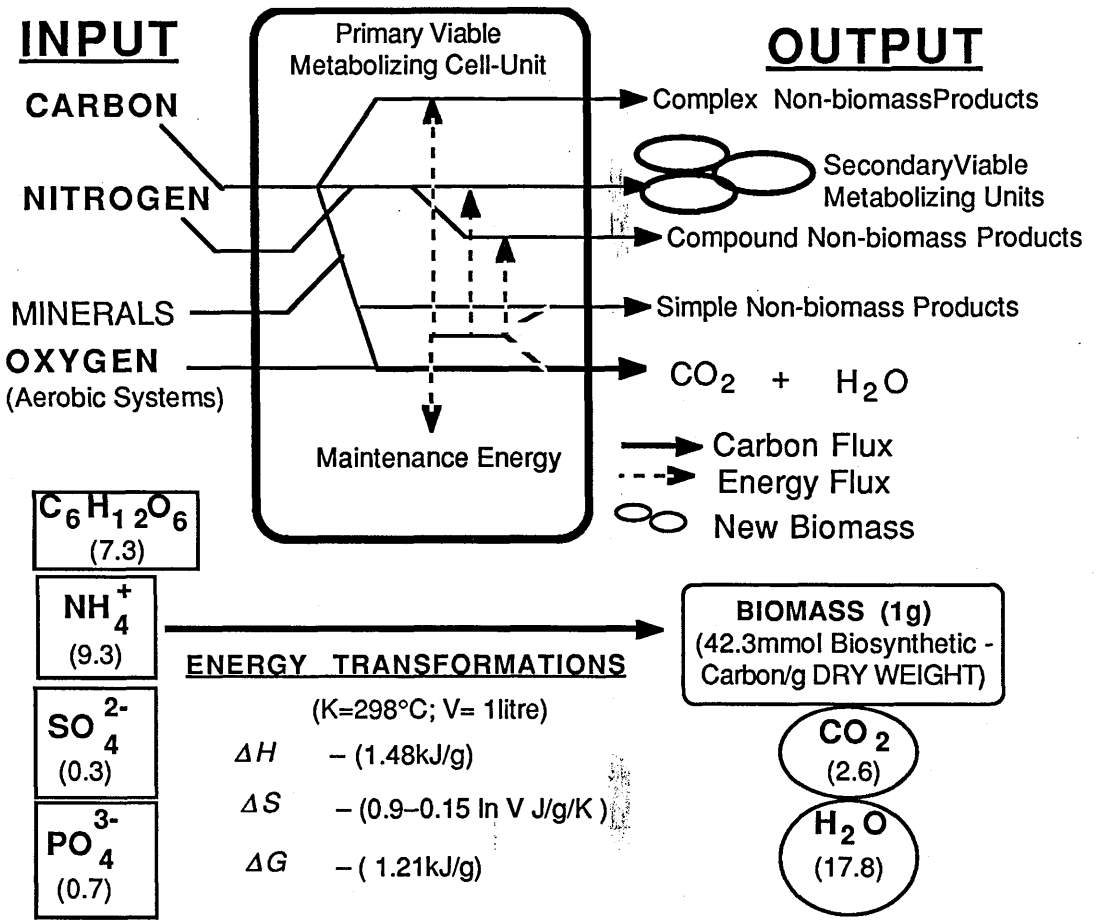


FIGURE 1.1 SCHEMATIC REPRESENTATION OF THE DYNAMICS OF A VIABLE ACTIVELY-METABOLIZING HETEROTROPHIC BACTERIAL CELL
(Based on Morowitz, 1978; Sinclair, 1987)

1.3.1 DERIVATION OF FLUXES AND ESTIMATION OF THROUGHPUT

When the source, course and fate of carbon input are known, fluxes can be computed (Dawes, 1986; Holms, 1987), while potential fluxes are also predictable (Holms, 1986a). The flux control coefficient of an enzyme during metabolism reflects its local effect on metabolic flux relative to variation in its concentration or activity (Sen, 1990). It can be determined from the net variation in specific input or output of flux (Holms, 1986a) and/or expressed in relative algebraic terms (Kacser and Burns, 1973; Acerenza and Kacser, 1990)*(Equation 1.1)*:

$$C_{e_i}^{J_j} = \frac{e_i}{J_j} \cdot \frac{\partial J_j}{\partial e_i}$$

WHERE J_j is a given unidirectional flux of input through a given enzyme, the concentration of which is (e_i).

∂ the degree of quantitative change of enzyme or flux *EQUATION 1.1*

The flux control coefficients of the enzymes associated with a common metabolic pathway vary relative to each other (Kell, vanDam and Westerhoff, 1989); the extent depends on the character of each pathway (Crabtree and Newsholme, 1985). Flux control coefficient is therefore a systemic phenomenon which is only poorly mimicked by the properties of enzymes determined *in vitro* (Kacser and Porteous, 1987).

Throughput can be estimated from the output of new cells (Holms, 1986b) which can then be expressed thermodynamically as the end-products of series of chemical fluxes (Morowitz, 1978; Sinclair, 1987) whose stoichiometry can be determined (Harrison, 1978; Stouthamer and vanVerseveld, 1987). The monomers which make up cells are functions of their growth rates and ecosystems (Snell, 1949; Lowry *et al.*, 1971; Neidhardt, 1987; Morita, 1988). Typical cell samples are neither uniform nor strictly representative of the composition of entire cell populations in cultures (Koch, 1988; Wanner and Egli, 1990). However, there is little dispute over the qualitative similarities in the composition of functional

protoplasm and low molecular weight molecules of cells (Snell, 1949; Morowitz, 1978). Fortunately too, the published amounts of constituent-monomers of cells of various strains of *Escherichia coli* were within acceptable working ranges of each other (Morowitz, 1968; Dennis and Bremer, 1974; Holms, 1986a; Mulder *et al*, 1988; Wanner and Egli, 1990). A few generalizations can therefore be made for the common features of biomass composition, especially if growth parameters were defined and values reflected the steady-state scenario (Neidhardt, 1987). Only eight or at most nine intermediate metabolites of the central pathways were required in precise proportions as common precursors for the biosynthesis of a variety of low molecular weight compounds (Holms, 1987), including 20 carbohydrates, 20 amino acids, 20 fatty acids, 5 purines, 5 pyrimidines and some cofactors (Morowitz, 1968; Dawes and Sutherland, 1976) (figures 1.2; 1.3). These are processed with increasing complexity into a variety of proteins, four types of ribonucleotides, an equivalent amount of deoxyribonucleotides and other organic macromolecules to produce cell structure (Dawes 1986; Neidhardt, 1987).

1.3.2 GROWTH YIELD AS A FACTOR IN FLUX ANALYSIS

Input, flux and growth are linked by simple reciprocal functions (Bauchop and Elsden,1960; Holms, 1986a; Hütter, 1987; Linton, 1990). The concentration of the substrate and yield of metabolic energy are also positively correlated (Stouthamer, 1976). The quantitative biotransformation of input to biomass is governed by a stable stoichiometry (Morowitz, 1968; Linton, 1990) which is usually reproducible even when genetic changes occur (Monod, 1949), provided nutrient/carbon source is standardized (Owens and Legan, 1987; Chesbro, 1988). In functional terms, this represents a substrate-specific *Molar Growth Yield* ($Y_{SUBSTRATE}$)(Stouthamer, 1976; Dawes, 1986; Ratledge, 1987):

$$Y_S = x\,(S_i - s_r)^{-1}$$

EQUATION 1.2

WHERE Y = Yield Coefficient (g.DRY WEIGHT mol. substrate^{-1})
x = Net output of new viable metabolizing units (g.dry weight)
S_i = Initial Concentration of input [M]
s_r = Residual Concentration of input [M]

For any flux, quantitative variation in a mediating enzyme can be expressed algebraically as a function of its effect on the amount of input,

catabolite or flux; hence, the *Concentration Control Coefficient (C)*(Sen, 1990), whose usefulness depends on empirical data (Walsh *et al.*, 1987):

$$C_{e_i}^{X_j} = \frac{e_i}{X_j} \cdot \frac{\partial X_j}{\partial e_i}$$

X_j represents the concentration of input/catabolite
e_i concentration of specific enzyme of flux

EQUATION 1.3

1.3.3 Efficiency-Index and Metabolic Fluxes

Molar growth yield is a conventional index of the efficiency of several pilot-scale or industrial metabolic processes which involve the growth of cells (Harrison, 1978, Andersen and vonMeyenburg, 1980; Olsen and Allermann, 1987; Luli and Strohl, 1990). Other parameters include the yield of ATP (Tempest and Neijssel, 1984; Stouthamer and vanVerseveld, 1987) and non-biomass outputs (Hollywood and Doelle, 1976; Landwall and Holme, 1977; Namdari and Cabelli, 1990). The amount of energy conserved by the degree of coupling between exergonic and endergonic fluxes is an additional index of efficiency (Holms, 1986a). In all instances, allowances are made for the fraction of energy output which is invested in endogenous metabolic demands. The *maintenance coefficient* neither results in new biomass, nor depends on the growth rate (Pirt, 1965; Harrison, 1978; Koch, 1988). A realistic index of efficiency would correlate positively with fluxes of carbon to biosynthesis, but negatively with fluxes to complete oxidation which can be monitored by the output of carbon dioxide (Holms, 1986a).

1.4. Fluxes and Economy in Biosynthesis

Bacterial metabolism has evolved into a purpose-designed mechanism, providing the cell with its biosynthetic and energy requirements (Gunsalus, 1949; Iuchi and Lin, 1988). With the rare exception of so-called *'futile cycles'* which decimate available metabolic energy of cells (Cannon and Nedergaard, 1985; Rogers *et al.*, 1988), bacterial metabolism is coordinated such that available nutrients can be utilized efficiently (Krebs, 1981) and as economically as possible (Davis, 1961; Battley, 1987).

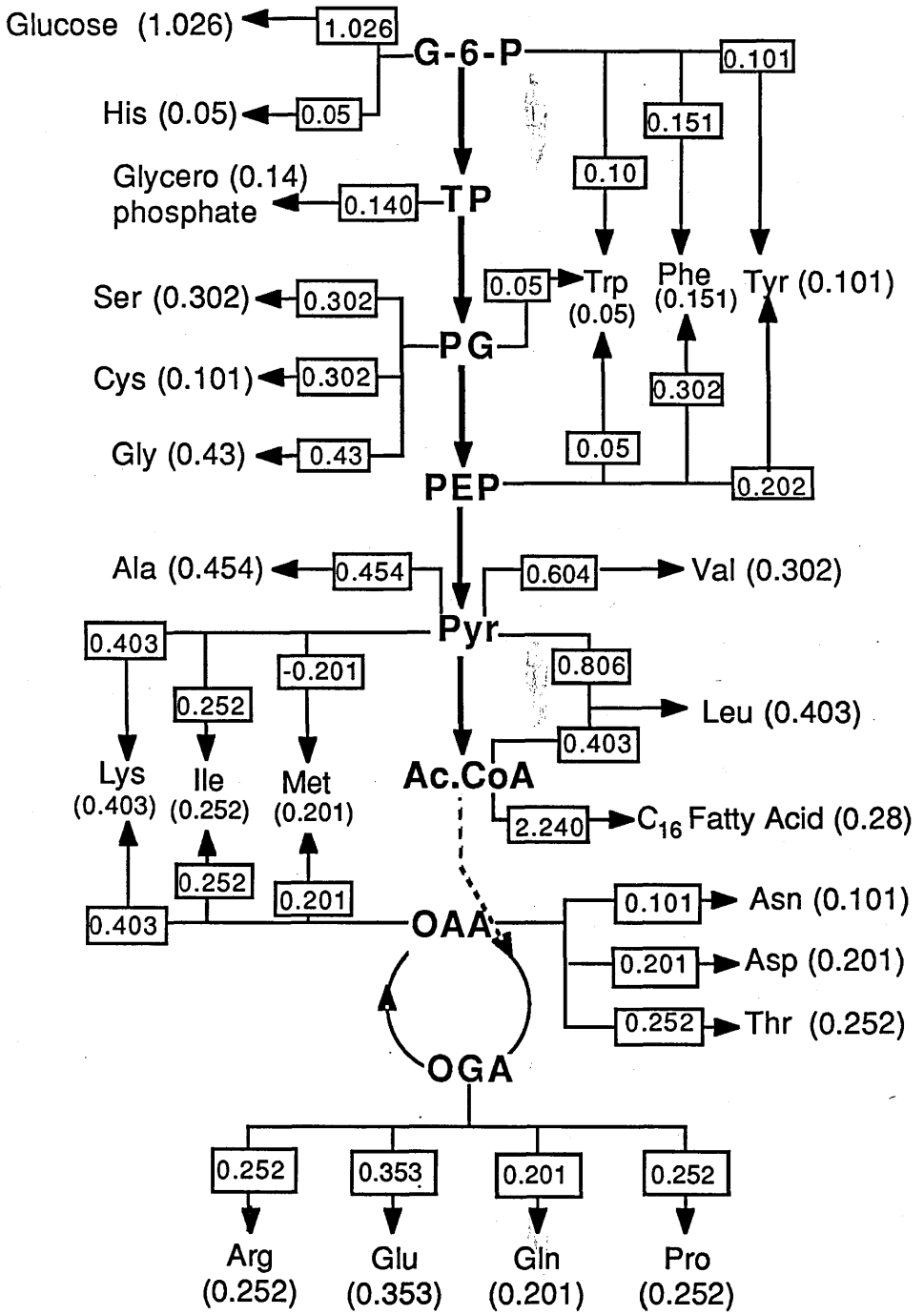


FIGURE 1.2 QUANTITATIVE DRAINAGE OF INTERMEDIATE METABOLITES FROM THE CENTRAL METABOLIC PATHWAYS TO BIOSYNTHESIS AND OUTPUTS OF AMINO ACIDS

Values are expressed in mmol. g. dry cell biomass^{-1}. Amino acid abbreviations are standard.

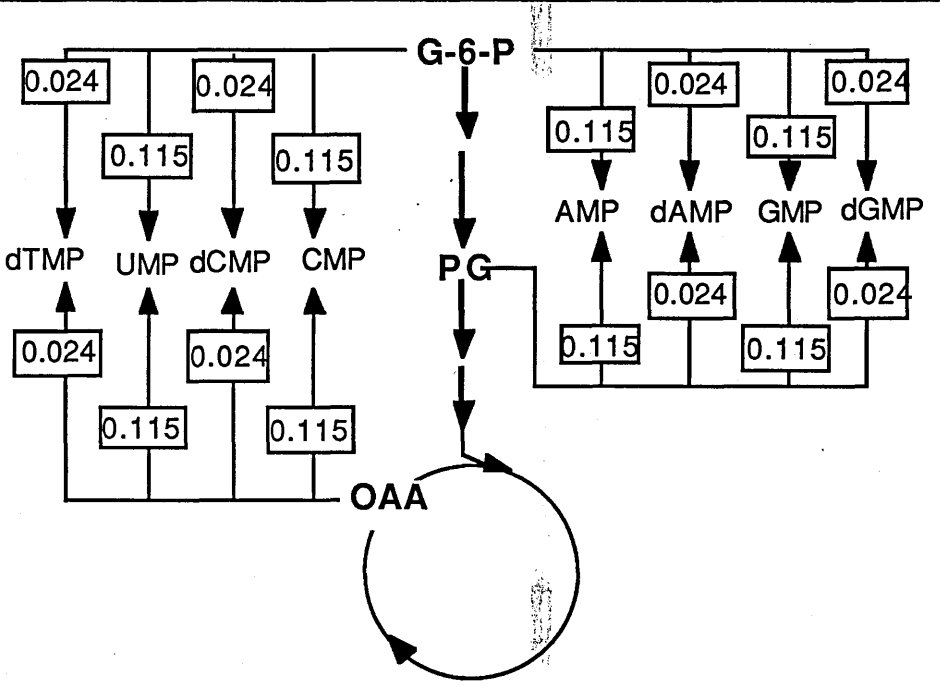


FIGURE 1.3

THROUGHPUTS OF INTERMEDIATE METABOLITES FROM THE CENTRAL METABOLIC PATHWAYS TO THE SYNTHESIS OF NUCLEOTIDES

Values as in fig 1.2; *(based on Morowitz, 1978; Holms, 1986a)*

1.4.1 COORDINATION OF METABOLISM

Bacterial systems exhibit a high degree of coordination (Baumberg, 1982; Postma, Brœkhuizen and Geerse, 1989) in controlling their metabolism (Chance, 1961; Cohen, 1986; Umbarger, 1961; 1969b, Holms, 1987). This is most clearly demonstrated in their ability to adapt basic cellular activities to fluctuations in exogenous supply of nutrients (Kliger, 1916; Lowry *et al.*, 1971;Tauchert, Jahn and Oelze, 1990). The growth of bacterial cells occurs by binary fission (Schlegel, 1988) which is a function of the parallel divisions of genetic and cellular matter (Chesbro, 1988). In nutrient-sufficient ecosystems, the growth of cells is *balanced* and essential macromolecules are synthesized proportionally (Pritchard and Tempest, 1982; Wanner and Egli, 1990). However, this is an unnatural *copiotrophic* state (Owens and Legan, 1987; Koch, 1988), in contrast to the less endowed, but more typical *oligotrophic* ecosystems (Morita, 1988) which can only support *unbalanced* growth (Chesbro, 1988).

1.5 CONTROL OF METABOLIC FLUXES

As a rule, metabolic fluxes are determined and changed according to the requirements of respective bioflux systems (Crabtree and Newsholme, 1987). The most elementary control of input operates at the physical level (Dawes and Sutherland, 1976). The peptidoglycan cell wall of Gram negative bacteria is freely permeable to small solutes, while the cytoplasmic membrane is selectively permeable (Rosen, 1978). A combination of periplasmic and transmembrane permeases selectively import compounds and ensure the accumulation of metabolically-important molecules in the cell (Ames, 1990). The transport mechanisms may be passive or energy-consuming (Futai, 1978; Moat and Foster, 1988) (figure 1.4). In special cases, conservation mechanisms ensure that energy-consuming transportation is coupled to initial catabolic reactions of transported molecules (Hunter, 1977; Hunter and Kornberg, 1979; Kornberg, 1981; 1986; Postma, 1987; Roseman, 1990). At least fifteen carbohydrates are translocated by the generalized *phosphoenolpyruvate: sugar phosphotransferase* (PTS) *system,* composed of about 20 distinct proteins (Chin *et al.*, 1987). During translocation, the substrates are phosphorylated (Geerse, Izzo and Postma, 1989); in most instances, this also initiates their catabolism (Saier and Chin, 1990) (Figure 1.4).

1.5.1 SPECIALIZED CONTROL OF METABOLIC FLUXES

Flux control has been most widely studied at the enzyme level. Metabolic fluxes may be sensitive to quantitative and qualitative changes in enzymes or their activities (Monod and Jacob, 1961; Cohen, 1965, Baumberg 1981; Acerenza and Kacser, 1990). The regulatory mechanisms may operate at physiological or molecular levels (Holms, 1986a; Spencer and Guest, 1987; Goodridge, 1990) usually through the interaction of regulators with key enzymes of fluxes (Crabtree and Newsholme, 1985).

In broad terms, fluxes to biosynthesis are sensitive to their respective outputs (Sanwal, 1970), while catabolic fluxes which are exergonic by nature, are regulated by energy compounds (Sanwal *et al.,* 1971; Thompson, 1987). The former includes several cases of *endproduct (negative feedback*) control mechanisms (Umbarger, 1961) while the latter has numerous examples of *catabolite repression* (Postma, 1986; Saier, 1989; Namdari and Cabelli, 1990).

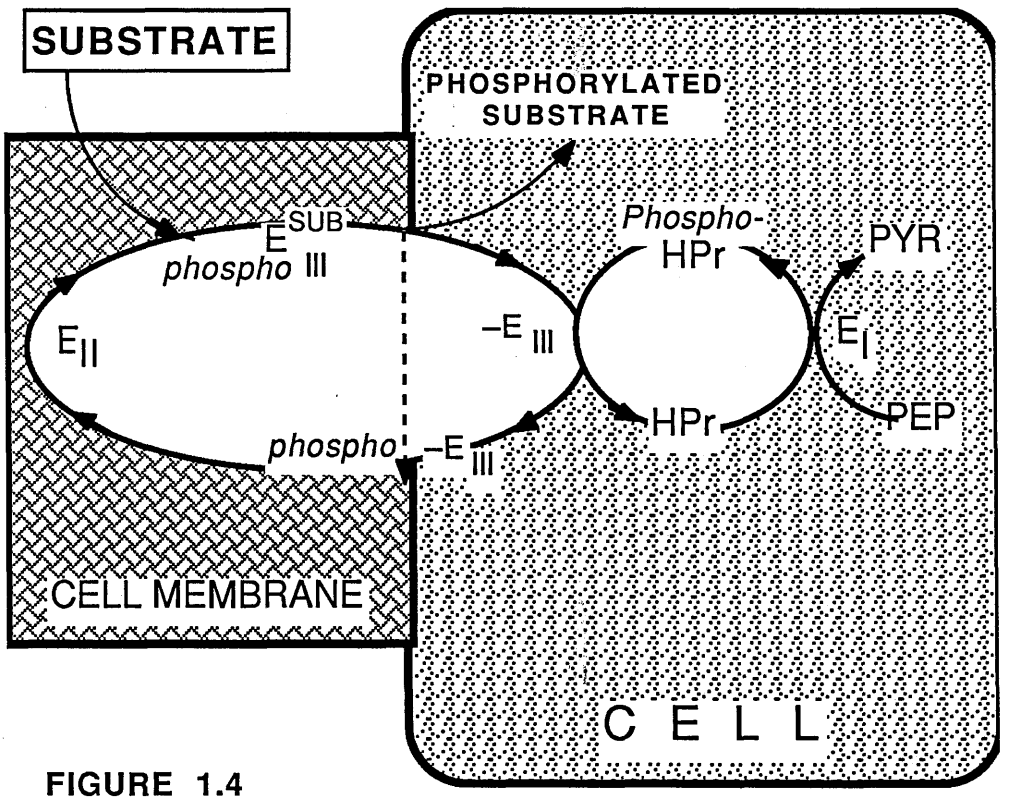


FIGURE 1.4

GENERALIZED PHOSPHO *ENOL* PYRUVATE: SUGAR PHOSPHOTRANSFERASE SYSTEM

Equilibrated reactions between HPr, Enzyme I and PEP have been abbreviated and fused.

SUBSTRATE	Exogenous Carbon/Energy Source	HPr	Heat-stable carrier protein
$E_{I, II, III}$	Phosphoenzymes	PEP	Phospho*enol* pyruvate
Phospho	Phosphorylated State	PYR	Pyruvate

(Based on Postma and Lengeler, 1985; Saier and Chin, 1990)

Fluxes associated with amphibolism are multifarious because they are bidirectional, bifunctional (Sanwal *et al.,* 1971) and include several divergent and convergent pathways (Umbarger, 1969a). Unique control mechanisms therefore abound in this group. Several enzymes of amphibolic routes are precursor-activated (Hess, Haekel and Brand, 1966). On the other hand, Paulus and Gray (1964) demonstrated a *multivalent endproduct inhibition* of entire enzymes of a chain of *communicating* (see Crabtree and Newsholme, 1987) metabolic fluxes by simultaneous regulation through *concerted feedback inhibition* (Datta and Gest, 1964). Alternatively, the initial catabolic flux may be regulated by the various outputs of peripheral fluxes deriving from it (Nester and Jensen, 1966).

A quantitative and uniform change in the enzymes of a metabolic pathway should effectively change the net flux in proportion to the magnitude of change of enzyme concentration (Kacser and Burns, 1973; Crabtree and Newsholme, 1985):

$$\frac{\partial e_i}{e_i} = \alpha = \frac{\partial F}{F}$$

α = margin of variation of concentration of enzymes
e_i = concentration of enzyme (s)
F = Specific flux (es) mediated by specific enzyme (e_i)
(Kacser and Burns, 1973) EQUATION 1.4

In practice however, this is not always the case since net change in flux does not always accompany quantitative changes in the enzymes pertinent to the given flux (Crabtree and Newsholme, 1987).

By far the largest influence on amphibolic fluxes are *effector-molecules* (Koshland, 1969; Umbarger, 1969a; Sanwal, 1970; Yoshinaga *et al.*, 1970; Teraoka *et al.,* 1974; and Saier, 1989). Effectors may inhibit or activate enzymes (Ratledge, 1987) and may change the catalytic properties of enzymes by physical modification of enzyme structure. The major influences include phosphorylation, adenylation, adenosine diphosphate ribosylation, or oxidation of thiol groups (Patel and Roche, 1990). Reduced *di*-phosphopyridine nucleotide (DPNH) or NADH, was found to be a

common control signal of convergent fluxes in *Escherichia coli* (Sanwal; 1970). Fluctuation in the ratio of NADH to NAD^+ was regulatory for several enzymes of metabolic fluxes (Snœp *et al.*, 1990). A *feedforward-feedback* mechanism also regulates cellular metabolism in response to the energy-state of the cell, as demonstrated by the regulation of the PTS system by the PEP:pyruvate ratio (Mason *et al.*, 1981). Other critical ratios include: NADP/NADPH (Reeves, O'Neil and Weitzman, 1983), ATP/AMP (Dawes, 1986) acetyl-CoenzymeA/CoenzymeA; ATP/ADP and the phosphorylation/ dephosphorylation cycle (Patel and Roche, 1990). The phosphorylation state of metabolic enzymes also influences their activities (Bennett and Holms, 1975). The extent of this regulatory mechanism is becoming even more widely recognized (Postma *et al.*, 1989; Saier, 1989). Regulation by reversible inactivation of enzyme activity through phosphorylation, has been demonstrated in enzymes at key junctions of amphibolic routes (Bennett, 1970; Borthwick, 1984; Holms, 1987; Saier, 1989).The sensitivity of any flux to fluctuating levels of enzymes and effectors of the flux, is believed to be corporate. Such *absolute-change sensitivity*, when expressed algebraically, equals unity (Kacser and Porteous, 1987) :

$$\frac{\partial F}{F} = \sum_{1 \rightarrow n} \left[\frac{\partial F}{F}\right] = \alpha \sum_{1 \rightarrow n} Z_i$$

WHERE n is the number of communicating enzymes or fluxes(*F*)
Z Sensitivity Coefficient (of specific flux to effector)

$$\sum_{1 \rightarrow n} Z_i = 1$$

THE FLUX CONTROL SUMMATION THEOREM *EQUATION 1.5*

Adapted from Kacser and Burns (1973); Kell et al. (1989); Sen (1990)

It has been argued that the summation theorem is limited in its application to steady-state systems and does not effectively convey the relative effects of changes in concentrations of enzymes, metabolites or effectors on fluxes (Crabtree and Newsholme, 1987). The potential influence of feedback effects also appears underestimated. Hence, a *relative* or *intrinsic* sensitivity has been proposed for the assessment of the generalized sensitivities of complex flux-systems (Crabtree and Newsholme, 1987). Signal flow graphs have also been proposed for easy

visual evaluation of the control structure of unbranched, unidirectional but communicating pathways (Sen, 1990).

1.6 Flux Control and Genetic Origins

All instances cited so far have presented regulatory mechanisms as functions of their respective metabolic fluxes or metabolites which communicate with fluxes. The classical work of Jacob and Monod (1961a), contained the framework for expressing the qualitative and quantitative activities of enzymes as functions of mechanisms controlling the expression of genes which code for them.

Most genes are regulated during transcription or translation (Gottesman, 1984). The model of Jacob and Monod (1961a) illustrated how the expression of structural genes is modulated by *regulator* genes at *operator* segments in the neighbourhood of the structural genes. The products of regulator genes are *regulatory* molecules whose targets are operator-genes. They are also capable of diffusing into the cytoplasmic milieu where their sensitivity to exogenous and endogenous compounds, such as inputs and outputs of metabolism, is an important key to the control of fluxes by the control of the synthesis of enzymes associated with them (Cohen, 1986; Hütter, 1986; Ratledge, 1987; Saier and Chin, 1990).

1.6.1 THE REGULATORY MOLECULES

Regulatory molecules may be *repressors* which interact with operators to prevent transcription (Mandelstam, 1962; Kornberg, 1986; Schlegel, 1988) or *activators* which activate transcription and initiate fluxes (Saier, 1989). In both instances, the ultimate goal is cell economy (Davis, 1961). *Operons* also facilitate the coordinate transcription of structural genes because of the common operators they share. The four genes coding for components of the fructose phosphotransferase system form an operon (Postma *et al,* 1989) and are jointly regulated by a single repressor protein, FruR (Geerse *et al.*, 1989b) which mimics catabolite repression (Saier and Chin, 1990). Jointly-regulated genes may be contiguous (Jacob and Monod, 1961a) or dispersed, as in the arginine regulon (Gorini and Maas, 1957). Many of the genes of the tricarboxylic acid cycle are scattered throughout the linkage map of *Escherichia coli* (Miles and Guest, 1987). Nonetheless, the operation of the cycle is highly coordinated and may involve common *communicating* signals (Spencer and Guest, 1987; Iuchi and Lin, 1988; Bell, Cole and Busby, 1990).

In summary, the entire bacterial genome represents a library of independent, highly coordinated molecular blueprints which determine the nature of metabolic fluxes (Jacob and Monod, 1961b; Hütter, 1986). The information it carries may also contain important signals for flux control.

1.6.2. CATABOLITE REPRESSION: A MECHANISM FOR FLUX CONTROL IN MULTIPLE-SUBSTRATE SYSTEMS

As a rule, bacterial systems utilize substrates in multiple-nutrient cultures preferentially and sequentially (Clark and Holms, 1976), with the possibility of diauxic growth patterns (Tauchert *et al.*, 1990). In general, substrates whose utilization demand the least input of metabolic energy, but whose capacity for supporting growth exceed others, are metabolized first (Robertson, 1970; Ratledge, 1987).

1.6.2.1 THE REGULATORY ROLE OF *CYCLIC* - ADENOSINE 3',5'-PHOSPHATE IN CELLULAR METABOLISM

Cyclic adenosine 3',5'-phosphate (*cyclic-AMP*) is a principal determinant of selective utilization of substrates in multiple-nutrient systems. It is synthesized from ATP *via* the enzyme-*adenylate cyclase* (deCrombrugghe and Pastan, 1973). In association with a specific receptor protein, *CRP* (or *protein kinase* in eukaryotic systems, Rogers *et al.*, 1988), cyclic-AMP (*c-AMP*) promotes the transcription of several metabolic genes (Postma *et al.*, 1989), secretion of macromolecules, functions of ion channels (Tai, 1990) and the catalytic activity of metabolic enzymes (Driessen and Konings, 1990; Goodridge, 1990). Cyclic-AMP controls transcription by binding to DNA in the form of a cyclic-AMP–CRP complex (Saier, 1989). Tests showed that individual components of the complex were not able to produce such effects (deCrombrugghe and Pastan, 1973; Namdari and Cabelli, 1990). The regulatory effect was found to be pleiotropic, as in the derepression of enzymes of the tricarboxylic acid (TCA) cycle under aerobic conditions to curtail the *Crabtree effect* in aerobic cultures (Cabelli and Namdari, 1989). The rationale for these activities lies partly in the positive correlation between the intracellular concentration of c-AMP and the *energy charge* of the cell (see below; Snœp *et al.*, 1990):

ENERGY CHARGE INDEX

$$= \frac{[ATP] + (0.5[ADP])}{[ATP] + [ADP] + [AMP]}$$

(Dawes, 1986; Ratledge, 1987) EQUATION 1.6

Moreover, adenylate (adenyl) cyclase is powerfully inhibited by catabolites of glucose (Saier, 1989) and free *Enzyme* III of the glucose PTS system (Meadow *et al.*, 1986). Consequently, operons whose transcription require the c-AMP–CRP complex are repressed competitively in the presence of glucose (Novotny *et al.*, 1985). A case in point is the utilization of glycerol. The enzymes required for its metabolism are coded by the *glp* regulon which is specifically induced by glycerol-3-phosphate (Sweet *et al.*, 1990) but is only expressed fully when c-AMP is present (Postma *et al.*, 1984). Phosphorylated PTS EnzymeIII^{Glc} activates adenyl cyclase allosterically but is unable to bind to the target permeases or allosteric sites of enzymes associated with the uptake of *"non-PTS"* sugars (Saier, 1989). The translocation of PTS sugars dephosphorylates EnzymeIII^{Glc} (Postma and Lengeler, 1985) which deactivates adenyl cyclase (Saier and Chin, 1990) and in the presence of glucose, results in *inducer exclusion* of glycerol-3-phosphate (Nelson *et al.*, 1986).

1.6.3 PLEIOTROPIC REGULATION

An important factor in the coordination of metabolism, is the possibility of coregulating several families of genes, operons or regulons (Saier and Chin, 1990). *Modulons* (Iuchi *et al.*, 1989) exert *global* controls on metabolic pathways by coordinately transcribing the gene systems with which they can communicate (Gottesman, 1984; Iuchi and Lin, 1988). In facultative anaerobes, a similar mechanism is thought to mediate the switch between aerobiosis and anaerobiosis (Gray, Wimpenny and Mossman, 1966) and the substantial repression of enzymes of the TCA cycle during anaerobic metabolism (Griffiths and Cole, 1987; Spencer and Guest, 1987). The products of the *arcA* &*B* genes act as modulons which repress genes of TCA cycle enzymes in anaerobic cultures (Iuchi *et al.*, 1989). Similarly, a common signal is believed to activate the nitrate reductase operon and repress the fumarate reductase and trimethylamine *N* -oxide

reductase operons simultaneously (Plas *et al.*, 1983; Iuchi and Lin, 1987; Miles and Guest, 1987; Jayaraman *et al.*, 1988). The repressor protein of the fructose operon (fruR) also regulates both glycolytic and gluconeogenic enzymes (Kornberg and Elvin, 1987; Geerse *et al.*, 1989). It is also suggested that the activity of adenyl cyclase might be under the control of the fructose operon (Postma *et al.*, 1989), while *enzyme III*^{Glc} may also be pleiotropic in action (Saier and Chin, 1990). Another pleiotropic factor operates in non-equilibrium, nutrient-limited systems where the very slow growth of bacteria under *starvation survival* (Owens and Legan, 1987) increases the likelihood of errors in the synthesis of macromolecules which carry genetic information (Morita, 1988; Wanner and Egli, 1990), with attendant risks of protein *mistranslation* (Chesbro, 1988). The *stringent response* of cells during starvation survival involves remedial nutritional adaptations and amplified proofreading of protein synthesis (Cashel and Rudd, 1987). Both austerity measures are controlled by a common nucleotide effector–*guanosine 5'-diphosphate, 3'-diphosphate* (ppGpp) (Chesbro, 1988) which also modulates the synthesis of antibiotics in Streptomycetes and Bacillus species. The universality of the latter effect is yet to be demonstrated (Hunter and Baumberg, 1989).

1.6.4 MUTANT-SYSTEMS AS TOOLS IN DETERMINATIVE FLUX STUDIES

Although a universal "ground plan" exists in bacterial metabolism (Umbreit, 1949) and functional protoplasm is relatively similar (Snell, 1949; Morowitz, 1978), very wide variations exist in the requirement for accessory nutrients among heterotrophic bacteria (Schlegel, 1988). Biosynthetic precursors, intermediate metabolites or peripheral outputs become *essential nutrients* (Woods, 1947) to organisms which have lost the ability to synthesize them (Gunsalus, 1948) from conventional input during normal metabolism (Snell, 1949). Such new phenotypes are often traits of altered genotypes (Richmond, 1968) which may affect the structure and function of enzyme proteins (Umbarger, 1961; Cohen, 1965). Since each transitional and terminal output of fluxes denotes specific throughputs (Holms, 1986a), controlled from specific gene loci (Demerec and Hartman, 1959), it is possible to isolate or design mutants which have become :

(a) defective in specific fluxes (Creaghan and Guest, 1970; Kohara *et al.*, 1987);
(b) incapable of yielding expected outputs (Mansi and Holms, 1989);
(c) incapable of metabolizing specific inputs (Lambden and Guest, 1976)

In nutritional mutants, characteristic dysfunction is a clue to function (Weitzman, 1981). This provides an important tool for flux studies. Certain

mutations relieve metabolic fluxes of the strict regulatory regimes under which they normally operate (Mandelstam, 1962). In such cases, the transcription of the structural gene is deregulated and becomes passive to effectors (Jacob and Monod, 1961b). The gene product is synthesized optimally, regardless of nutrient status and cell requirements (Holms, 1966), and therefore *constitutively* (Mortlock, 1982; Schlegel, 1988). A combination of these mutant-systems are conventionally used to resolve the patterns of metabolic fluxes (Herbert and Guest, 1968; Gibson and Cox, 1973; Spencer and Guest, 1982; Nimmo and Cohen, 1987; Geerse *et al.*,1989; Luinenburg and Coleman, 1990); course of inputs (El-Mansi *et al.*, 1986) and sources of outputs (Sugita and Komatsubara, 1989; Park and Toka, 1990). Genetic-based metabolic research has become a field in itself, supported by a plethora of literature and works which transcend the scope of the most ambitious review.

To complement the use of mutants in research, isotopic methods (Woods, 1947) involving the radiolabelling of elemental components of input (Gunsalus, 1948; Monod, 1949) are used routinely to facilitate the determination of the course (Krebs and Eggleston, 1940; Mosbach and King, 1950; Dawes and Holms, 1958), and fate (Eisenberg and Gurin, 1952; Douglas and King, 1953; Entner and Doudoroff, 1952) of inputs. Metabolic analogues or competitive inhibitors have also proved invaluable in the study of intermediary metabolism (Hochster and Quastel, 1963).

1.7 Predictive Flux Analysis in Selective Optimization of Throughput and Yield

Historically, microbes have featured in industry as agents of biotransformation and commerce: leavening was a common tradition of Biblical times and contemporary industry continues to exploit the favourable aspects of microbial metabolism (Morris, 1983), perhaps heeding the contention of Haldane -"Why trouble to make compounds when a bug will do it...?"(J. B. S. Haldane,1929 as cited by Prentis, 1985). Mechanisms which potentially enhance productivity by manipulating metabolic fluxes are always welcome in industry (Phelps and Clarke, 1983).

One of the major obstacles to improved productivity in microbial systems is the inherently higher activity of degradative processes relative to biosynthesis (Hütter, 1986). As a result, the sum of Flux Control Coefficients of degradative and biosynthetic fluxes nearly always exceeds unity and

biosynthesis does not fully exploit the outputs of catabolism (Andersen and von Meyenburg, 1980; Neijssel and Tempest, 1979; Kell *et al.*, 1989); (equation 1.7):

$$\left(\sum C_{e_{(1,2...n)}^{cat}}^{J_{(1,2...n)}^{cat}}\right) + \left(\sum C_{e_{(1,2...n)}^{ana}}^{J_{(1,2...n)}^{ana}}\right) > 1$$

cat. catabolic fluxes *ana. anabolic fluxes*

CUMULATIVE FLUX CONTROL COEFFICIENTS
(SEE equation. 1.1) *EQUATION 1.7*

The cellular mechanisms which couple exergonic to endergonic fluxes are not foolproof (Gunsalus and Shuster, 1961). In economic terms, carbon and energy supply in cells exceeds their demand (El-Mansi and Holms, 1989), resulting in overflow reactions (vanUrk *et al.*,1990) which may dissipate excess carbon as carbon dioxide (Tempest and Neijssel, 1984), or transform it into storage compounds (Sherwani and Fixter, 1989; Wanner and Egli, 1990). In *Escherichia coli*, energy reserves or storage products are not common or quantitatively significant (Mulder *et al.*, 1988; Wanner and Egli, 1990). It is more usual for superfluous intermediate metabolites to be extruded directly or after metabolic modification in transit (Hütter, 1986; Driessen and Konings, 1990), while unused energy may be dissipated as heat (Stücki, 1980).

1.7.1 THE BACTERIAL *CRABTREE* EFFECT

The theoretical outputs of aerobic glucose metabolism by *E. coli* are biomass and gaseous byproducts (carbon dioxide and water vapour). In practice, this probably never occurs and microbes in addition, excrete a variety of products (Holms and Bennett, 1971; Hollywood and Doelle, 1976; Wanner and Egli, 1990.). Most of these are outputs of inefficient ancillary fluxes which arise from the limited respiratory capacity of the cell (Andersen and vonMeyenburg, 1980) or disproportionate throughputs to the known biosynthetic precursors (Holms, 1987). A number of low molecular weight outputs are excreted by bacteria, including varying amounts of organic

acids (Landwall and Holme, 1977) of which acetate preponderates (Britten, 1954). The phenomenon of *aerobic acidogenesis* has been attributed to the *Crabtree effect* (Crabtree, 1929) or "aerobic fermentation" which is thought to result from the inhibition of enzymes which pertain to aerobic metabolism (Hollywood and Doelle, 1976; Iuchi and Lin, 1988), with near-stagnation of the activity of the TCA cycle (Doelle, Hollywood and Westwood, 1974; Spencer and Guest, 1987; Namdari and Cabelli, 1989). Flux through glycolysis is not as severely inhibited; in fact, there is evidence that it may be enhanced under the *Crabtree effect*(vanUrk *et al*., 1990).

1.7.2 OCCURRENCE AND SOURCE OF ACETATE

By convention, acetate is associated with anaerobic metabolism (Wood, 1961). However, as early as the turn of the century, it had been demonstrated in aerobic cultures (Harden, 1902). Resting cells from an aerobic culture of *E. coli* excreted 78*mol* ACETATE per 100*mol* GLUCOSE (Stokes, 1949).

Metabolism at the level of pyruvate plays a principal role in the *Crabtree* effect and acetate excretion (Holms, 1986a; vanUrk *et al.*, 1989; Snœp *et al.*, 1990). Pyruvate is a general purpose metabolic *hub* from which multifarious fluxes diverge (Morowitz, 1978; El-Mansi *et al.*, 1986). The partition of carbon flux at pyruvate depends on its concentration, the activities and kinetics of enzymes which mediate the fluxes (vanUrk *et al.*, 1989). In several anaerobic systems, acetate was a ubiquitous output from pyruvate; in a few instances, it was the sole non-gaseous product (Koepsell and Johnson, 1942). A common feature of most fluxes from pyruvate (regardless of the source) was the pivotal oxidation of pyruvate into acetyl-CoA by a three-step carbon flux through the pyruvate dehydrogenase multienzyme complex (Snœp *et al.*, 1990). The flux is modulated powerfully by the energy charge of the cell (Patel and Roche, 1990), and changes in the concentration of acetyl-CoA (Dawes and Large, 1982) or phospho*enol*pyruvate (Sanwal, 1970).

1.7.3 IMPLICATION OF THE EXCRETION OF ACETATE IN FLUX CONTROL ANALYSIS

Acetate excretion is a symptom of carbon and energy *leakage* (Holms, 1986a; Kell *et al.*, 1989; Namdari and Cabelli, 1990) from a superfluous glycolytic flux (vanUrk *et al.*, 1990). Acetic acid lowers the pH of unbuffered culture (Park and Toda, 1990) and acidification may attain suicidal proportions in a few instances (Namdari and Cabelli, 1989). In general,

growth is limited (Andersen and von Meyenburg, 1980; Pan, Rhee and Lebeault, 1987), imposing a constraint on the yield of microbial products of industrial value (Anderson, Grulke and Gerhardt, 1984), which depend on high cell density (Luli and Strohl, 1990). The reduced growth yield could indicate the absence of the right proportions of the required cocktail of biosynthetic precursors (Holms, 1986b), given that the synthesis of those from glycolytic origin did not necessarily cease while the activity of the TCA cycle and the precursors from it, diminished progressively (Griffiths and Cole, 1987; Miles and Guest, 1987).

From a different perspective, excretion of acetate is the price for accelerated rate of metabolite turnover (Owens and Legan, 1987); in a competitive natural ecosystem, this is advantageous (Koch, 1988). In addition, subsidiary fluxes from pyruvate regenerate the pool of free CoenzymeA (El-Mansi and Holms, 1989), yield ATP, and may be vital in chemiosmotic coupling (Sone, 1990) by maintaining the proton motive force (Repaske and Alder, 1981). Excreted organic acids also act as alternative electron-acceptors (Anderson *et al.*,1984). Finally, acetate is a type of extracellular reserve nutrient which may become a secondary carbon source as soon as the primary source is exhausted (Holms, 1987; Namdari and Cabelli, 1989).

On the whole however, acetate is typical of nuisance-outputs which complicate downstream processing of bioreactors where microbial biomass is a desirable output (Olsen and Allermann, 1987; Schmidt-Kastner and Gölker, 1987). Moreover, it represents wasteful efflux of raw materials which invariably diminishes productivity (Anderson *et al.*, 1984; Hütter, 1986).

1.8 Optimization of Throughput and Genetic Manipulation of Metabolic Fluxes

Optimization of yield would necessarily involve an increase in carbon conversion coefficient (Harrison, 1978). In an ideal scenario, the concentrations of all pertinent biosynthetic or catabolic enzymes are changed simultaneously and proportionally (Kacser and Burns, 1973), by *coordinate control operation* (overleaf). Theoretically, this is feasible only through generalized repression and induction in inverse proportions (Acerenza and Kacser, 1990). The central metabolic pathways would require manipulations which diminish the potential difference between flux control coefficients in favour of fluxes to biosynthesis, while maintaining growth rate as close to optimum as possible (Koch, 1988; Kell *et al.*, 1989).

In practice this would seem highly unrealistic. Indeed, it is argued that such generalized systemic changes could be achieved simply by *relative changes* at points of *intrinsic sensitivity* (Crabtree and Newsholme, 1985).

$$E_{j,\alpha} = \alpha E_{j,r}$$

WHERE $E_{j,\alpha}$ = net change in time course due to simultaneous quantitative change[$E_{j,r}$] in the concentration of enzymes of pathway by[α]

COORDINATE CONTROL OPERATION *EQUATION 1.8*

(Acerenza and Kacser, 1990)

Since all reactions of any one flux *communicate* to a greater or lesser extent (Crabtree and Newsholme, 1987), only a few potential sites of regulation need be manipulated with discretion, to attain another more desirable steady state. Hence, effective practical approaches need to target distributive junctions of metabolic fluxes, unidirectional fluxes to limiting key biosynthetic precursors (Holms, 1987), centres which determine cellular energy charge (Thompson, 1987; Snœp *et al.,* 1990) or metabolic switches between aerobiosis and anaerobiosis in facultative anaerobes (Gray *et al*, 1966; Griffiths and Cole, 1987; Spencer and Guest, 1987; Iuchi *et al.,* 1989).

Input, output or throughput can be selectively altered in three broad ways to enhance productivity in microbial systems :

1) variation of catabolic flux by modulating transcription and gene expression;
2) vertical expansion and contraction of pathways to accommodate new inputs;
3) horizontal contraction or expansion of pathways to streamline or diversify intermediate and terminal outputs

(Lehrbach and Timmis, 1983; Wöhrl *et al.*, 1990).

A critical but limiting flux can be enhanced by constitutive derepression or amplification of genes pertinent to its enzymes (Schlegel, 1988; Wöhrl *et al.,* 1990). The latter can be achieved either by screening natural populations for characters of interest which occur spontaneously (Mortlock, 1982; Okungbowa, 1983; Elander, 1987; Ochman and Wilson, 1987), or engineering the genome and genotype to obtain desired phenotypes (Lehrbach and Timmis, 1983; Murray, 1987). The strategy adopted would however be dictated by intent, nature of primary input, energetics (Stouthamer and vanVerseveld,1987) target output (Morris, 1983), and the *"bioflux"* system (Prentis, 1985). Spontaneous desirable changes are low frequency, often unpredictable events in natural populations (Selander, Caugant and Whittam, 1987). Hence they are often introduced by genetic manipulation as new genetic information (Lehrbach and Timmis, 1983). Process development exploits a combination of physiological, technological and genetic techniques to enhance productivity (Hütter, 1986).

1.8.1 MECHANISMS OF GENETIC MANIPULATION OF FLUXES: RECOMBINANT DNA TECHNOLOGY

Activities of specific enzymes of pathways can be increased by enhanced efficiency of transcription and translation using promoters and altered ribosomal binding sites (Gold and Stormo, 1987; Murray, 1987) or numerical increase of gene copies (Walsh *et al.,* 1987). The desired genes can be cloned by routine methods (Wilde, Jeyaseelam and Guest, 1986; Kushner, 1987; Urena *et al.,* 1987). In *Escherichia coli*, a plethora of selectable phenotypes (Vinopal, 1987) and fourteen phylogenetic groupings of mutants have been derived as auxotrophic cloning hosts (Bachmann, 1987). Plasmids (Thompson, 1988), cosmids (Dillon *et al.*, 1985) and bacteriophage (Kohara *et al.*, 1987) are routine cloning vectors which possess characteristic phenotypes for counter-selection, in addition to carrying the clones (Stanisich, 1988) and replicating independent of host chromosomes (Grinsted and Bennett, 1984). The gene of choice can also be obtained by nucleic acid hybridization (Maniatis, 1981) using radioactively/immunologically-labelled DNA or RNA probes (Prentis, 1985) to pinpoint complementary gene sequences or cell populations which carry them (Pritchard and Holland, 1985; Sambrook, Fritsch and Maniatis, 1989). Finally, genes may be transferred as cell-free recombinant entities (Smith and Danner, 1981; Elander, 1987) in association with a phage (Margolin, 1987) or by mating (Willetts,1988).

1.8.2 CURRENT OPTIONS IN PROCESS DESIGN AND ALTERNATIVE APPROACHES

Most contemporary methods for minimizing acetate excretion are not based on flux control analysis, but on physical and mechanical fine tuning of culture conditions. The control of acetate output by limiting substrate input in glucose cultures is a popular option (Doelle *et al*, 1974). Reduction of growth rate also reduced acetate excretion (Hollywood and Doelle, 1976). In continuous culture, acetate was eliminated at 75% of optimal growth rate in *E. coli* ML308 (Holms, 1986a). Mechanical removal of acetate from culture by dialysis (Landwall and Holme, 1977a) or microfiltration (Anderson *et al.,* 1984) enhanced growth yield on the basis that growth is inhibited when acetate accumulates in high concentrations (Pan *et al.,* 1987; Namdari and Cabelli, 1989). Increased aeration was not effective against acetate excretion (Andersen and vonMeyenburg, 1980; Namdari and Cabelli, 1990).

Wide variations in quantitative acetate excretion among strains of *E. coli* suggests that intrinsic differences exist in the relative abilities of strains to control the quantitative excretion of acetate. The very factor of exogenous accumulation of a potential nutrient reflects an inherent property which varies with strains and composition of culture (Hütter, 1986; Pan *et al.,* 1987). Mutants of *E. coli* which carried lesions for enzymes of acetate formation did not excrete acetate but lactate in some instances. Regulation of pyruvate supply also limited the excretion of acetate as well as growth rate in continuous culture (El-Mansi and Holms, 1989). It should therefore be possible to address the question of acetate excretion by manipulating metabolic flux at the genetic level.

1.9 THESIS

The metabolism of facultatively anaerobic bacteria such as *Escherichia coli*, generates intermediate metabolites from catabolic fluxes for anabolic processes. On several substrates however, catabolic throughputs to the cocktail of biosynthetic precursors (figures 1.2; 1.3) are not commensurate with their respective biosynthetic demands in precise relative ratios. Consequently, catabolic outputs are not fully exploited during biosynthesis and productivity is sub-optimal. This scenario encourages the intracellular accumulation of superfluous intermediate metabolites and the development of overflow reactions to dispose of them (Hütter, 1986). An example is the throughput of carbon to the metabolic hub, pyruvate, at which catabolic fluxes converge. The accumulation of pyruvate does not preclude its pivotal decarboxylation to acetyl-CoA, especially when throughputs to other biosynthetic precursors are limiting. The acetyl moiety is excreted eventually after conversion to acetate along with a variety of other organic acids in smaller amounts (Britten,1954). The disproportionate throughputs to biosynthetic precursors also reduces growth yield. The resulting excretion and sub-optimal growth yield, are indexes of an inefficiently-controlled metabolic system. Since growth and productivity may be limited, not necessarily by the total absence of biosynthetic precursors, but by their disproportionate outputs in sub-optimal ratios, a simple approach to optimization of process efficiency is the selective manipulation of carbon flux to balance the relative ratios of the known cocktail of intermediate metabolites required as biosynthetic precursors (Holms, 1987). Strategies for optimization would involve the identification of sensitive fluxes whose manipulation could effect a better balance in throughputs of carbon to biosynthetic precursors (Crabtree and Newsholme, 1985). The partition of carbon flux at PEP offered an attractive control-junction for this purpose, with the prospect of contrasting but desirable effects on carbon fluxes through glycolysis and the TCA cycle.

The branch-point at phospho*enol* pyruvate (PEP) is central to the intracellular concentration of pyruvate (figure 1.5). Reactions of PEP include divergent fluxes through pyruvate kinase and the PTS as well as throughputs to biosynthesis (figure 1.2) and carbon fixation to provide carbon skeleton for biosynthesis from the TCA cycle (figures 1.2; 1.3). The first two reactions encourage the excretion of acetate while the third sustains fluxes of PEP to the synthesis of aromatic amino acids but on the whole, does not lead to a net balance in the relative ratios of throughputs to

other biosynthetic precursors. The achievement of this balance was most likely through the flux which fed carbon directly into the TCA cycle from glycolysis (figure 1.5). The carboxylation of PEP yields oxaloacetate which is a major intermediate metabolite for amino acid and nucleotide biosynthesis (figures 1.2; 1.3). Furthermore, its condensation with acetyl-CoA reduces the accumulation of acetyl-CoA and enhances the operation of the TCA cycle upon which the throughputs of carbon to 2-oxoglutarate and succinyl-CoA (other precursors for acid biosynthesis) depend. Concurrent enhancement of throughputs of carbon to oxaloacetate, 2-oxoglutarate and succinyl-CoA appeared possible by amplification of the activity of phospho*enol* pyruvate carboxylase which mediates the conversion of PEP to oxaloacetate.

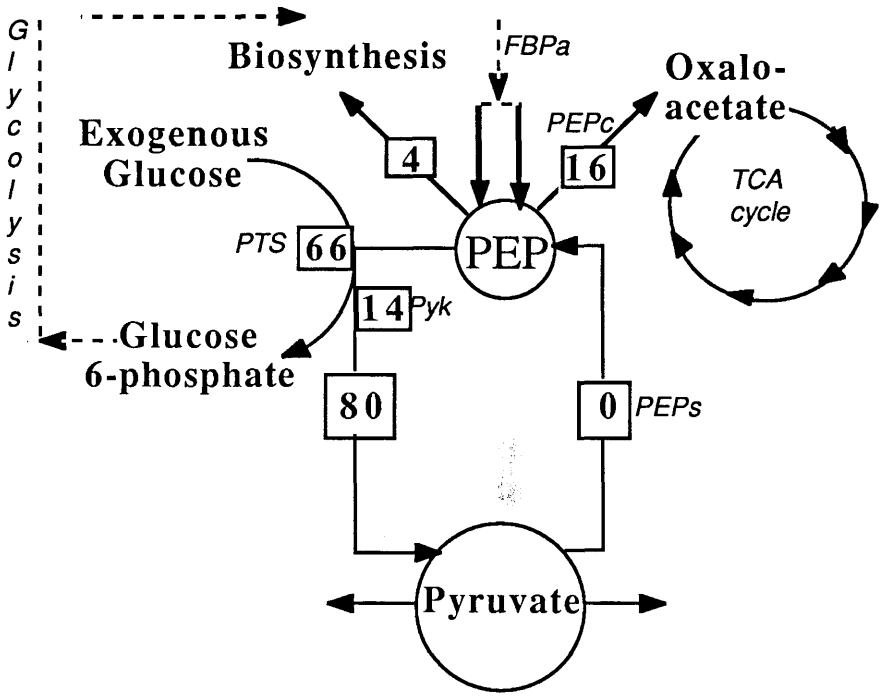


FIGURE 1.5
PARTITION OF CARBON FLUX AT PHOSPHO*ENOL* PYRUVATE

Values (%) are typical of the distribution of carbon flux at PEP during aerobic metabolism of a PTS sugar by a representative strain of *Escherichia coli* ; given that 100% flux of *input* carbon to PEP is equivalent to 16(±1)*mmol. g. dry biomass.*$^{-1}$. *h.*$^{-1}$. at μ= 0.92(± 0.02) *h.*$^{-1}$

FBPa Fructose-1,6-*bis* phosphate aldolase
PEPc Phospho*enol* pyruvate carboxylase *PEPs* Phospho*enol* pyruvate synthase
PTS Phosphotransferase multienzyme system

1.9.1 SUBJECT, SCOPE & STRATEGIES

The hypothesis was tested primarily in *E. coli* ATCC15224 (ML308; NCIB 9553). Previous work in this strain has contributed to the understanding of mechanisms which govern aerobic metabolism and enzyme function in facultatively anaerobic systems (Pardee Jacob and Monod, 1959; Mandelstam, 1962; Holms, 1966). A comprehensive metabolic flux of the strain has been developed, largely as a result of studies undertaken in this laboratory which addressed :

a) the effect of constitutive expression of its derepressed *lactose* operon on its metabolism (Holms, 1966);

b) control of its ß-galactosidase activity *vis-a-vis* catabolite repression (Holms, 1966; Robertson, 1970);

c) the mechanisms controlling its ability to discriminate between substrates in multiple-substrate cultures resulting in the sequential utilization of glucose and fructose (Clark, 1974; Clark and Holms, 1976);

d) the impact of maintenance energy on biomass yield on various substrates (Wallace, 1975; Wallace and Holms, 1986);

e) the principles of energy conservation and active transport in carbohydrate metabolism (Hunter, 1977).

A unique finding from the study of the metabolism of *E. coli* ATCC15224, was the control of carbon flux at metabolic branch-points to accommodate the multifarious demands on such junctions as carbon sources changed (Holms and Bennett, 1975). A case in point was the adjustment required to switch between glucose metabolism and the subsequent utilization of acetate which accumulated, and was excreted while glucose was being used (Bennett, 1970; Bennett and Holms, 1971; Borthwick, 1984; El-Mansi *et al.*, 1985; 1986). The information pool from these and other studies, provided a ground-plan for analyzing the transmission and regulation of carbon and energy fluxes in aerobic bacterial systems (Holms, 1986a). The model was based on the universal requirement for certain known intermediate catabolites as mandatory precursors for biosynthesis of macromolecules, cell structure and new actively-metabolizing cell units. The relative biosynthetic ratios of these precursors were found to be precise and critical to growth yield. The occurrence of overflow reactions and excretion of metabolites could then be explained partly by a disproportionate partition of carbon flux to these biosynthetic precursors (Holms, 1987). Thus, the model formed a general

basis for optimization of biotechnological and industrial outputs. Application of the model would involve manipulation of fluxes between biosynthetic and overflow metabolic channels, usually at sensitive centres (Crabtree and Newsholme, 1985) such as branch-points. A realistic and cost-effective manipulation of metabolic flux would depend on an understanding of the metabolism of the *bioflux* system and definition of the desired output.

Aim of Study

The primary aim of the present study was the manipulation of flux with a view to influencing the efficiency of substrate utilization, using *E. coli* ATCC15224 as a model. The possibility of controlling acetate excretion and enhancing biomass yield was of particular interest. Among the branch-points which arose during the metabolism of various carbon and energy sources, the partition of carbon flux at phospho*enol* pyruvate (PEP) offered the most attractive potential for achieving both objectives concurrently. Throughput of carbon to PEP not only yielded aromatic amino acids, but also determined relative fluxes to half of other biosynthetic precursors during glycolysis or gluconeogenesis. A major determinant of the efficiency of substrate utilization was the partition of carbon fluxes between phosphorylated and carboxylated routes (Holms, 1986a). A higher flux through the former and a lower flux through the latter, was capable of producing a metabolic *bottleneck* and a potentially inefficient system, which partly explained the phenomenon of excretion of acetate in aerobic metabolism by bacteria (Hütter, 1986; Luli and Strohl, 1990; Namdari and Cabelli, 1990).

Partition of flux at PEP offered a potential site for manipulation of flux in favour of the carboxylated section of metabolism to enhance the generation of carbon skeleton which may be limiting to biosynthesis. In particular, flux through phospho*enol*pyruvate carboxylase (PEPc) which has been described as *anaplerotic* for several substrates (Kornberg, 1966), introduced carbon into intermediary metabolism by fixing carbon dioxide (Bandurski and Greiner, 1953; Wood and Utter 1965; Ashworth and Kornberg, 1966). The flux also formed a unidirectional bridge between the largely phosphorylated and the carboxylated sections of the amphibolic pathways and converted PEP to oxaloacetate in competition with fluxes which converted it to pyruvate. The work reported includes the enhancement of throughput of carbon to oxaloacetate by increasing the activity of PEP carboxylase.

Amplification of flux was attainable, either by derepression or gene amplification to augment the activities or levels of enzymes (Mortlock, 1982). The latter was more predictable and reliable and therefore used during this study. To enhance enzyme activity by gene amplification, it was first necessary to clone the gene encoding the enzyme. The cloning of the *ppc* gene by complementation of the PEPc lesion of a *ppc*–mutant from a gene bank, is reported (chapter 3). Overexpression of phospho*enol*pyruvate carboxylase activity in *E. coli* ATCC15224 and its effect on the metabolism of selected carbon and energy sources was subsequently investigated (chapters 4, 5 and 6). To understand the implication of the effects recorded in the test organism, PEPc activity was also overexpressed in other strains, including mutants whose characteristic metabolic lesions restrained carbon fluxes through specific enzymes, thereby allowing comparative metabolic studies to be carried out (chapter 7). Attempts have been made to offer possible explanations and suggestions for the observed effects (chapter 8).

CHAPTER 2

MATERIALS AND METHODS

TABLE 2.1

RELEVANT GENOTYPES AND PHENOTYPES OF BACTERIAL STRAINS USED

ORGANISM	STRAIN	§GENOTYPE	‡PROPERTIES	SOURCE
Escherichia coli	**PA342** derivative of Paris -strain –P678	*pro, his, arg, leu, thr, ppc,*	Carries lesion for PEP-carboxylase; auxotrophic for C_4-dicarboxylic acids; unable to utilize glucose as sole carbon and energy sources	I. S.Hunter and A. P. Jessop (Genetics, Glasgow)
	ATCC 15224 (*a.k.a.* **ML308; NCIB 9553**)	i^-, z^+, y^+, a^+	Constitutive for lactose operon produces blue colonies on *BCIG* plates; requires no supplement in defined media	W. H. Holms (Biochemistry Dept., Glasgow)
	K10 derivative of strain K-12	Hfr, *tonA22, garB10, ompF627, relA1, pit-10 spoT1, metB*$^+$, $T2^r$, PO2A	Requires only thiamine supplementation in defined medium	H. L. Kornberg (Biochemistry Dept., Cambridge)

‡"Properties" include characteristic genotypes or phenotypes pertinent to the present study.

§Gene symbols are consistent with their usage in Bachmann(1983; 1987) and Vinopal(1987)

TABLE 2.1 (continued)

ORGANISM	STRAIN	§GENOTYPE	‡PROPERTIES	SOURCE
	K2-1t (derivative of strain K-12/K2)	*his, arg, leu, thr, pps*	Carries lesion for PEP-synthase; unable to utilize pyruvate as sole carbon and energy sources	H. L. Kornberg (Biochemistry Dept. Cambridge)
Escherichia coli	**HW0760** (derivative of K-12)	F^-, λ^-, *tyrA* del::*Km* ^{R}	Parent strain / Positive control for *pyruvate kinase* lesion in HW1387; utilizes both PTS & non-PTS sugars	Nutrasweet Company Chicago, USA
	HW1387 (derivative of HW0760)	F^-, λ^-, *tyrA* del:: *Km* ^{R}, *pykF* :: Tn5 *pykA*	Carries lesions for pyruvate kinases A and F; unable to utilize non-PTS sugars as sole carbon and energy sources	Nutrasweet Company

‡"Properties" include characteristic genotypes or phenotypes pertinent to the present study.

§Gene symbols are consistent with their usage in Bachmann(1983; 1987) and Vinopal(1987)

2.1 BIOCHEMICALS & REAGENTS

2.1.1 SUBSTRATES (CARBON & ENERGY SOURCES)

ß-D(-)fructose, D-gluconate, D-glucuronate, pyruvate (sodium salts) (Sigma Chemical Co., St. Louis, USA); glucose (Formachem,Research Inter'l. Ltd., Strathaven, Scotland); glycerol (B.D.H. Ltd., Poole, England).

2.1.2 AMINO ACIDS, ANTIBIOTICS AND OTHER SUPPLEMENTS OF MEDIA

L-arginine mono HCl, L-aspartic acid, L-histidine, L-threonine (T. J. SAS & Sons Ltd., Holborn, England); ampicillin hydrochloride, chloramphenicol, kanamycin, L-leucine (BDH); L-proline, thiamine hydrochloride, L-tyrosine, 5-*bromo*- 4-*chloro*-3-*indolyl* -β-galactoside (BCIG) (Sigma).

2.1.3 ENZYMES

Restriction enzymes and enzymes for molecular biology were obtained from Bethesda Research Laboratories (BRL), Paisley, U.K.; Lysozyme (Sigma); malate dehydrogenase (BDH).

2.2 MISCELLANEOUS PREPARATIVE METHODS

2.2.1 STERILIZATION

Heat-stable materials were sterilized by heat. Working tops were disinfected with *iso*-propanol, hibitane or 70%(v/v) ethanol.

2.2.1.1 STERILIZATION BY MOIST HEAT

Sterilization by moist heat was by autoclaving at 115°C for 60 minutes in an Autoclave chamber (Manlove-Alliot and Co Engineerings Ltd., Nottingham, England) saturated with steam generated in Speedlec electrode boiler (Bastian & Allen Co. Ltd., Harrow, England) at 50 *pounds per square inch* (psi). Portable bench-top *Prestige* (*Hi-dome*) pressure cookers were used for smaller volumes at 115°C for 15 to 30 minutes.

2.2.1.2 STERILIZATION BY DRY HEAT

Most glassware was sterilized at 160°C for at least 2 hours in a sterilizing oven (Laboratory Thermal Equipment, Greenfield, NR Oldham, England). Inoculating tools were sterilized by red heat (e.g. loops and needles) or by flaming (e.g.glass-rods, glass pipette tips and scalpels).

2.2.1.3 STERILIZATION OF HEAT-LABILE MATERIALS: EXPOSURE TO ETHYLENE OXIDE GAS

A Sterilizing chamber package (*Anprolene* AN 74) was used (H. W. Andersen Products Inc., Oyster Bay, New York, USA). Samples were packed

loosely in *Anprolene Seal & Peel* bags (AN850, 5" x 200' roll stock) and exposed to ethylene oxide (supplied as sterilizing gas ampules AN 79) overnight in a suitably-ventilated fume chamber.

2.2.1.4 FILTER STERILIZATION: SMALL VOLUMES (UP TO 20ML)

Samples were taken in disposable syringes (*Plastipak,* Becton-Dickinson, England) and forced through *Millex*-GS sterilizing units (pore size=0.22μm) (Millipore Corp., Bedford, Mass. USA) at not more than 60*psi.*

LARGER VOLUMES (OVER 30ML)

Samples were poured into the upper chambers of autoclaved *Millipore* filtering units (100ml or 250ml sizes) and filtered (by connecting to a vacuum line) into lower sterile chambers through sterile Millipore-GS membrane filters (HAWP 004700; pore-size = 45μm; diameter = 47mm). Aliquots were stored in *Sterilin* containers and frozen at -20°C where appropriate.

2.2.1.5 INDICATORS FOR STERILIZATION

AUTOCLAVE	i)	Heat/pressure sensitive autoclave-tapes (3M,St. Paul, MN, USA)
	ii)	*Browne* Sterilizer Control tubes, Type1 Black spot (Albert Browne Ltd.,Leicester, England)
OVEN	i)	*Browne* Type 3 Green spot
ETHYLENE OXIDE	i)	Exposure Indicator stickers (*Anprolene* AN 85)

2.2.2 METHODS FOR DRYING OF SAMPLES

Samples were dried at 60/105°C in the drier (Baird & Tatlock, Essex).

2.2.1.1 VACUUM DRYING

Small volumes were dried *in vacuo* with spinning in a 40-tube capacity *Speedvac* Concentrator (Savant Instruments Inc., Hicksville, New York, USA). Vacuum was generated using *Speedvac* High Vacuum Pump (Edwards High Vacuum Ltd., Sussex, England).Polypropylene vacuum dessicators with polycarbonate caps were used for larger sample volumes.

2.2.3 CENTRIFUGATION

Desired speeds or relative centrifugal fields (RCF) were determined from a Beckman chart which correlated rotor size, RCF and centrifuge speed.

2.2.3.1 MINI-VOLUMES

Samples between 1μl and 1.5ml were centrifuged in *Eppendorf* –microfuge tubes by Bench top microfuges (*Eppendorf* 3200; 5414(S) or *Beckman* Microfuge E) at 10,000-12,000 x *g*.

2.2.3.2 MEDIUM & LARGER VOLUMES

Corex tubes (15 or 30ml) or *Beckman* centrifuge tubes (10, 50 or 250ml capacities) were used together with *Beckman* rotors (JA 14 & 20 types) and

the *Beckman* centrifuge (model J2-21).

2.2.3.3 ULTRA-CENTRIFUGATION

Beckman range of ultracentrifuges (L2-65, L5-65, L7-55, L8-55) were used with suitable centrifuge tubes (*Beckman* polyallomer *Quick-seal* tubes- 13 x 51mm & 16 x 76mm (Beckman Instruments Inc., Ca. USA).

2.2.4 pH MEASUREMENTS

TRIZMA E6634 glass electrode probe (Sigma) fitted to a *Kent* pH meter 7010 (Kent Electronic Instruments Ltd., Chertsey, England) was immersed in samples to determine pH.

2.2.4 1 CALIBRATION & STANDARDIZATION

Commercial buffer tablets for pH 4.0, 7.0 and 9.2 (BDH) were dissolved in 100ml sterile distilled water as standard solutions for calibration.

2.2.4 2 pH ADJUSTMENT

The pH of samples was adjusted to desired values by adding standard solutions of HCl (5.0M, 1.0M, 100mM and 10mM) or corresponding standard solutions of NaOH, depending on initial pH.

2.2.5 PIPETTING

2.2.5 1 VOLUMES BETWEEN 1.0 and 25ml

Standard pipettes of 1 to 25ml capacities (E-mil B, Corning Ltd., Staffordshire, England) were used. Glass pipettes were sterilized by dry heat while plastic pipettes were sterilized by ethylene oxide.

2.2.5 2 VOLUMES BETWEEN 1µL AND 1ML

Gilson (Pipetman) automatic pipettes (P1000, P200, P100 and P20)(Gilson Medical Electronics, S. A., France) were used together with corresponding micropipette tips which had been autoclaved in racks.

2.3 MISCELLANEOUS PREPARATION

(in distilled water except stated otherwise)

2.3.1 STOCK SOLUTIONS

2.3.1.1 Sodium Chloride (NaCl)

NaCl (8.77g); water (to 1 litre)Final concentration = 150mM. Final pH = 7.0

2.3.1.2 Glucose Stock Solution

D-glucose (20g); distilled water (to 100ml)
Sterilized at 105°C. Final pH 7.0. Stored at room temperature

2.3.1.3 Stock Solutions of Amino Acids and Growth Factors (g. 50ml.$^{-1}$)

L-arginine (0.5); L-histidine (0.5); L-leucine (0.5); L-proline (0.5); L-threonine (0.5); L-tyrosine (0.5); L-aspartate (2.5); thiamine. HCl (0.03).

Sterilized by filtration. Stored at 4°C. Final concentrations were 10mg. $ml.^{-1}$. Amino acids except for L-aspartate (50mg ml^{-1}), thiamine (600µg ml^{-1}). pH 7.

2.3.1.4 Stock Solution of Ampicillin

Ampicillin ($C_{16}H_{18}N_3O_4S$. Na. $3H_2O$) [1.0g]; water (to 50 ml). pH adjusted to 8.0 with 5M NaOH. Final concentration 20 mg ml^{-1}. Filter-sterilized and stored at -20°C. Used at 0.5% (v/v).

2.3.1.5 5-*bromo*-4-*chloro*-3-*indolyl* -β-Galactoside (BCIG) STOCK

BCIG (12mg); Dimethyl Formamide (2ml).
Stored in dark bottle. Final Concentration 6mg *ml.* $(Formamide)^{-1}$

2.3.1.6 Standard Protein Solution

Bovine Serum Albumin (F) [0.1g]; NaCl stock (100ml)
Final concentration 1mg BSA ml $(150mM\ NaCl)^{-1}$

2.3.1.7 Sodium dodecyl Sulphate (SDS) Stock

SDS (10g); distilled water (to 100ml). Sterilized by autoclaving.
Stored in *Falcon/Beckman* polypropylene bottles at room temperature.
Final concentration 10% [w/v]

2.3.1.8 Tris-HCl

Tris (Hydroxymethyl)aminomethane crystals (Boehringer Mannheim GmbH, W. Germany)(121.1g); distilled water (to 1 litre).
Final concentration=1M. Final pH=7.6. Sterilized by autoclaving

2.3.1.9 EDTA

Ethylene diaminetetra-acetic acid, Na^+. $2H_2O$ (93.06g); distilled water (to 1 litre). Final concentration=250mM. pH=7.6. Sterilized by autoclaving

2.3.1.10 Potassium Acetate ($CH_3COO.K$) Stock

$CH_3COO.K$ (123g); distilled water (to 250ml).
Sterilized by autoclaving. Final concentration=5.0 M

2.3.1.11 Lysozyme

Lysozyme (50mg); Buffer (1ml). Final concentration = 50mg/ml; stored at -20°C

2.3.1.12 Ethidium Bromide (EtBr)

EtBr (100mg); distilled water (to 10ml). Final concentration = 10mg/ml. Stored in dark brown aluminium-coated reagent bottle at room temperature

2.3.1.13 Ethidium Bromide Neutralizing Solution

500mM $KMnO_4$; 1.25M HCl; water (to 1 litre).
Neutralizes ethidium bromide at 1:1 (v/v) dilution

2.3.1.14 70%(v/v) Ethanol

Absolute Ethanol (70ml); distilled water (30ml). Final volume 100ml.
Stored at room temperature

2.3.2 BUFFERS

2.3.2.1 Tris-EDTA (TE) Buffer

Tris-HCl Stock (1ml); EDTA stock (400µl); sterile distilled water (to 100ml).
Final Concentrations: 10mM Tris-HCl; 1mM EDTA. pH 7.6

2.3.2.2 Tris-buffered Phenol (pH 7.6)

AnalaR Grade Crystalline phenol (100g); Tris-HCl stock (1.2ml); water (26ml).
Left to liquefy overnight at room temperature. Stored in dark bottle

2.3.2.3 STET Buffer

8%(w/v)Sucrose; 5% (v/v)Triton x100; 50mM Tris-HCl; 50mM EDTA
water (to 100 ml). Final pH=8.0

2.3.3 *DOLY* REAGENTS FOR PREPARATION OF PLASMIDS.

2.3.3.1 Doly I (pH 7.6)

50mM glucose; 25mM Tris-HCl; 10mM EDTA; distilled water (to 100ml)
Glucose solution was autoclaved at 115°C. Stored at room temperature.

2.3.3.2 Doly II (Alkaline-SDS Solution)pH 12.5

1% [w/v]SDS; 200mM NaOH; sterile distilled water (to 100ml)
Stored in *Falcon* polypropylene bottles at room temperature.

2.3.3.3 Doly III (pH 4.8)

3M Potassium acetate; Glacial acetic acid (11.5ml); sterile distilled water (to 100ml). Stored in reagent bottle at room temperature.

Ratios 1 [*Doly* I+ (1mg [Lysozyme) ml^{-1})] : 2 [*Doly* II] : 1.5 [*Doly* III] (see 2.5.5; 2.5.6)

2.3.4 BUFFERS FOR ELECTROPHORESIS AND MOLECULAR BIOLOGY

2.3.4.1 Tris-Borate EDTA Buffer (10x)

890mM Tris-HCl ; 890mM Boric acid; 25mM EDTA.
Final pH = 8.3. Used at 10^{-1} dilution

2.3.4.2 Loading Buffer/Tracking Dye (in water)

50% [v/v]Ficoll; 200mM EDTA; (0.001%, w/v) Bromophenol blue

2.3.4.3 10x Mung Bean Nuclease Buffer (in water)

300mM Sodium acetate; 500mM NaCl; 10mM $ZnCl_2$; 50% [v/v] glycerol. Final pH 4.6. Stored at -20°C. Used at 10^{-1} dilution.

2.3.4.4 10x S1 Nuclease Buffer (in water)

500mM Sodium acetate; 1.5M NaCl; 5mM $ZnSO_4$. $7H_2O$.
Final pH 4.6. Stored at -20°C. Used at 10^{-1} dilution.

2.3.4.5 S1 Nuclease "Stop" Buffer

4M-$CH_3COO.NH_4$; 50mM-EDTA (pH 8.0); 20% (v/v) SDS stock; *t*- RNA (10μg. ml^{-1}). Final pH 8.0. Filter-sterilized and stored at 4°C.

2.3.4.6 10x *Klenow* Buffer pH 7.6

500mM-Tris-HCl; 10mM-$MgCl_2$. $6H_2O$; 1mM-Dithiothreitol (DTT); 500mM-NaCl. Final pH 7.6. Filter-sterilized, stored at -20°C and used at 10^{-1} dilution.

2.3.4.7 10x Ligation Buffer for T4-DNA Ligase

660mM–Tris-HCl; 66mM-$MgCl_2$. $6H_2O$; 100mM-DTT; 600μM-ATP. Final pH 7.6. Filter-sterilized, stored at -20°C and used at 10^{-1} dilution.

2.3.4.8 Loading Buffer/Tracking Dye for Electrophoresis

50% (v/v) glycerol; 200mM-EDTA; 10μg. ml^{-1} Bromophenol Blue. Stored at 4°C and used at 10% (v/v) concentration.

2.3.5 Complex and Semi-synthetic Media

Dehydrated commercial stock of preformulated *Oxoid* Nutrient broth and Nutrient agar (Oxoid Ltd., Basingstoke, England) or *Difco* Luria-broth and L-agar (Difco Laboratories Inc., Detroit, Michigan, USA) were used at concentrations recommended by the manufacturers except where indicated otherwise and sterilized by autoclaving (2.2.1.1).

2.3.5.1. Custom-made Complex Media

Sterile complex agar media were made differential for *E. coli* ATCC15224 (constitutive for the *lac* operon) by addition of 50μg. BCIG/ml and selective for ampicillin resistant strains by addition of 100μg. ampicillin/ml. both at 50°C.

2.3.5.2 Defined Minimal Salts Media

Basal Salts Medium: The basal medium was made of the following salts which were added in amounts which gave the following concentrations in the final medium of 100ml or 800ml : –

40 mM-KH_2PO_4; 10 mM-$(NH_4)_2 SO_4$;

2 mM-$MgSO_4$.$7H_20$; 10 μM $FeSO_4.7H_20$.

Formulation

To make one litre, a basal medium of the phosphate, nitrogen and sulphate sources (PNS) was prepared separately in distilled water (as part A) and the pH was adjusted to 7.0 with 5M-NaOH before making up final volume to 600ml. A solution of $MgSO_4$ and the carbon source in distilled water was made up to a final volume of 388ml after adjusting the pH to 7.0 with 100mM-NaOH as part B). $FeSO_4$ was dissolved in distilled water (as part C) to a final concentration of 0.833μM. pH was adjusted to 2.0 with 1M HCl. Components

A, B and C were autoclaved separately to prevent magnesium salts from precipitating. Final medium was made up of 60ml A; 38.8ml B; 1,2ml C. All concentrations given are relative to this final medium.

Carbon Sources

4.0mM D-glucose [10mM];	4.0mM D-fructose [10mM];
4.0mM D-gluconate [10mM]	4.0mM D-glucuronate [10mM];
7.5mM pyruvate [20mM];	5.0mM glycerol [20mM];
7.5mM lactate [20mM]	12 mM.L-arabinose

Amounts in parentheses are concentrations required for adapting cells to growth on specific carbon sources during the preparation of inoculum.

2.3.5.3 Custom-Made Defined Media (see 2.3.1 for stock solutions)

i) Complete medium + 1.5% [w/v] Bacteriological agar no. 1
ii) Complete medium + 0.5% [v/v] ampicillin stock
iii) Complete medium + 0.5% [v/v] amino acids/Growth factors stock
iv) Complete medium + 0.5% [v/v] BCIG stock

2.4 Apparatus for Replica-plating

The replica-plating set consisted of a 50mm-high solid aluminium block with cylindrical top (83mm diameter x 20mm), neck (50mm diameter x 30mm) and base (100 mm square). Sterile circular velvet cloths (diameter=130mm) were secured over the circular frame by rings (diameter=87mm).

2.5 Materials and Methods for Cloning the *ppc* Gene

2.5.1 Cloning Host Culture and Maintenance

E. coli PA342 (*ppc*) was used as specific host for cloning the *ppc* gene. The culture was obtained as an impregnate on filter paper and revived in 10ml L-broth at 37°C for 18 hours, streaked on L-agar plates to confirm purity and isolate individual colonies. Several plate cultures of individual colonies were prepared. .The strain was preserved as agar slant cultures at 4°C for up to 6 months. Sterile glycerol (20%, v/v) was added for prolonged storage of liquid cultures at -20°C or -70°C.

2.5.2 Procedure for Testing Auxotrophy

L-broth cultures grown for 18 hours were serially-diluted to obtain about 400 colonies per plate of L-agar at 37°C. and replicated on plates of glucose minimal salts agar deficient in the following supplements :

a)	L-proline	d)	L-leucine
b)	L-histidine	e)	L-threonine
c)	L-arginine	f)	thiamine.HCl
g)	L-aspartate	(a-f)	Supplemented with L-aspartate

Complete media were defined or complex. Plates were incubated for up to 72 hours.

2.5.3 General Procedure for the Induction of Artificial Competence in Cells

A combination of the methods of Mandel & Higa (1970), Dagert & Ehrlich (1979) and Saunders & Saunders (1988) was adopted. Prewarmed 100ml L-broth in 1-litre flasks were inoculated from an 18-hour stationary culture of *E. coli* PA 342 to initial optical density values of 0.015-0.02 (OD_{600nm}). Cultures were grown with vigorous shaking at 37°C. A flask was withdrawn into ice for treatment after 60, 80, 90, 100 and 120 minutes respectively. Cells were harvested by centrifugation at 1,500x*g* for 5 minutes, rinsed in 50ml cold 10mM Tris-HCl before suspending in 1ml 10mM Tris-HCl.

Direct resuspension in $CaCl_2$ usually produced poorly dispersed, permanently clumped cells. Cells were mixed with cold $CaCl_2.6H_20$ in 10mM Tris-HCl (pH 7.5)to a final volume of 50ml. Different concentrations of $CaCl_2$ were tested (10, 50, 75 & 100mM). Calcium-treated cells were incubated in ice for 30, 45 & 60 minutes respectively, before recovery by centrifugation (1,500x*g* for 5 minutes). Cells were resuspended in 50, 75 or 100mM $CaCl_2.6H_2O$ [10mM Tris-HCl] to a final volume of 1, 2 or 5ml. Options of immediate transformation, prolonged incubation at 4°C or storage at -70°C were tried.

2.5.3.1 Storage of Calcium-Treated Cells

Sterile glycerol was added to competent cells to 20%(v/v). 200µl aliquots in *Eppendorf* tubes were flash-frozen in liquid nitrogen and stored at -70°C.

2.5.3.2 Determination of Transformation Efficiency

Frozen cells were thawed on ice, pelleted in the microfuge (12,000x*g*) for 30 seconds and suspended in 100µl cold $CaCl_2$ before mixing with 10µl (2.5*ng/µl*) pBR322 plasmid vector. Cells were incubated on ice for 30, 45 or 60 minutes respectively, and heat-shocked at 42°C for a minute or two, then mixed with L-broth to 1ml. Suspensions were incubated at 37°C in a water-bath with or without shaking for 45 minutes to express. Options included prolonged incubation for 60, 90 and 120 minutes for full expression. 100µl expression mixture was plated with appropriate dilutions (100µl, 10^{-1}, 10^{-2}, on ampicillin plates; 100µl, 10^{-4}, 10^{-5} and 10^{-6} on L-agar to determine the viability of cells).

2.5.3.3 Controls

Negative controls consisted of 100µl of untransformed thawed cells. Serial dilutions of 10^{-1} to 10^{-7} were plated on L-agar to determine total viable

competent cells. Plates were incubated at 37°C for 24 hours and colonies counted. Incubation beyond 24 hours encouraged the growth of satellite colonies of ampicillin-sensitive cells which masked transformants).

2.5.3.4 Units for Expression of Viability and Transformation Frequency

Total number of viable cells was expressed relative to 1ml of cell suspension. Transformation frequency was expressed as a function of plasmid concentration :

TRANSFORMATION EFFICIENCY	} }	Total Number of Transformed Cells μg^{-1} of Reference Plasmid DNA

2.5.4 Customized Method for Transformation

A combination of the most favourable parameters from test transformations were used to induce competence in *E. coli* PA342. 1-litre of prewarmed L-broth was inoculated with 18-hour stationary culture of *E.coli* PA 342.to an initial optical density of 0.025 at (OD_{600nm}). Samples of 100ml were withdrawn at OD_{600nm} 0.1, 0.2, 0.3, 0.4 and 0.5. and harvested at 3,000x*g* , 4°C, for 5 minutes. Cells were suspended in 1ml 10mM Tris-HCl (pH 8.0) and incubated on ice.

2.5.4.1 Calcium chloride Treatment

The cell suspension was mixed gently with 40ml ice-cold, 50mM $CaCl_2$ buffered with 10mM Tris-HCl (pH 8.0) and incubated in ice for 45 minutes before harvesting. The cells were resuspended in 2ml ice-cold, $CaCl_2$.

2.5.4.2 Transformation and Expression

200µl of treated cells was mixed with 10µl 2.5*ng µl* $(pBR322)^{-1}$ and incubated for 60 minutes on ice. Cells were heat-pulsed at 42°C for 90 seconds only and re-incubated on ice for another 5 minutes. Cells were removed from calcium chloride by centrifugation at 10,000x*g*, 4°C for 30 seconds, resuspended 1ml of L-broth and incubated for 90 minutes in 37°C water-bath without shaking except for periodic mixing every 20 minutes. Undiluted and serially-diluted samples were plated and incubated as described previously. Transformants (cft) were counted to determine transformation efficiency and cell viability after 24 hours.

2.5.5 Isolation of Plasmids from Transformants

A combination of methods from Birnboim & Doly (1979); Dillon *et al.* (1985) and Sambrook *et al.* (1989) were adopted for rapid microscale plasmid preparation. All manipulations were performed at room temperature; exceptions are stated in the text.

2.5.5.1 Method 1

Cells grown in 1.5ml L-broth, recovered at 12,000x*g.* for 30 seconds and washed in 1ml of 10mM Tris-HCl (pH 8.0), were suspended in 100µl *Doly* reagent I and 2µl [10mg/ml] lysozyme and allowed to stand for 5 minutes. 200µl *Doly* II was added with incubation for another 2 minutes before neutralizing with 150µl *Doly* III for 10 minutes. The ratios of volumes of *Doly* reagents to each other were [1:2:1.5]. The supernatant was recovered by centrifugation at 25,000x*g*, 4°C for 20 minutes and deproteinized with TE-buffered phenol (1:1, v/v). Isopropanol (1:1, v/v, room temperature) or ethanol (1:2, v/v, -20°C) was added to the supernatant and incubated for 10 minutes to precipitate the DNA. The precipitate was recovered by centrifugation at 25,000x*g* for 15 minutes and washed in 1ml, 70%(v/v) ethanol then dried *in vacuo* for 2 minutes and dissolved in 50µl TE buffer.

2.5.5.2 Method 2

(As above, without *Doly* Reagents) washed cells were suspended in 50µl *STET* buffer; 5µl (10mg/ml) lysozyme was added and left to stand for 5 minutes before plunging in boiling water for 45 seconds. The pellet from the precipitate was recovered by centrifugation (25,000x*g* for 5 minutes) and discarded. The supernatant was then treated as described above (2.5.5.1).

2.5.6 PREPARATION & PURIFICATION OF PLASMIDS IN BULK

Larger amounts of plasmids were prepared, recovered and purified from 200 to 500ml of culture by adapting the method of Grinsted & Bennett (1988). Cells were first recovered at 8,000x*g* at 4°C for 5 minutes. 5ml *Doly* I, 10ml *Doly* II and 7.5ml *Doly* III were used. The supernatant was recovered by centrifugation at 25,000x*g* , 4°C for 20 minutes and decanted over glasswool into fresh centrifuge tubes. The sample was deproteinized with an equal volume of TE-buffered phenol at room temperature. Phenol-resistant tubes were used. The upper aqueous phase was recovered by centrifugation at 25,000x*g* 20°C for 2 minutes and added to 15 ml isopropanol at room temperature. After incubation for 20 minutes to precipitate the DNA, the sample was centrifuged at 25,000x*g* at 20°C for 10 minutes to recover the pellet. The pellet was washed with 2ml, 70%(v/v) ethanol, recovered at 25,000x*g* , for 5 minutes and dried *in vacuo* .

2.5.6.1 Dye-Bouyant Density Equilibrium Centrifugation

The dry pellet was dissolved in 3.5ml, TE buffer (pH 7.6). 3.7g cæsium chloride (CsCl) was added with mild vortexing to dissolve. The density of the solution was 1.75*g./ml.* The addition of 200µl ethidium bromide reduced the density to 1.58*g./ml.* and brought the final volume was 5ml. The solution was

transferred to 13 x 51mm *Quick-seal* polyallomer centrifuge tubes (Beckman, Instruments Inc., California, USA). Larger volumes were loaded onto 16 x 76 mm tubes. The tubes were topped up with liquid paraffin where necessary before sealing by heat (Beckman heat sealer).

2.5.6.1.1 Cæsium chloride / Ethidium bromide Gradient

Tubes were loaded into Beckman vertical rotor (VTi65) to balance each other and centrifuged *in vacuo* at 55,000rpm at 22°C for 5 hours. The larger samples were centrifuged in Beckman titanium fixed angle rotor (Ti 70.1) at 49,000rpm for 16 hours. In both cases, the Beckman series of grade 'H' ultracentrifuge (L5-65; L7-55; L8-55) was used.

Tubes were removed from the rotor with minimum agitation and illuminated with ultraviolet light to visualize the bands. Two bands were usually visible. The lower band of supercoiled, covalently-closed circular plasmid DNA was recovered by piercing the side of the tube below the band with a 21 gauge hypodermic needle attached to a syringe. Total volume was usually between 700µl and 1.5ml.

2.5.6.2 Extraction of Plasmids from Cæsium chloride / Ethidium bromide Solution

The solution was diluted three-fold with 10mM-Tris-HCl (pH 7.6) and distributed in 800µl aliquots into *Eppendorf* tubes. 70µl of 5M-potassium acetate (pH 4.6) and 500µl *iso*propanol were added to each tube. The tubes were vortexed, then centrifuged in a benchtop microfuge at room temperature. The pellet was resuspended in 40% [v/v] isopropanol solution containing 50µl, 5M-potassium acetate (pH 4.6) and centrifuged to remove residual CsCl. The pellet was washed in 1ml, 95% [v/v] ethanol. Pellets from the same batch were combined into single volumes of 800µl, 10mM-Tris-HCl and deproteinized with 800µl buffered phenol. The mixture was vortexed and centrifuged for 1 minute at 12,000x*g* . The upper aqueous phase was recovered and mixed with 70µl, 5M-potassium acetate and 500µl *iso*propanol and incubated at for 10 minutes at –20°C. The precipitate was recovered by centrifugation at 12,000x*g* and washed in 1ml, 70% [v/v] ethanol before drying *in vacuo*. The pellet was dissolved in 250µl TE buffer (pH 7.6). All manipulations were carried out at room temperature except where stated otherwise.

2.6 Analysis of Plasmids

Plasmids were screened by electrophoresis and restriction analysis, after Grinsted & Bennett (1988).

2.6.1 RESTRICTION ANALYSIS

DNA samples were usually digested in 500µl-*Eppendorf* tubes at 37°C for 60 minutes. Where necessary, 1µl of RNAse was added after initial incubation for 40 minutes.

2.6.1.1 Restriction Enzyme Cocktail

Typical Cocktail for Digestion with a Single Restriction Enzyme

Plasmid sample	3-5µl
Ready-made (*REact*)Buffer (10x)	2µl
Concentrated Restriction Enzyme (10 U/µl)	1µl
Distilled water	to 16µl
RNAse A (0.5µg./µl)	1µl
FINAL VOLUME	20µl
Loading Buffer	3µl (post-digestion)

TYPICAL COCKTAIL FOR DIGESTION WITH DOUBLE RESTRICTION ENZYME

Plasmid sample	3-5µl
Common *REact* Buffer (10x)	2µl
Concentrated Restriction Enzyme I (10 U/µl)	1µl
Concentrated Restriction Enzyme II (10 U/µl))	1µl
Distilled water	to 16µl
RNAse A (0.5µg./µl)	1µl
FINAL VOLUME	20µl
Loading Buffer	3µl (post-digestion)

Undigested samples consisted of the plasmid in distilled water and loading buffer only.

2.6.2 ELECTROPHORESIS

DNA samples were electrophoresed in horizontal agarose gels (Agarose *Ultra pure* electrophoresis grade, Bethesda Research Laboratories, Gaithersburg, MD, USA). The voltage was generated from a regulated DC power supply (0.25-250*volts*, 300*mA*) (Kikusui Electronic Corporation).

2.6.2.1 Gel Making Apparatus

Agarose was used at concentrations of 0.8% (w/v) in single strength TBE buffer (pH 8.3). 800mg agarose in 100ml TBE buffer was dissolved in the microwave for 2 minutes at full power and allowed to cool to 50°C. 10µl, ethidium bromide (10mg/ml) was added before pouring into suitable gel casts. Two sizes were used: (a) minigel (5 x 8cm; 8-sample well capacity); (b) medium-sized gel (11 x 13cm; 12 to 17-sample capacities).

2.6.3 DETERMINATION OF MOLECULAR SIZES

The sizes of native or restricted DNA samples were estimated relative to the mobility of standardized fragments generated by digesting bacteriophage λ with restriction enzyme, *Hind* III except where stated otherwise.

2.6.4 ESTIMATION OF THE CONCENTRATION OF PLASMID

The concentration of plasmid was estimated spectrophotometrically. The absorbance of 1%(v/v) preparation was measured at λ_{260nm}. The absorbance of samples was also determined at $\lambda_{270, 280, 208nm}$ respectively to determine the presence of extraneous matter which may interfere with values at λ_{260nm}.

2.6.4.1 Interpretation of Absorbance Readings of DNA Samples

λ_{260nm}	absorbance due to DNA in sample
λ_{270nm}	absorbance due to contaminating Phenol
λ_{280nm}	absorbance due to residual protein in sample
λ_{208nm}	absorbance due to other extraneous matter
$\lambda_{260nm}1.0$	was equivalent to 50μg (double stranded DNA) ml^{-1}

2.6.4.2 Determination of Relative Purity of DNA Preparations

The sample was considered relatively free of proteins if the $\lambda_{260}/\lambda_{280}$ ratio was above 1.8 and relatively free of RNA if the ratio was less than 2.2. In addition, a $\lambda_{270}/\lambda_{260}$ ratio of 0.8 or less represented relatively phenol-free samples (Dillon *et al.*, 1985).

2.7 SCREENING OF TRANSFORMANTS

Cells which contained relevant plasmids were first identified by selecting for characteristic antibiotic-resistant phenotypes which the plasmids conferred on them. Plasmids were subsequently isolated, purified and analysed by electrophoresis (see section 2.6). Hybrid plasmids containing desired genes were detected by complementation analysis. Specific enzyme activity was also assayed in extracts of cells carrying such plasmids

2.7.1 ASSAY OF ENZYME ACTIVITIES:

PHOSPHO*ENOL* PYRUVATE CARBOXYLASE

100ml prewarmed minimal medium containing 10mM glucose was inoculated with an 18-hour culture of test organism on glucose to an initial OD_{420} of 0.2. and grown for 2 hours to OD_{420} (0.8) and harvested at 8,000x*g*, 4°C for 10 minutes. The pellet was washed in 50ml, 10mM Tris-HCl buffer (pH 8.0) and suspended in cold, 5ml, 50mM Tris-HCl-5mM $MgCl_2$ (pH 8.0). Cells were disrupted by sonication to obtain crude extracts.

2.7.1.1 Preparative Methods and Ultrasonication of Cells

Pre-cooled *Dawe* soniprobe Type 7532A was inserted into 3ml of cell suspension in a 6ml-sample bottle on ice to sonicate at 80 watts (Lucas Dawe

Ultrasonics, London) in 4 cycles of 30 seconds each at intervals of 30 seconds. The sonicate was cleared of cell debris by centrifugation at 25,000x*g*, 4°C, for 20 minutes.

2.7.2 ENZYME ASSAY

A typical assay cocktail contained the following (per *ml.* of buffer) :-

Ultrasonic extract (in buffer)	10, 20, 30...60µl
500mM, $NaHCO_3$	20µl (saturating)
10mM acetyl CoA	10µl
10mM NADH	10µl
Malate dehydrogenase	1µl (undiluted)
100mM PEP	20µl
Buffer (50mM Tris-HCl, 5mM $MgCl_2$)	to 1ml (pH 8.0)

The assay-cocktail without PEP was equilibrated at 27°C and assayed for intrinsic oxidase activity for 4 minutes at λ_{340nm}. The reaction was started by addition of PEP. Extinction of NADH was monitored spectrophotometrically, at A_{340} for 4 minutes.The coupled reaction involved is illustrated (figure 2.1).

2.7.2.3 Expression of Enzyme Activity

Specific activity of enzyme was expressed in *µmol.* of NADH oxidized *mg.*(Protein)$^{-1}$ minute^{-1}.

2.7.3 ASSAY OF PROTEIN IN ULTRASONIC CELL EXTRACTS

Protein was determined after Bradford (1976). 1, 10 & 100µl of clarified ultrasonic cell extracts and serially-diluted standard protein solutions (10-50µl, 1mg (BSA) ml.$^{-1}$ in 100µl buffer) were added to 5ml *Bradford.* Reagent and incubated at 37°C for 10 minutes. Absorbance was read at λ_{595nm} against a reaction blank containing only buffer and reagent. The unknown protein concentration was estimated by extrapolation from a standard curve of BSA concentration and λ_{595} (figure 2.2).

2.7.3.1 Bradford Reagent

A	Coomasie Brilliant Blue	100mg
(ii)	95%(w/w) Ethanol	50.0ml
B	85%(w/w)*ortho* -phosphoric acid	100ml
C	Distilled water	(to 1 litre)

(Filtered using filter paper-lined Buchner Flask)

BUFFER: 50mM Tris-HCl, 5mM $MgCl_2 \cdot 6H_2O$ (pH 7.5)

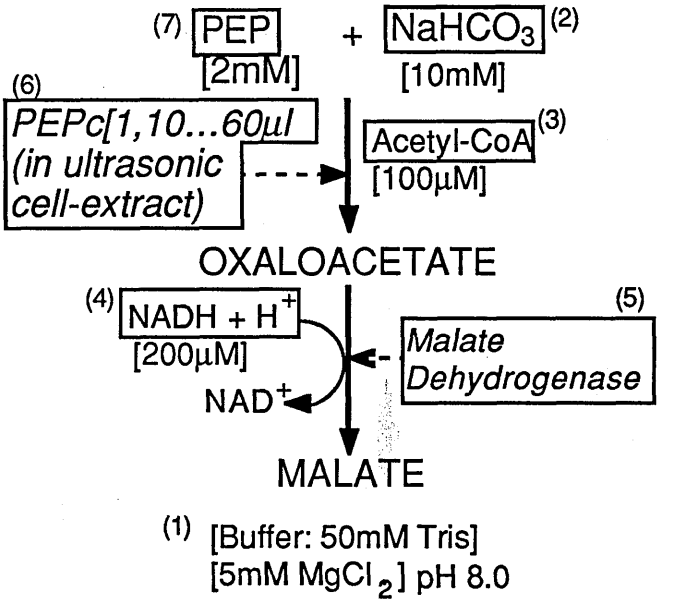


FIGURE 2.1 COUPLED REACTION FOR ASSAY OF PHOSPHO-*ENOL*PYRUVATE CARBOXYLASE ACTIVITIES IN ULTRASONIC EXTRACTS OF CELLS OF *Escherichia coli*

Reactants (boxed) are numbered in the order in which they were added to the reaction cocktail.Most reactants in 1ml of reaction cocktail were present at saturating concentrations. The activity of PEPc was monitored spectrophotometrically by determination of the rate of oxidation of NADH at λ_{340nm} after the addition of PEP. Acetyl-CoA was necessary for the activation of PEPc in cell extracts

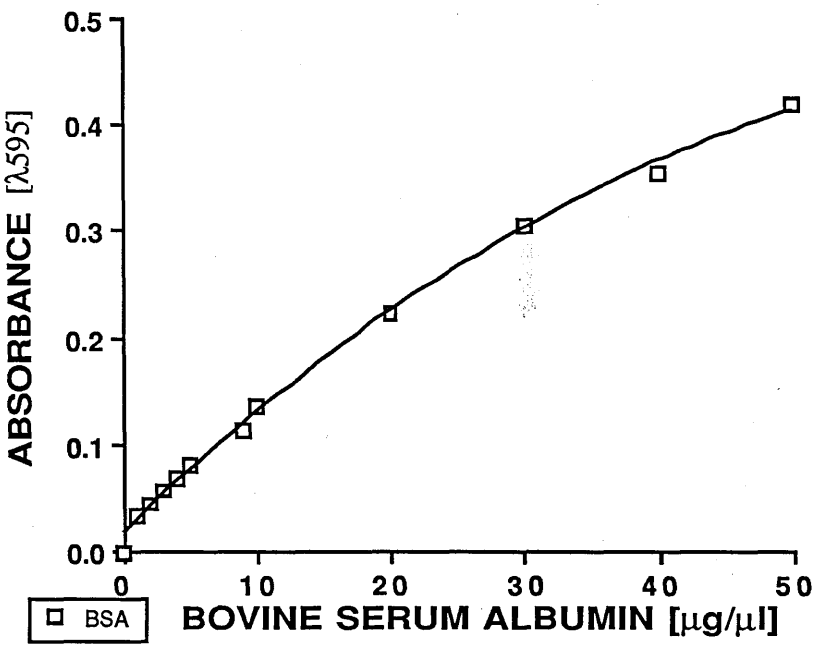


FIGURE 2.2

CALIBRATION CURVE FOR ESTIMATION OF PROTEIN CONCENTRATION IN ULTRASONIC EXTRACTS OF CELLS

2.8 PHYSIOLOGICAL STUDIES

Growth studies were carried out in batch culture containing a specified carbon and energy source in minimal medium. The medium was contained in 1-litre flat-bottomed flasks with side arms.

2.8.1 PREPARATION OF INOCULUM

A single colony from a plate culture of the test organism was inoculated into 100ml of minimal medium containing a specific carbon and energy source (pH 7.0) in 250ml-*Erlenmeyer* flasks. The concentration of the substrate was equivalent to 720*ppm* of organic carbon. The culture was grown over 10 generations and diluted 1:100 in fresh medium similar to the first. The subculture was also diluted after 10 generations as described above and grown for the same length of time as the second subculture. The final subculture was harvested at 4,000x*g* , 4°C for 5 minutes and washed in carbon-free minimal medium before suspending in fresh, carbon-free minimal medium at 4°C. The procedure described above was standard for adapting cells to growth on specific substrates.

2.8.2 CULTURE FOR METABOLIC STUDIES

800ml, minimal medium in a 1-litre flask was inoculated with cells which had been conditioned to growth on the specific substrate. The concentration of the substrate was equivalent to 288*ppm* organic carbon or 4mM glucose. The initial cell-density expected was OD_{420}=(0.05). A sample was taken immediately after inoculation to determine basal state of the culture. The apparatus for growth was set up and operated as described by Harvey *et al.* (1968) and Holms & Hamilton (1970).

2.8.2.1 Frequency of Sampling

5ml of culture sample was withdrawn for analysis at intervals, for 7 to 12 hours. In the first hour, samples were taken 30 minutes apart. Sampling time was reduced to 20, 10 and 5 minutes respectively in 3 succeeding hours; the order was reversed for subsequent hours.

2.8.2.2 Determination of Growth Rate and Mean Generation Time

Increase in cell density was the index of growth of cells and was measured turbidimetrically as apparent optical density (OD) at λ_{420nm} in a single beam flow cell *Philips Pye Unicam* PU8610 (uv/vis)Kinetics spectrophotometer, against a blank of uninoculated medium. OD was determined after a ten-fold dilution when the relationship between cell density and optical density became non-linear after OD_{420} 0.5. Dilution kept all values below OD_{420} (0.5). Apparent OD was then computed by multiplying the OD_{420}

value by the dilution factor.

Growth was defined by the (bio)mass doubling time (mean generation time) in the log phase. The mean generation time was calculated from semilogarithmic plots of cell density, as the time required for the concentration of cells to change two-fold. The specific growth rate of exponentially-growing cells (" μ ")was derived as a function of the generation time determined experimentally:

$$\mu = \frac{(\ln 2)}{MGT}$$

μ = growth rate $(hour)^{-1}$
(ln2) = natural log of 2 (or 0.693)
MGT = mean generation time (hour)

2.8.3 MEASUREMENT OF BIOMASS AND DETERMINATION OF FACTOR FOR CONVERSION OF OPTICAL DENSITY TO DRY WEIGHT OF CELL BIOMASS

Optical density values were converted to bacterial dry weight by determining the relationship between optically-determined density and actual cell density. Apparent optical measurement of cell density at OD_{420} was calibrated for the spectrophotometer used, by a combination of the recommendations of Mallette (1969), Koch (1981) and Gerhardt (1981).

A four-litre glucose culture of *Escherichia coli* was concentrated 100-fold in ice-cold distilled water after harvesting at 5,000x*g*, 4°C, for 10 minutes and two successive washes in equal volumes of cold distilled water. The OD of the thick suspension was determined by serially diluting to a working value of less than 0.4 at λ_{420} which was then multiplied by the dilution factor. The cells were harvested at 8,000x*g*, 4°C, 10 minutes, dried to constant weight at 105°C on aluminium foil of known weight and cooled in a dessicator. The difference in the final weight of the dried sample and the foil, was the dry weight of cells of known OD which was expressed as $\mu g.$ dry weight. $ml.^{-1}$ of initial culture. An OD_{420} of 1.0 was equivalent to 205 ($\pm$7)$\mu g.$ dry bacterial biomass $ml.^{-1}$ of liquid culture.

2.8.4 PROCESSING OF CULTURE SAMPLES

Turbidity of each sample was read at λ_{420} before centrifugation at 8,000x*g*, 4°C for 5 minutes. The cell-free supernatants were frozen at -20°C until analyzed. Alternatively, 1.5ml of each culture-sample was taken in sterile *Eppendorf* tubes with labels and centrifuged at 20,000x*g*, 4°C for 1 minute in a microfuge, then frozen immediately for subsequent analysis. The turbidity was determined from a different fraction. When required for analysis, each tube was thawed, mixed and centrifuged to concentrate the cells and obtain cell-free supernatant.

2.9 ANALYSIS & ANALYTICAL METHODS

The following were determined :-

1 Cell biomass in culture
2 Concentration of Residual Substrate
3 Other metabolic products in culture medium

Estimation of Cell biomass has been described (*see* 2.11.2.3).

2.9.1 ESTIMATION OF THE CONCENTRATION OF RESIDUAL SUBSTRATES

2.9.1.1 Glucose

Glucose was determined by a modification of glucose Oxidase-Perid method (Boehringer Mannheim GmbH) as described by Werner et *al* (1970):

Half-strength *Reagent* II (see below) was made up by dissolving 7g in 1 litre distilled water. 200µl of culture supernatant or a ten-fold dilution, was added to 5ml, Reagent II and incubated at room temperature for 25 minutes. Colour development was monitored at λ_{610nm} in *Philips Pye Unicam* PU8610 (uv/vis) Kinetics spectrophotometer, against a blank containing only reagent II and carbon-free minimal medium.

REAGENT II

(1) 100*mmol*l [phosphate buffer]$^{-1}$;
(2) 0.8*U*ml [peroxidase]$^{-1}$
(3) 10*U*ml [glucose oxidase]$^{-1}$;
(4) di-ammonium-2, 2'-azino-*bis*-3-ethylbenzothiazoline-6-sulfonate (ABTS)

STANDARDS

Standard glucose solutions of 0, 0.5, 1.0, 1.5...5mM were made in basal minimal salts medium. 200µl of each sample was treated as shown above (2.9.1.1). The intensity of colour development was related to glucose concentration in a standard curve (figure 2.3) Concentration of residual

glucose in culture samples was determined by extrapolation of corresponding absorbance values from the standard calibration curve.

Residual substrate concentration was also determined as total organic carbon (TOC). After making allowances for other sources of organic carbon in medium (predetermined by specific analytical methods), TOC due to substrate carbon was determined by difference.

2.9.1.2 Determination of Total Organic Carbon (TOC)

TOC was determined using *Shimadzu* Total Organic Carbon Analyzer (TOCA) model TOC-500 (Shimadzu Corporation, Kyoto, Japan) under the following analytical conditions:

Carrier Gas	1kg/cm2 compressed air
Mass Flow rate	150ml/min
Measuring Range	x 10
Injection volume	10µl
Measuring unit	parts per million (ppm)
Standard solution (for calibration)	0.850g l^{-1}, Potassium hydrogen phthalate (2-(COOH)C_6H_4COOK)

CALIBRATION

10µl standard solution of 2-(COOH)C_6H_4COOK was analyzed in the TOCA This yielded the equivalent of 400µg carbon/ml (or 400ppm organic carbon). TOC of samples was estimated relative to 400ppm carbon/ml of standard. Thus, 1mM glucose corresponded to 72ppm; 1mM acetate, 24ppm and 100µg/ml [27µm] ampicillin was 70-ppm.

2.9.1.3 Estimation of Residual Fructose

Fructose was determined by adapting the Inulin assay method of Heyrovsky (1956) or by TOCA.

REAGENT STOCK

1) 500mg ß-Indolyl acetic acid (ß-IAA) in 100ml, 95%(v/v) ethanol; stored in dark brown bottle at room temperature.
2) Concentrated HCl [37%(w/v)].
3) Standards (0.1, 0.2 ...1, 2...5 mM fructose)
4) Blank (distilled water or carbon-free medium)

METHOD

100µl sample was mixed in equal volumes with ß-IAA, 4ml HCl was added and samples were incubated at 37°C for 60 minutes. Development of colour was monitored at λ_{530nm} against a reaction blank. Residual fructose in culture supernatant was estimated by extrapolation from a standard curve of fructose concentration and absorbance (λ_{530})(figure 2.3).

2.9.1.4 Estimation of Residual Pyruvate

Pyruvate was determined by HPLC (see below) and detected as described below after a retention time of 6 minutes 20 (±10) seconds (column 300x7.8mm, see below) on the small column and three times longer on the large. Alternatively, it was assayed by TOCA.

2.9.1.5 Estimation of Residual D-Gluconate and D-Glucuronate

D-gluconate was determined by HPLC and detected at a retention time of 5 minutes 15 (±10) seconds.D-glucuronatewas determined by HPLC and detected at a retention time of 4 minutes 15 (±10) seconds. Residual concentrations were estimated from a standard curve of peak height against authenticated standards of each carbon source (figure 2.4).

2.9.2 Analysis Of Metabolic Products

The most significant metabolic by-product in culture was *acetate*. In this study, acetate was assayed by chromatography.

2.9.2.1 Analysis of Samples by High Performance Liquid Chromatography (HPLC)

Apparatus

Gilson HPLC system was used; the set consisted of :

1) 2 pumps (model 303)
2) Manometric module pump pressure gauge (model 802C)
3) Holochrome detector and data master (model 620)
4) Apple IIe computer monitor and printer
5) Chromatographic column

Columns

Biorad , HPLC Fast Organic Acid Analysis Columns were used (HPAH, 100x7.8mm; Aminex Ion Exclusion, HPX-87H, 300x 7.8mm) (Biorad, Richmond, Ca. USA) together with *Biorad* Microguard (Cation H) column.

Mobile Phase and Mobile Phase Velocity

The mobile phase was filtered 25mM H_2SO_4 (M & B, Pronalys AR, Ltd., Dagenhan, England [density=1.84g/ml]) at a flow rate of 1ml/min and a back pressure of 1.05K*psi* (70Bar).

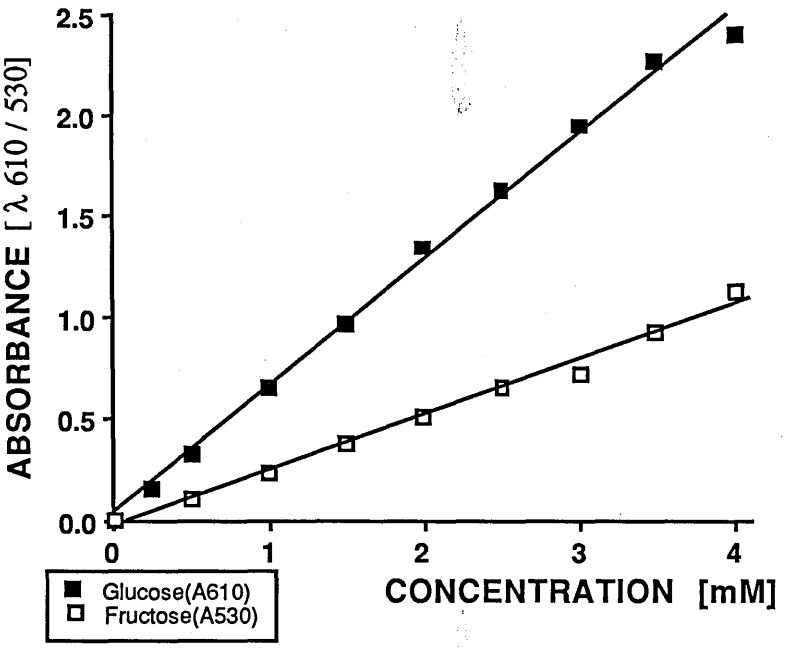


FIGURE 2.3

CALIBRATION CURVE FOR ESTIMATION OF D-GLUCOSE AND D-FRUCTOSE CONCENTRATIONS

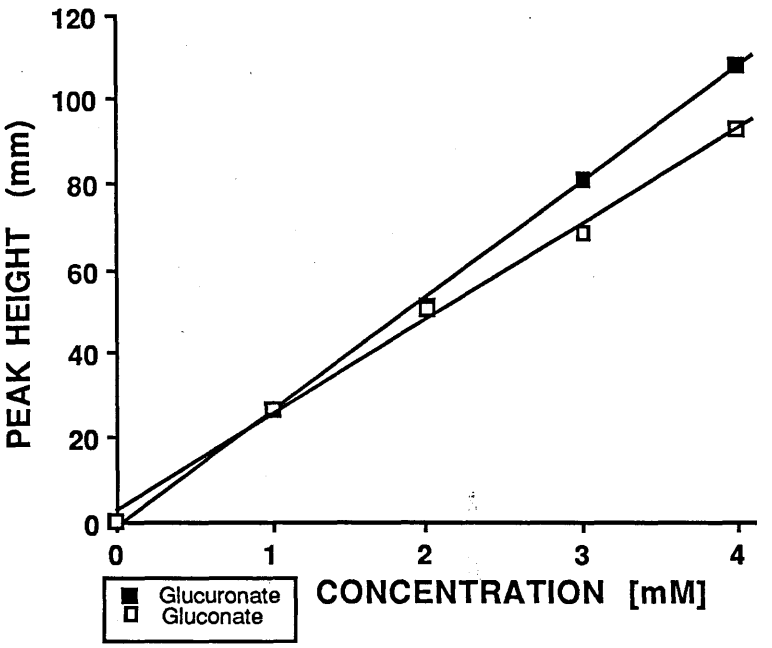


FIGURE 2.4

CALIBRATION CURVES FOR THE ESTIMATION OF D-GLUCONATE AND D-GLUCURONATE CONCENTRATIONS BY HPLC

Loading of samples

A known volume of sample was loaded into the sample pot using a 1ml-syringe with a blunt-tipped needle.For practical purposes, the actual volume processed was ultimately determined by a range of fixed-volume sample loops (20-100µl).

Qualitative Detection of Fractions

Sample fractions were resolved into discrete peaks at λ_{210nm} and detected at peak-sensitivity of 0.05. The retention time for each peak was characteristic. The total peak area was a quantitative measure of the fraction detected. When the width of peaks was held constant, the height of peaks was positively correlated with concentration of sample fraction.

2.9.2.2 Calibration

Standard solutions of the substance under investigation were loaded in equal volumes into the HPLC. A standard curve relating concentration to peak area/height was generated to determine unknown quantity of the component of interest.

2.9.2 3 Qualitative & Quantitative Acetate Estimation

100µl cell-free culture supernatant was analysed by HPLC. The peak corresponding to acetate was previously identified using an authenticated sample (13.61mg, $CH_3COONa.\ 3H_2O$ in 10ml, sterile distilled water or 10mM. diluted to 01, 0.2, 0.3... 2mM). Acetate was detected after a retention time of 3 minutes 15 (± 5) seconds or 9 minutes 15 (±10) seconds (larger column). The concentration of acetate was estimated by extrapolation from a standard curve of peak height and acetate concentration (figure 2.5).

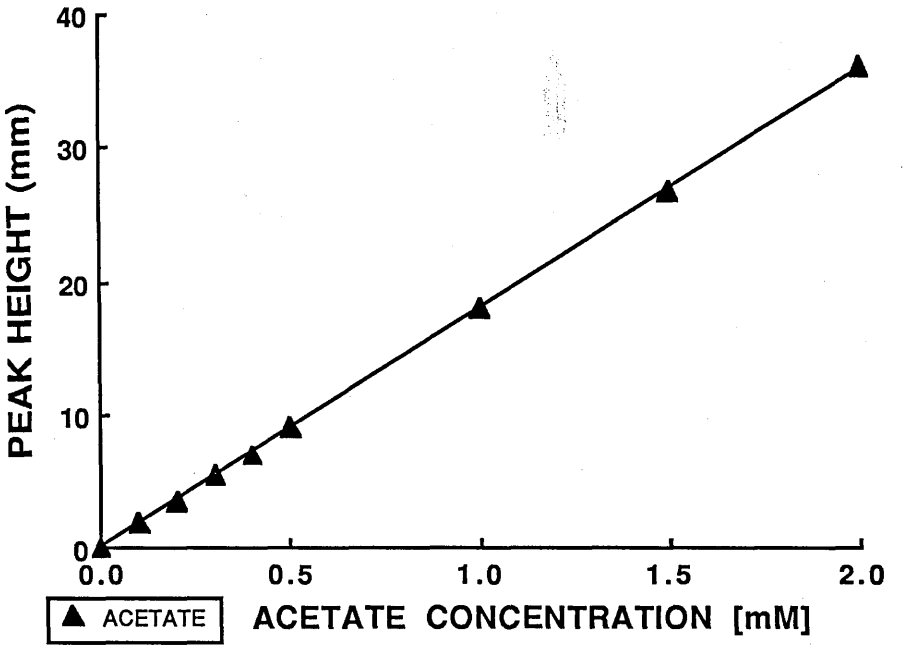


FIGURE 2.5

CALIBRATION CURVE FOR ESTIMATION OF THE CONCENTRATION OF ACETATE IN CULTURE FROM HPLC TRACES

Peaks were detected at a sensitivity of 0.05, λ_{210} . The retention time for acetate in the organic acid column (300x7.8mm) was three times more than the column of 100x7.8mm

RESULTS

CHAPTER 3

CLONING OF THE *ppc* GENE AND ASSAY OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE ENZYME ACTIVITIES

3.1 INTRODUCTION:

THE ROLE OF PHOSPHO*ENOL* PYRUVATE CARBOXYLASE IN AEROBIC BACTERIAL METABOLISM

During carbohydrate metabolism, the fixation of carbon dioxide (CO_2) serves to introduce non-substrate carbon into intermediary metabolism (Wood and Utter, 1965). This reaction is unidirectional in enterobacteria (Sanwal *et al.*,1971) and mediated by the enzyme–phospho*enol* pyruvate carboxylase (PEPc) in *Escherichia coli* (Cánovas and Kornberg, 1965; Yoshinaga *et al.*, 1974; Sugita and Komatsubara, 1989). During the process, phospho*enol* pyruvate (PEP) the acceptor-molecule for CO_2, is carboxylated into oxaloacetate (Bandurski and Greiner, 1953) which subsequently condenses with acetyl-CoA to produce citrate and sustain the TCA cycle (Luinenburg and Coleman, 1990). This reaction has been identified as the unique anaplerotic step for the synthesis of C_4-dicarboxylic acids during the metabolism of 3-carbon compounds or their precursors (e.g. hexoses) in several bacterial systems (Ashworth and Kornberg, 1966; Kornberg, 1966; Sanwal and Maeba, 1966; Quayle, 1969), and is analogous to pyruvate carboxylase in *Bacillus spp.*(Fisher and Magasanik, 1984), yeasts and mammalian systems (Wood and Utter, 1965).

3.1.1 THE ENZYME AND ITS GENE

Phospho*enol* pyruvate carboxylase–ortho-phosphate: oxaloacetate carboxy-lyase (phosphorylating—EC 4.1.1.31) is an allosteric enzyme-protein specified by the *ppc* gene (Phillips *et al.*, 1987). Its presence in animal tissues has not been demonstrated (Lane *et al.*, 1969). The principal molecular properties of PEPc have been determined and published. Its molecular weight of 361,000 is the sum of four identical subunits, each of which contains a single polypeptide chain, with serine residue at the amino terminus (Yoshinaga *et al.*, 1974). The enzyme assumes multiple conformational states relative to a multiplicity of allosteric effectors (Table 3.1) which interact with at least four regulatory sites on each subunit (Teraoka *et al.*, 1974).

The determined nucleotide sequence of the structural gene, which is located at *89.45 minute* on the linkage map of *E. coli* (Bachmann, 1983), contained approximately 3,100 base pairs (Fujita *et al.*, 1984). Among the clones of the *Clarke-Carbon* colony/gene bank, which consisted of synthetic colE1-*E. coli* hybrid plasmids in *E. coli* JA200 (F^+, *thr, leu, lac* YI, *trp* Δ*E5, recA1* ; Guest, 1981a), the *ppc* gene was borne on plasmid pLC20-10 (Phillips *et al.*,1987) and strain JA200|pLC20-10 (Izui *et al.*, 1981)

ALLOSTERIC EFFECTORS

POSITIVE (ACTIVATING)	NEGATIVE (INHIBITING)
Acetyl-CoenzymeA	L-aspartate
Fructose-1,6-*bis* phosphate	L-malate
Long Chain Fatty Acids (LCFA)	Fumarate
CoenzymeA derivatives of LCFA	Nucleotides
Organic Solvents	
Dioxane	
Alcohols	

TABLE 3.1 MULTIPLE ALLOSTERIC EFFECTORS OF THE ENZYME PHOSPHO*ENOL* PYRUVATE CARBOXYLASE OF *E. coli*
(Cánovas & Kornberg, 1966; Sanwal, 1970; Yoshinaga *et al*, 1970; 1974)

The critical metabolic role of the enzyme is underscored by the inability of *Escherichia coli* PA342, a phospho*enol* pyruvate carboxylase-negative mutant (table 2.1), to grow on glucose, glycerol or pyruvate as sole carbon and energy sources in minimal media. Ashworth and Kornberg (1966) associated this phenotype with an *anaplerotic* requirement for the output of the PEPc flux in the replenishment of the tricarboxylic acid (TCA) cycle. The auxotrophy was relieved when C_4-dicarboxylic acid intermediates of the TCA cycle or their derivatives were supplied as nutritional supplements.

The flux mediated by the enzyme is a direct link between glycolysis and the TCA cycle under aerobic conditions. It is also a unidirectional bridge between the phosphorylated and carboxylated intermediates of the central pathways. The enzyme was therefore a naturally-attractive candidate for modulating the efficiency of carbohydrate utilization by bacteria.

Quantitative analyses of the level and specific activity of PEPc in *E.coli* revealed that both were low (Cánovas and Kornberg, 1965; Izui, Sabe and Katsuki, 1981). As a remedy, Wöhrl *et al.*(1990) recommended the amplification of pertinent genes to enhance low side enzyme activity. Clarke and Carbon (1975) described a procedure for studying specific

gene systems and their phenotypes by cloning portions of the genome of *Escherichia coli* associated with the desired gene and product. It involved the cloning of essential genes by direct selection for complementation of specific chromosomal mutations in bacterial hosts from a comprehensive bank of genes (Clarke and Carbon, 1976). The bank would normally consist of chromosomal fragments on a vector with a characteristic selectable marker (Kohara *et al*, 1987).

This chapter reports the cloning of the *ppc* gene, amplification of the activity of PEPc *in vivo* and evaluation of the effect of increased enzyme activity on aerobic bacterial metabolism of selected carbon sources. A primary gene library containing pBR322–*E. coli* genomic DNA hybrid plasmids was screened to isolate the *ppc* gene.

3.1.2. Notes on the Construction of a Chromosomal Gene Bank of *Escherichia coli* on a Plasmid

The genomic library was constructed from 10µg chromosomal DNA isolated from *Escherichia coli* strain JEF8 (Hfr, *thr, car* B, *met* B); (Clugston, 1986). Partially-digested fragments were generated randomly with varying concentrations of class II restriction endonuclease enzyme, *Sau*3A which cuts at a tetranucleotide recognition sequence with high frequency, to generate fragments with single-stranded, over-hanging cohesive ("sticky") ends (USB manual, 1990; Figure 3.1).

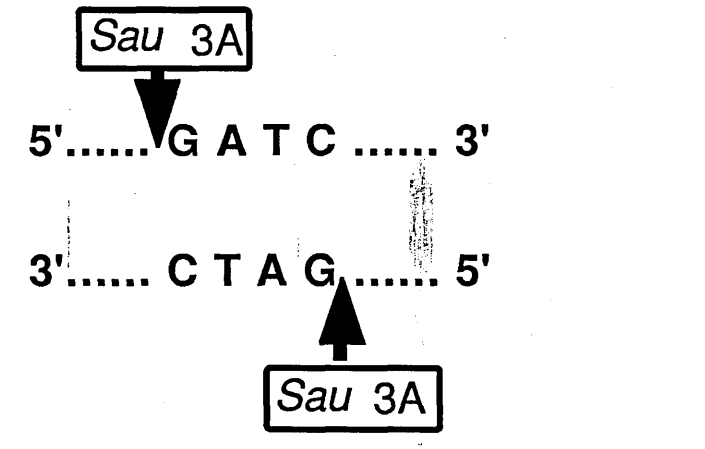


Figure 3.1 Recognition Sequence and Target Site of Restriction Enzyme *Sau* 3A

The partial digests were electrophoresed for 9 hours on a 0.8% (w/v) horizontal agarose gel at 60v. Fragments corresponding to 5-10 kilobases were extracted and purified.

Plasmid pBR322 (copy number:30±10) was used as cloning vector by complete digestion of 2.0µg with *Bam*HI restriction enzyme to generate cohesive ends which were compatible with corresponding ends on the genomic fragments, to facilitate the annealing of fragments to vector. The partial *Sau*3A fragments were ligated to the linear vector. The ligate was transformed into *E. coli* 902 (*rec*A). The presence of inserts in the plasmids was assessed by random analysis of plasmids from 15 ampicillin-resistant colonies of transformants of the bank DNA— all contained inserts. Plasmid DNA was prepared from colonies recovered from plate cultures by aseptic washing. Each microlitre of library-DNA preparation produced as many transformants as 20ng of plasmid pBR322 or about 3.0×10^3 *cft.*|*µl.*.

3.1.3 SCREENING OF THE GENE BANK

Escherichia coli strain PA342 (*ppc,arg, his, leu, pro, thr*) was the host to screen the gene library by complementation of its *ppc* lesion.

3.1.3.1 MECHANISM OF GENE TRANSFER

Transformation was considered the most practical mechanism for introducing plasmid cloning vectors containing DNA inserts into the host. Host bacterial cells "permeable" to exogenous DNA molecules are said to be in a state of *competence*. Several bacterial species (e.g. *Streptococcus sp., Bacillus subtilis, Neisseria sp., Acinetobacter calcoaceticus , Haemophilus influenzae , Pseudomonas stutzeri, Azotobacter vinelandii and some Cyanobacteria spp* ; Saunders & Saunders, 1988) are intrinsically competent at some point in their growth cycle. However, no strain of *E.coli* is known to be naturally transformable. For strain PA342, competence was induced by a combination of physical and chemical treatments.

3.1.3.2 INDUCTION OF ARTIFICIAL COMPETENCE

A number of extrinsic factors which induced competence are known (Saunders *et al* , 1984). However, their effectiveness varied widely, even within a species. Some of these factors were investigated to maximize competence in the the test-strain.

A typical cloning host needed to be sufficiently competent relative to a reference plasmid such as pBR322 DNA. This guaranteed that the colonies of transformants were representative of every one of about 3,000 genes envisaged in the gene bank of *Escherichia coli* with an estimated genomic size of 4700kb (Clarke and Carbon, 1975; Tabata *et al* ,1989). The probability ***(P)*** of finding a desired sequence in a collection of genes and the relative number of clones which must be screened ***(n)*** were derived by a simple algebraic function (Clarke and Carbon, 1976):

$$P = 1-(1-L/M)^n$$ (Burt and Brammar, 1985)

L = Average size of genomic fragments (max.=10kb; min.=5.0kb; ∴mean ≅7.5kb)

M = Total Genomic size (about 4.7×10^3 kilobase pairs (kb)in *E. coli)* (Kohara *et al*, 1987)

The mandatory pool-size of clones **(n)** was computed by :

$$n = ln\,(1-P)\; ln\,(1-L/M)^{-1}$$ (Clarke and Carbon, 1976)

According to the prediction, at least 2.884×10^3 recombinants would give 99% probability ($P = 0.99$) of obtaining the gene of interest from the gene bank. In addition, 1µl of bank-DNA mix would require competent cells with a minimum transformation efficiency of 1.44×10^5 transformants $\mu g.$ reference plasmid $pBR322^{-1}$.

3.2 TRANSFORMATIONS

Initial attempts to induce competence in *E. coli* PA342 produced low frequencies of transformed cells. However, the results revealed a correlation between transformation efficiency and growth phase of cells (Figure 3.2, i). Treatment of cells from the early log phase with calcium chloride ($CaCl_2$) produced relatively higher frequency of transformants. Thereafter, transformation efficiency decreased steadily. Another short cycle of competence was initiated in the mid-exponential phase (Figure 3.2, ii). Beyond the mid-log phase, further increase in apparent optical density did not lead to a corresponding increase in the total number of cells surviving treatment with calcium chloride (Figure 3.3).

FIGURE 3.2 (i)

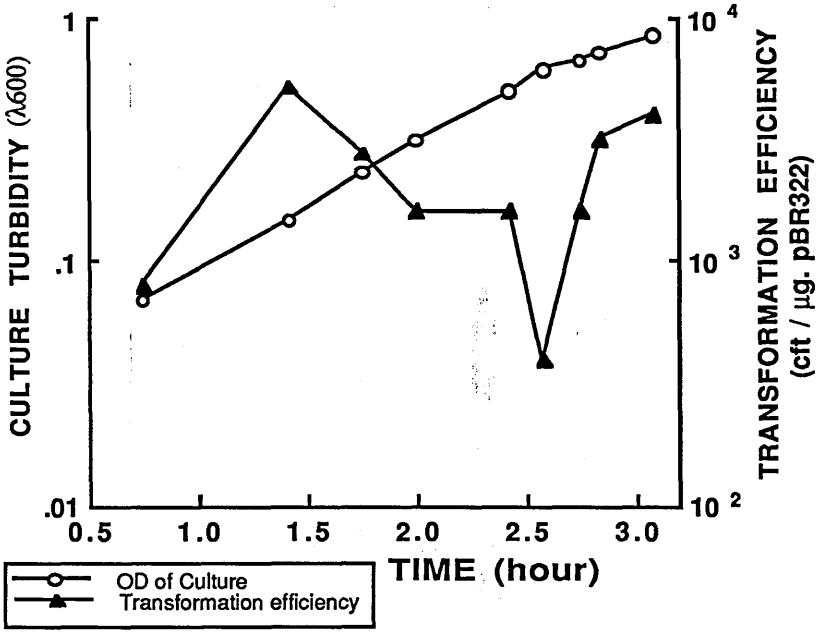


FIGURE 3.2 (ii)

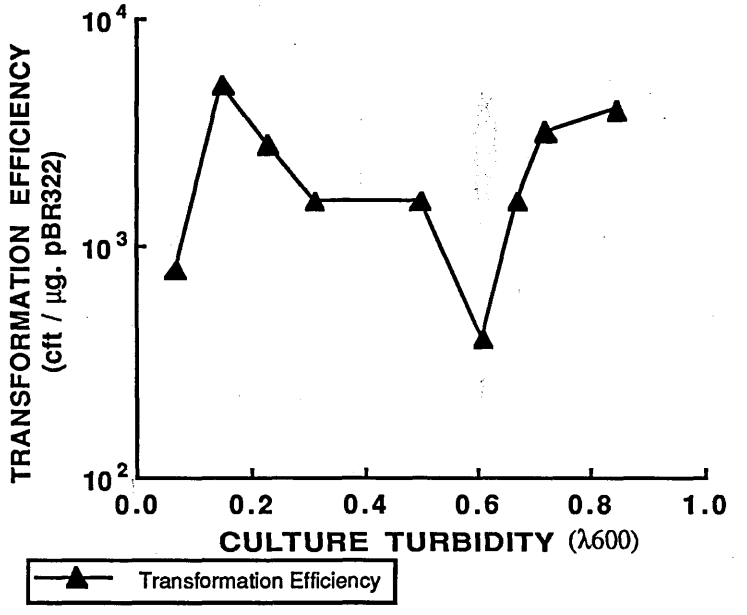


FIGURE 3.2 RELATIONSHIP BETWEEN GROWTH PHASE AND TRANSFORMATION EFFICIENCY OF *E.coli* PA 342

3.2.1 EFFECT OF VARIATION OF SOME PARAMETERS ON TRANSFORMATION EFFICIENCY

Competent cells were transformed as described previously but the duration of expression time after heat pulse was varied. The cells used were thawed after freezing in 20% glycerol at -70°C; their competence had been determined previously by transformation with pBR322 (figure 3.2). Samples of cells were plated after 45, 60, 90 & 120 minutes of expression time in L-broth following heat-shock and their total transformants were compared (Figure 3.4)

As expression time increased, the number of transformants also increased, mainly due to daughter cells of primary transformants and to a lesser extent, newly expressed transformants. The total number of survivors also increased with prolonged expression time. Transformation efficiency was not affected significantly by the initial volume of medium (Figure 3.5).

3.2.2 TRANSFORMATION OF *E. coli* PA342

The most favourable parameters from experimental transformations carried out previously were combined to induce competence in *E. coli* PA342. The detailed protocol has been described in section 2.5.4. Colony-forming transformants *(cft)* were counted to determine transformation efficiency per µg of DNA and viability of competent cells (Table 3.2).The batches of cells shown in table 3.2 were based on the points at which the growing cells were harvested to be made competence.

All samples gave at least 1.0×10^5 *cft. µg.* reference plasmid DNA.$^{-1}$. Transformation efficiency after the first hour of growth (batch 105) was as high as 4.08×10^6 *cft. µg.* (pBR322)$^{-1}$. Cells obtained between the first and second hours of growth (batches 157 to 316) remained highly competent to varying degrees (Figure 3.6).

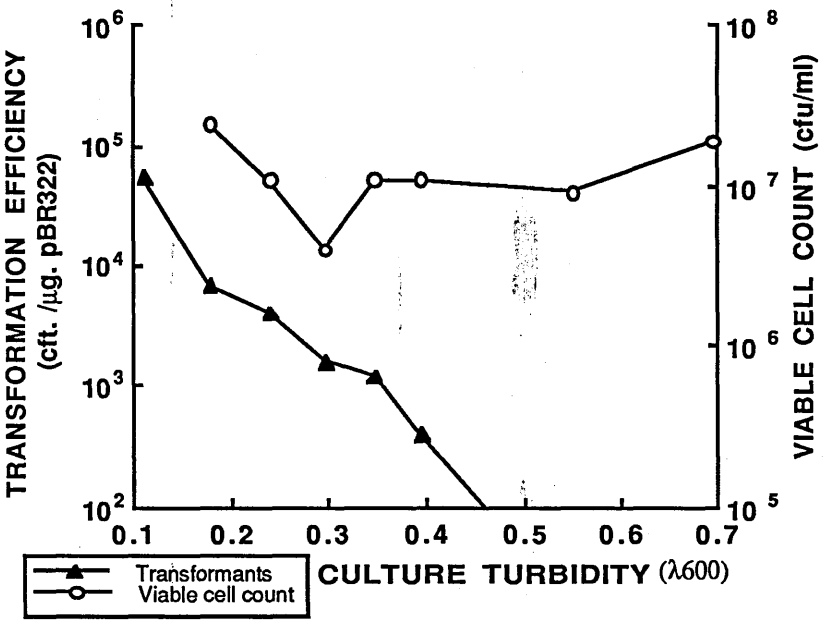


FIGURE 3.3
RELATIONSHIP BETWEEN GROWTH, VIABILITY AFTER CALCIUM CHLORIDE TREATMENT AND TRANSFORMATION EFFICIENCY

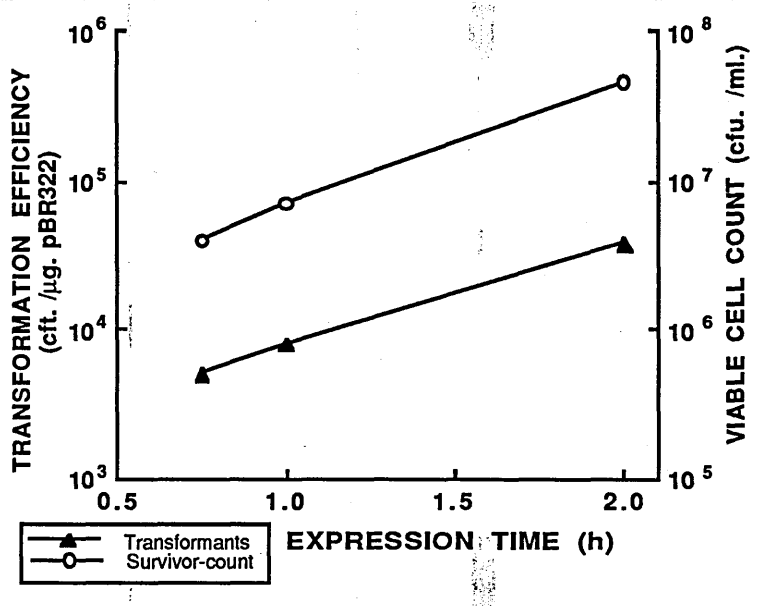


FIGURE 3.4
EFFECT OF EXTENDED EXPRESSION TIME ON TRANSFORMATION OF COMPETENT CELLS OF *E.coli* PA342

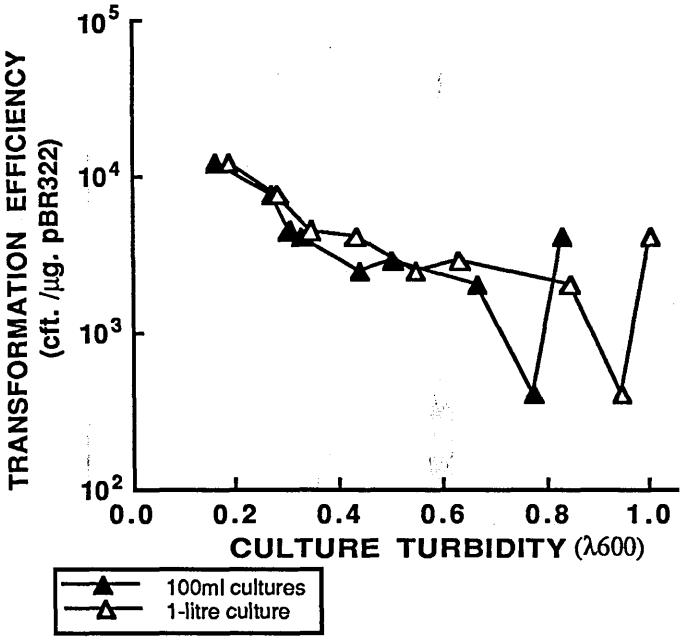


FIGURE 3.5

EFFECT OF CULTURE VOLUME ON CELL DENSITY AND COMPETENCE OF *E. coli* PA342

BATCH "A" (shaded symbols)

10 x 100ml L-broth cultures in 250ml Flasks grown at 37°C with orbital shaking.

BATCH "B "(open symbols)

l-litre L-broth culture in 2-litre flask grown at 37°C with magnetic stirring and additional aeration with 200cc compressed air min^{-1}

The apparent optical density, viable cell count and transformation efficiency were compared in both culture systems.

TIME (min)	TURBIDITY (OD_{600})	BATCH NUMBER	TRANSFORMANTS on L-amp (cft) 10^0	TRANSFORMANTS on L-amp (cft) 10^{-1}	VIABLE COUNT (cfu) 10^{-6}	EFFICIENCY (cft µg (Ref. Plasmid DNA)$^{-1}$
0	0.025	25	0	0	-	0
61	0.105	105	TNC	1020	206	4.08×10^6
81	0.157	157	TNC	330	143	1.32×10^6
91	0.183	183	TNC	90	139	3.52×10^5
105	0.236	236	nd	51	157	2.04×10^5
120	0.316	316	TNC	10	135	4.04×10^5

TABLE 3.2 TRANSFORMATION EFFICIENCIES OF *E. coli* PA342 TREATED AT DIFFERENT STAGES OF GROWTH

Incubation time represented the duration of growth before cells were harvested from the primary culture. Zero time was at inoculation with 18-hour overnight culture in L-broth. Expression time after heat shock was uniform (see 2.5.4).

cft	Colony-forming transformants	nd	not determined
cfu	Colony-forming Units	TNC	Too Numerous to Count (≥1000 colonies)
Ref.	Reference (plasmid)	min	time in minutes
OD	Apparent Optical Density	10^0	undiluted samples
λ_{600}	Optical Density of culture at wavelength of 600nm		
XFE	Transformation Efficiency (colony-forming transformants/µg of plasmid DNA)		

FIGURE 3.6 (i)

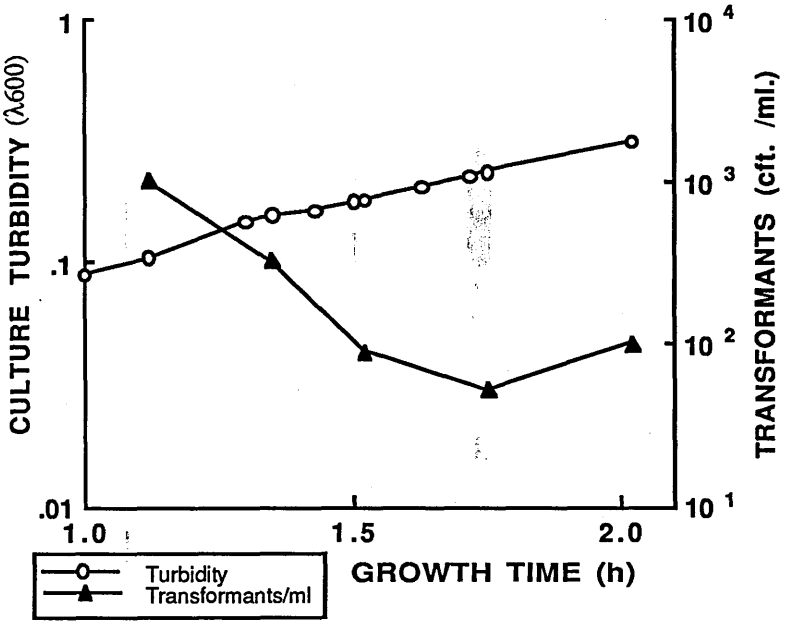


FIGURE 3.6 (ii)

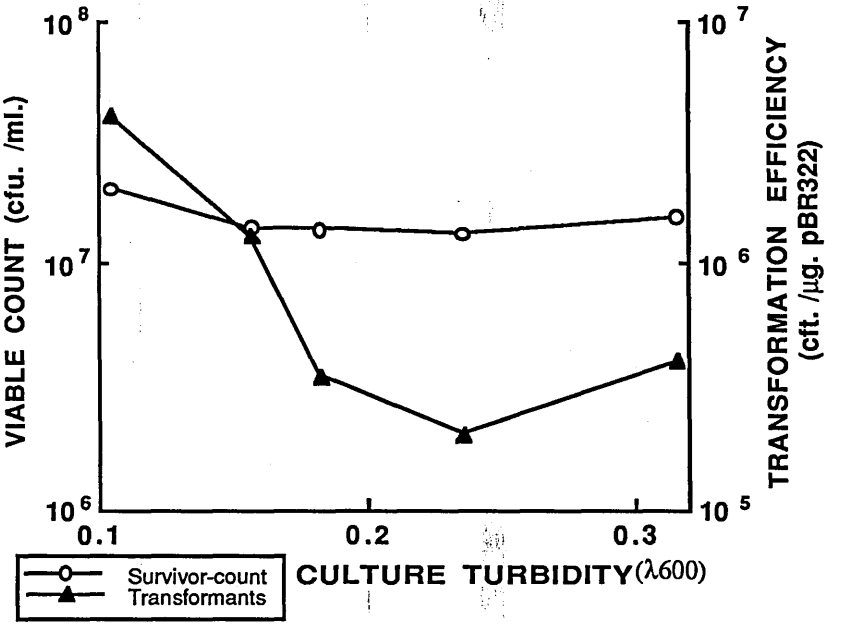


FIGURE 3.6 GROWTH, VIABILITY AND TRANSFORMATION EFFICIENCY OF *E. coli* PA342 BY ADAPTED METHOD

3.2.3 SCREENING THE GENE BANK FOR THE *ppc* GENE USING COMPETENT CELLS OF *E. coli* PA342

The gene bank was screened for the complementation of batch 105 of competent cells (transformation efficiency = 4.08×10^6 *cft.* µg. reference plasmid pBR322^{-1}). The competent cells were transformed with 1ul of Bank DNA mix (transformation efficiency≡20ng pBR322). The entire expression mix was plated in 100µl aliquots on selective media. A second batch of cells was also transformed with the vector plasmid simultaneously as a control/reference transformation (Table 3.3).

TOTAL TRANSFORMANTS	**3216 *cft* µl (Bank DNA-mix)$^{-1}$**
MANDATORY MINIMUM POOL-SIZE (p=0.99)	**2884 recombinants**
TRANSFORMATION EFFICIENCY	**1.48×10^6 *cft.* µg (pBR322)$^{-1}$**
TOTAL VIABLE COUNT	**4.6×10^7 *cfu.*(ml·expression mix)$^{-1}$ (after freezing & thawing)**
LOSS OF VIABILITY DUE TO FREEZE-THAWING	**22%**

TABLE 3.3

SUMMARY OF TRANSFORMATIONS OF COMPETENT CELLS OF *E. coli* PA342 WITH HYBRID PLASMIDS OF THE GENE BANK AND PLASMID VECTOR pBR322

3.2.4 Analysis of Recombinant Isolates for Complementation of *ppc* Lesion of *E. coli* PA342

Master-plates of colonies of recombinant isolates on L-agar ampicillin were replica-plated on a batch of selective media to test for:

1) ampicillin resistance
2) relief of nutritional auxotrophy, obviating the need for supplementation with C_4-dicarboxylic acids of the TCA cycle or their derivatives
3) requirement for marker-amino acids due to characteristic lesions in parent
4) viability on non-selective medium

A plate of *E.coli* PA342 transformed with pBR322 was used as negative control. All recombinants were viable on complete media (L-agar *ampicillin* or glucose plates supplemented with L-aspartate and other vital nutrients). The majority retained auxotrophy for the essential amino acids. The replica-plate test was repeated for 95 presumptive recombinants which were deficient in at least two supplements of the glucose media. Colonies were marked against a square-grid for easy identification and plates were incubated for 24, 48 and 72 hours. Twenty two recombinants were retained for further testing after comparing with positive and negative controls. Of these, 18 were either poorly complemented or were contaminants. Only Isolates *JO* 1, 2, 3 & 4, needed to be screened further.

3.2.4.1. Fine Analysis of Isolates Growing on Aspartate-free Glucose medium: Isolation & Analysis of Plasmids

Plasmid DNA was isolated from 200ml L-Broth-ampicillin cultures of isolates which appeared to grow on aspartate-free glucose plates in the presence of ampicillin (described as *glucose*$^+$). *E. coli* PA342. Batch 157 of competent cells of *E. coli* PA342 (transformation efficiency, 1.32×10^6. µg. (plasmid pBR322)$^{-1}$; Table 3.2) was transformed with 5µl of each plasmid (Table 3.4). Recombinant plasmids containing the *ppc* gene should retransform all competent cells of strain PA342 (*ppc*) to *ppc*$^+$ genotypes. After expression, each 1ml culture was standardized after initial plating to obtain about 400*cfr* per master-plate for replication. Plasmid preparation from Isolate*JO*1 (pJOE1) failed to complement (*ppc*) lesion of other *E. coli* PA342 transformants. Complementation by plasmids from Isolates *JO*2 and *JO*3 (pJOE 2&3) was weak even after extended incubation. Transformants of recombinant plasmid pJOE4 from *isolate JO4*, grew on glucose plates after 24 hours, making this the most likely candidate (Table 3.4).

PLASMIDS IN TRANSFORMANTS	REPLICA - PLATES (Deficient Amino acid)						
	Glucose	Pro	His	Arg	Leu	Thr	Complete Medium
pJOE1	–	–	–	–	–	±	++
pJOE2	-+	–	–	–	–	±	++
pJOE3	-+	–	–	–	–	±	++
pJOE4	+	–	–	–	–	±	++

TABLE 3.4
NUTRITIONAL TEST OF *E. coli* PA342 RE-TRANSFORMED WITH HYBRID PLASMIDS FROM PRESUMPTIVE GLUCOSE+ ISOLATES

Expression culture was diluted or concentrated (as applicable) to obtain about 400 colonies per plate. The colonies were replicated on test plates to determine degree of complementation and characteristic nutritional requirements.

GLU Glucose-*ampicillin* medium supplemented with Amino acids but not L-aspartate
COMPLETE Glucose-*ampicillin* medium supplemented with Amino acids and L-aspartate

ABBREVIATIONS FOR DEFICIENT AMINO ACIDS: Proline (Pro); Histidine (His); Threonine (Thr); Arginine (Arg); Leucine (Leu)

nd	not determined	(*cfr*)	colony-forming recombinants
-+	weak replication (poor growth)	-	negative (no growth)
±	(leaky mutation?)	+/++	positive /normal growth

pJOE1,2,3,4 are codes for hybrid plasmids recovered from transformants of *E. coli* PA342

3.2.5 Restriction & Gel Electrophoretic Analysis of Hybrid-plasmids pJOE2, pJOE3 & pJOE4

Plasmids pJOE2, pJOE3 and pJOE4 were analyzed for genomic-fragment inserts containing the structural gene for phospho*enol* pyruvate carboxylase by single digestion with selected restriction enzymes. Double digestion was also carried out using a combination of enzymes in suitable common buffers. Hybrid plasmids pJOE2 and pJOE4 contained unique targets for restriction enzymes *Sal* I and *Eco*RI respectively. Plasmid pJOE3 contained two (figure 3.7). The vector, pBR322 (size= 4.363 kb) had unique sites for both enzymes (*Sal*I at 0.651/4.363kb and *Eco*RI at 4.361/4.363kb of the published nucleotide sequence for pBR322). The published sequence of the *ppc* gene contained two characteristic *Pvu*II recognition sites separated by about eleven hundred base pairs while the vector, pBR322 also had a single site (2.066/4.363kb). Hence, a hybrid plasmid containing the *ppc* gene must contain at least three *Pvu*II sites. Hybrid plasmid pJOE3 had up to three bands after digestion with *Pvu*II while recombinant plasmid pJOE2 had two (figure 3.7). Restriction of recombinant plasmid pJOE4 with *Pvu*II generated 3 fragments estimated at 1.1, 3.5 and 4.8kb respectively (figure 3.8).

3.2.6 Detailed Analysis and General Picture of the Recombinant Plasmid pJOE4

Hybrid plasmid pJOE4 was examined in greater detail because it bore some characteristics of the *ppc* gene. Regions of the hybrid plasmid which corresponded to the vector plasmid pBR322 were determined by comparative analysis of the vector and the hybrid plasmid (Figure 3.9). The relative positions of all restriction targets were defined by comparing single and double digests from restriction enzymes (Figure 3.10).

Linear molecules of recombinant plasmid pJOE4 were generated by *Sal* I, *Pst* I, and *Eco*RI, which cleaved only the vector at single targets. The sizes of the linear molecules produced by each, was estimated at 9.36 (±0.1) kilobase pairs. Given that the vector accounted for 4.363 kb of this, the size of the segment of cloned bacterial DNA was 5 (±0.1)kb. The recombinant plasmid contained two *Bam*HI sites and three *Pvu*II sites, two of which were located on the genomic-insert. The insert had no recognition sites for *Sal*I, *Pst*I, *Eco*RI or *Acc*I. The presence of two *Eco*RI sites on plasmid pJOE3 (figure 3.7) suggested that it was unlikely to be a potential *ppc* clone based on the published sequences of the gene and pBR322.

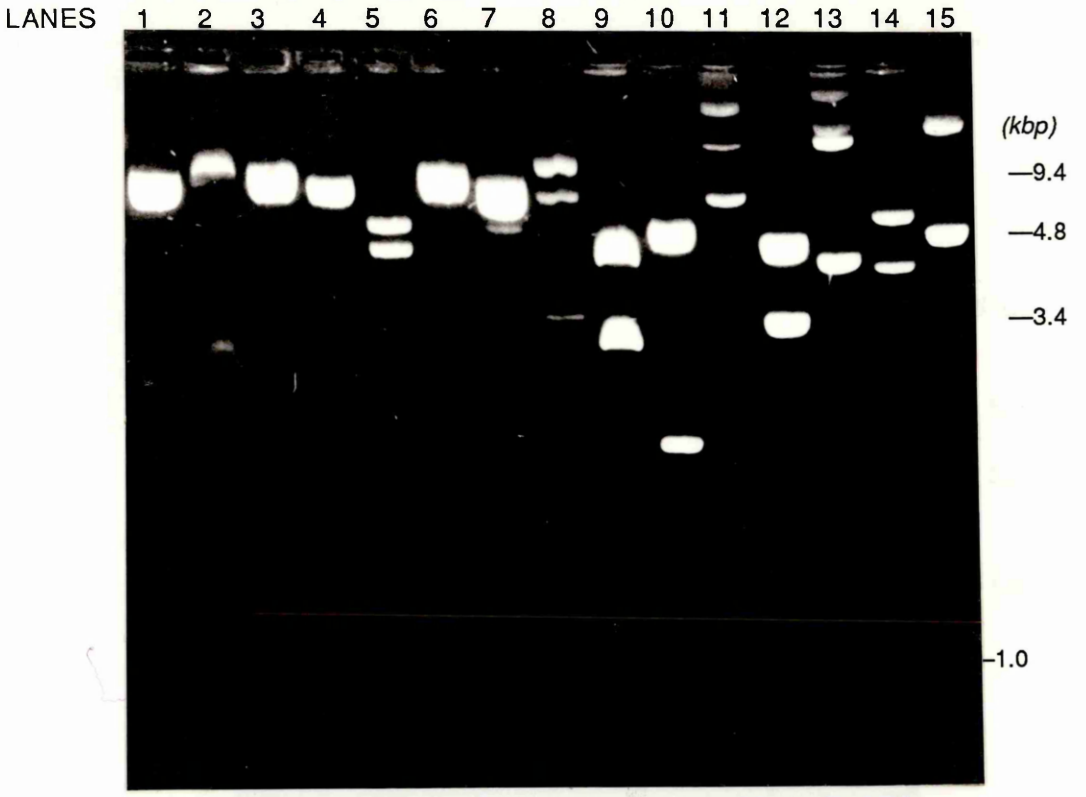


FIGURE 3.7

SINGLE ENZYME DIGESTION OF HYBRID PLASMIDS pJOE2, pJOE3 & pJOE4 WITH *Sal* I *Eco*RI, *Pvu*II & *Bam*HI

Individual hybrid plasmids were digested with the single restriction enzyme stated at 37°C for 1hour. Fragments were analysed by horizontal (0.8%, w/v) agarose gel electrophoresis in TBE buffer at 70V. Samples in lanes 13 to 15 were not digested with restriction enzymes.

LANE 1	pJOE2 treated with *Sal* I	LANE 8	pJOE3 treated with *Pvu*II
LANE 2	pJOE3 treated with *Sal* I	LANE 9	pJOE4 treated with *Pvu*II
LANE 3	pJOE4 treated with *Sal* I	LANE 10	pJOE2 treated with *Bam*HI
LANE 4	pJOE2 treated with *Eco*RI	LANE 11	pJOE3 treated with *Bam*HI
LANE 5	pJOE3 treated with *Eco* RI	LANE 12	pJOE4 treated with *Bam*HI
LANE 6	pJOE4 treated with *Eco* RI	LANE 13	Untreated Plasmid pJOE2
LANE 7	pJOE2 treated with *Pvu*II	LANE 14	Untreated Plasmid pJOE3
		LANE 15	Untreated Plasmid pJOE4

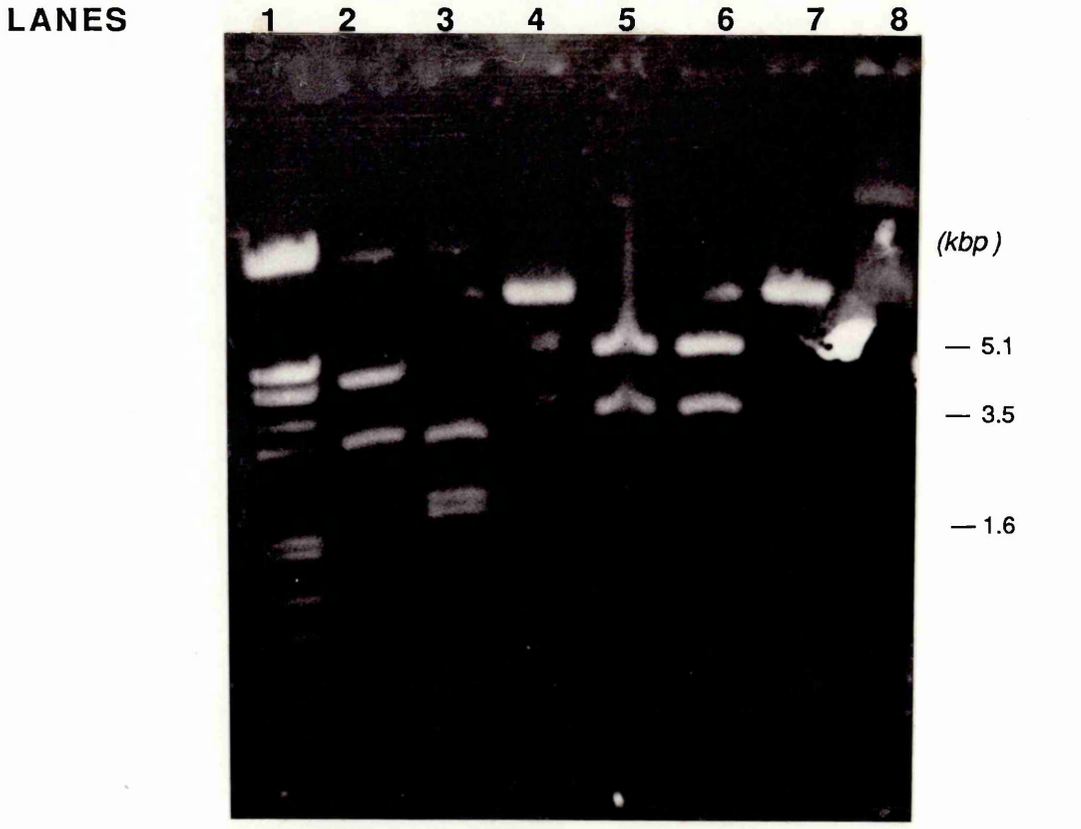


FIGURE 3.8

ANALYSIS OF RECOMBINANT PLASMID pJOE4 :

SINGLE AND DOUBLE SIMULTANEOUS DIGESTION WITH RESTRICTION ENZYMES *Bam*HI, *Eco*RI, *Hin*dIII AND *Pvu*II

Electrophoresis of single digests of plasmid pJOE4 with *Bam*HI, *Eco*RI, *Hin*dIII & *Pvu*II, and double digests with *Bam*HI/*Pvu*II, *Eco*RI/*Pvu*II and *Bam*HI/*Eco*RI on (0.8%, w/v) horizontal agarose minigel. Sizes of fragments were determined by comparison with bacteriophage λ treated with *Hin*dIII and *Eco* RI. There is a slight upward bend from samples 1 to 8 probably due to a disproportionate flow of current.

LANE 1	Molecular weight marker (λ *Eco* RI +*Hin*dIII)		
LANE 2	*Pvu*II	LANE 5	*Bam*HI
LANE 3	*Pvu*II /*Eco*RI	LANE 6	*Bam*HI /*Eco*RI (see also 3.10, lane 1)
LANE 4	*Eco*RI	LANE 7	*Hin*dIII
		LANE 8	Untreated plasmid pJOE4

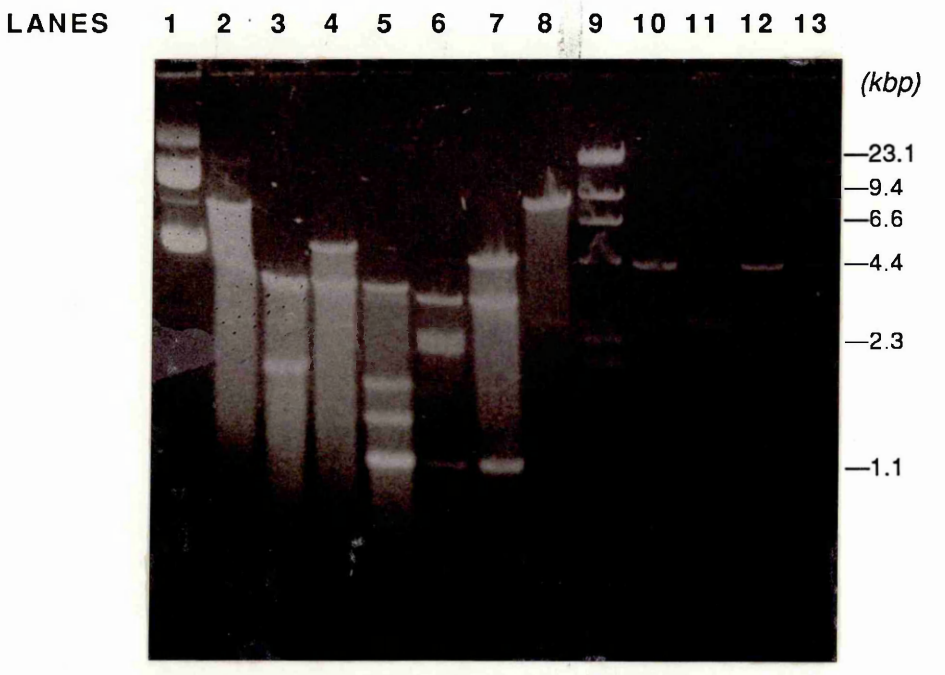


FIGURE 3.9

ANALYSIS OF RECOMBINANT PLASMID pJOE4 :
COMPARATIVE RESTRICTION ANALYSIS WITH PLASMID-VECTOR pBR322

Recombinant Plasmid pJOE4 (1-8) and vector pBR322 (10-13) were digested with *Pst* I, *Pvu*II, *Eco*RI and *Bam*HI, and analysed by (0.8%, w/v) agarose minigel electrophoresis in TBE buffer. There is a slight upward bend of bands especially between lanes 1 to 8 probably due to a disproportionate flow of current.

LANE 1		Untreated plasmid	LANE 8		*Eco*RI
LANE 2	Plasmid }	*Pst* I	LANE 9	Phage λ Treated with *Hin*dIII	
LANE 3	pJOE4 }	*Pst* I /*Bam*HI		(Molecular Weight Marker)	
LANE 4	{lanes 2-8)}	*Bam*HI	LANE 10	Plasmid}	*Pvu*II
LANE 5	digested}	*Pvu*II /*Bam*HI	LANE 11	pBR322}	*Pvu*II/*Bam*HI
LANE 6	with	*Pvu*II /*Eco*RI	LANE 12	digested with}	*Bam*HI
LANE 7		*Pvu*II	LANE 13		Untreated

FIGURE 3.10

ANALYSIS OF RECOMBINANT PLASMID pJOE4 :

DETERMINATION OF RELATIVE POSITIONS OF ENZYME RECOGNITION SITES AND ORIENTATION OF INSERT BY RESTRICTION ANALYSIS

Hybrid plasmid pJOE4 was digested with a combination of restriction enzymes to determine the relative position of restriction sites and orientation of the cloned segment of *Escherichia coli* genome. Samples were electrophoresed on 0.8% agarose minigels at 70V

FIGURE 3.10a

The first and third of the six bands generated by *Bam*HI / *Pvu*II double digest (lane 6) are products of a partial digest. The same is applicable to the first (faint) band of lane 7.Sizes were determined relative to fragments from single *Pvu*II digest (lane 7) which had been calibrated previously by comparison with λ*Hin*dIII/*Eco*RI size-marker (figure 3.8). Most of the lanes are relatively overloaded.

LANE 1	*Bam*HI / *Eco*RI	LANE 5	*Pst*I / *Pvu*II
LANE 2	*Bam*HI / *Pst* I	LANE 6	*Bam*HI / *Pvu*II
LANE 3	λ*Hin*dIII (size-marker)	LANE 7	*Pvu*II
LANE 4	*Hin*dIII / *Pvu*II		

FIGURE 3.10b

LANE 1	untreated sample	LANE 5	λ*Hin*dIII (size-marker)
LANE 2	*Bam*HI / *Mlu*I	LANE 6	*Mlu*I / *Pvu*II
LANE 3	*Mlu*I	LANE 7	*Acc*I/*Pvu*II
LANE 4	*Acc*I		

The first visible bands of lanes 4, 6 represent partial digests. Sizes were estimated relative to lane 5.

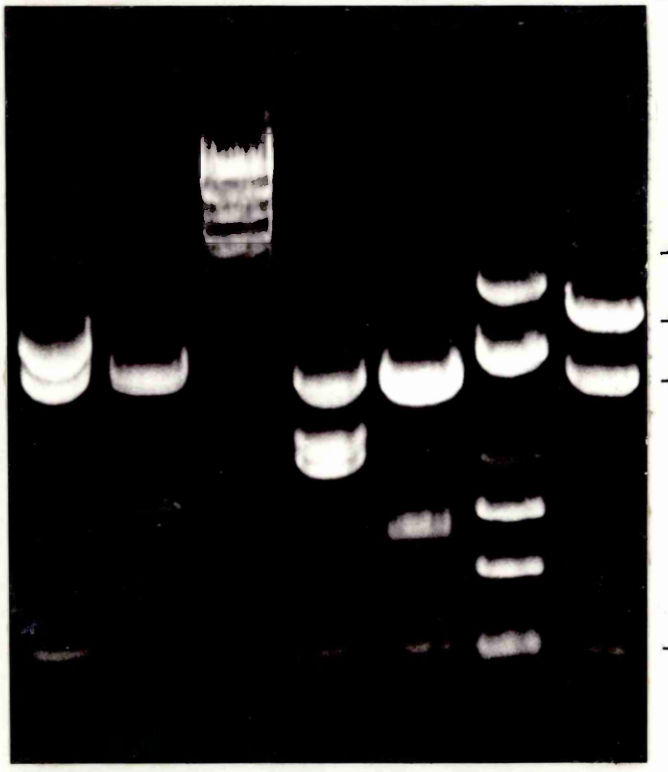


FIGURE 3.10a

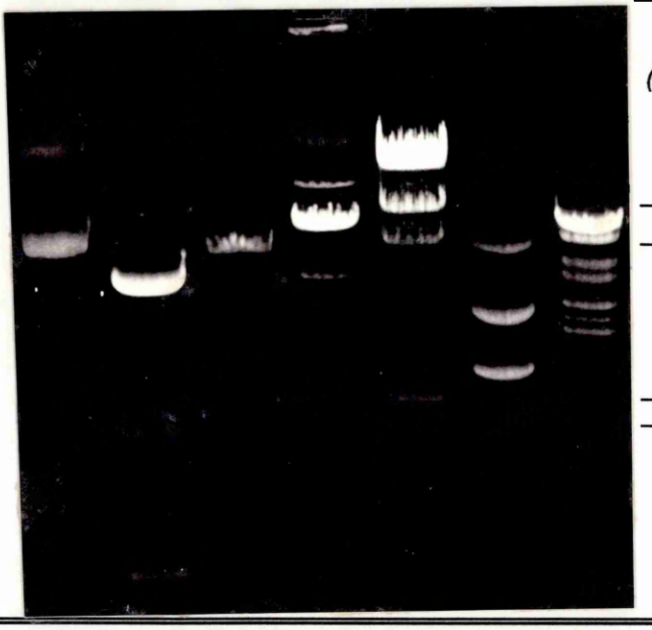


FIGURE 3.10b

3.2.6.1 ORIENTATION OF INSERT ON THE PLASMID

Restriction of recombinant plasmid pJOE4 with enzyme *Pvu*II produced three fragments. Their sizes were estimated at 1.1 (±0.05), 3.5(±0.1) and 4.8 (±0.1) kilobase pairs respectively (figure 3.8). Simultaneous double digestions with *Pst*I/*Pvu*II, *Hin*dIII/*Pvu*II, or *Eco*RI/*Pvu*II, *Mlu*I/*Pvu*II, *Acc*I/*Pvu*II showed that each enzyme cut within the 4.8kb *Pvu*II fragment (figures 3.8; 9 and 3.10a & b) while leaving the 1.1kb fragment intact (figure 3.10a). In the *Eco*RI/*Pvu*II digests, the largest *Pvu*II-generated fragment was fragmented into two new units of approximately 2.35 and 2.45 kb respectively (figure 3.8). The corresponding fragments from the *Hin*dIII/*Pvu*II double digest were about 2.3 and 2.4kb each (figure 3.10). These observations justified the location of the unique restriction sites for enzymes *Eco*RI, *Hin*dIII and *Pst*I (all on the vector) on the biggest *Pvu*II-generated fragment of the recombinant plasmid. The *Bam*HI recognition sequence located at 375/4363*bp* on the vector should have been disrupted by interposition of the genomic fragments generated with *Sau*3A (*see* 3.1.2). Nevertheless, digestion of hybrid plasmid pJOE4 with *Bam*HI alone produced two fragments (figures 3.7, lane 12; 3.8, lane 5; 3.9 lane 4). Since the published sequence contained only a single *Bam*HI site, it is likely that another hexanucleotide recognition sequence for *Bam*HI was reconstituted when the insert was ligated to the vector. Results from simultaneous digestion with *Bam*HI/*Eco*RI, *Bam*HI/*Pst*I (figure 3.9) *Bam*HI/*Pvu*II and *Bam*HI/*Mlu*I (figure 3.10) suggested that the *Bam*HI site located on the insert together with the new site should produce two fragments after digestion with *Bam*HI, whose sizes differed by about 1.6kb. In order to accommodate these fragments and the restriction patterns which were evident from figures 3.7 to 3.10, the new *Bam*HI sequence needed to be about 3.9kb away from the preexisting site and about 5.4 kb from position zero on the *Eco*RI recognition sequence.

Although three *Mlu*I sites were published for the *ppc* gene, only one band was visible after electrophoresis of *Mlu*I-digest of plasmid pJOE4. The size of the fragment was about 3kb short of the estimated size of the entire plasmid (figure 3.10b). Since pBR322 does not contain any *Mlu*I site, two possible locations have been deduced from double digests involving *Mlu*I. The third site which would enable *Mlu*I to cut within the 1.1kb fragment produced by *Pvu*II could not be accounted for (figure 3.10b, lanes 3 & 6).

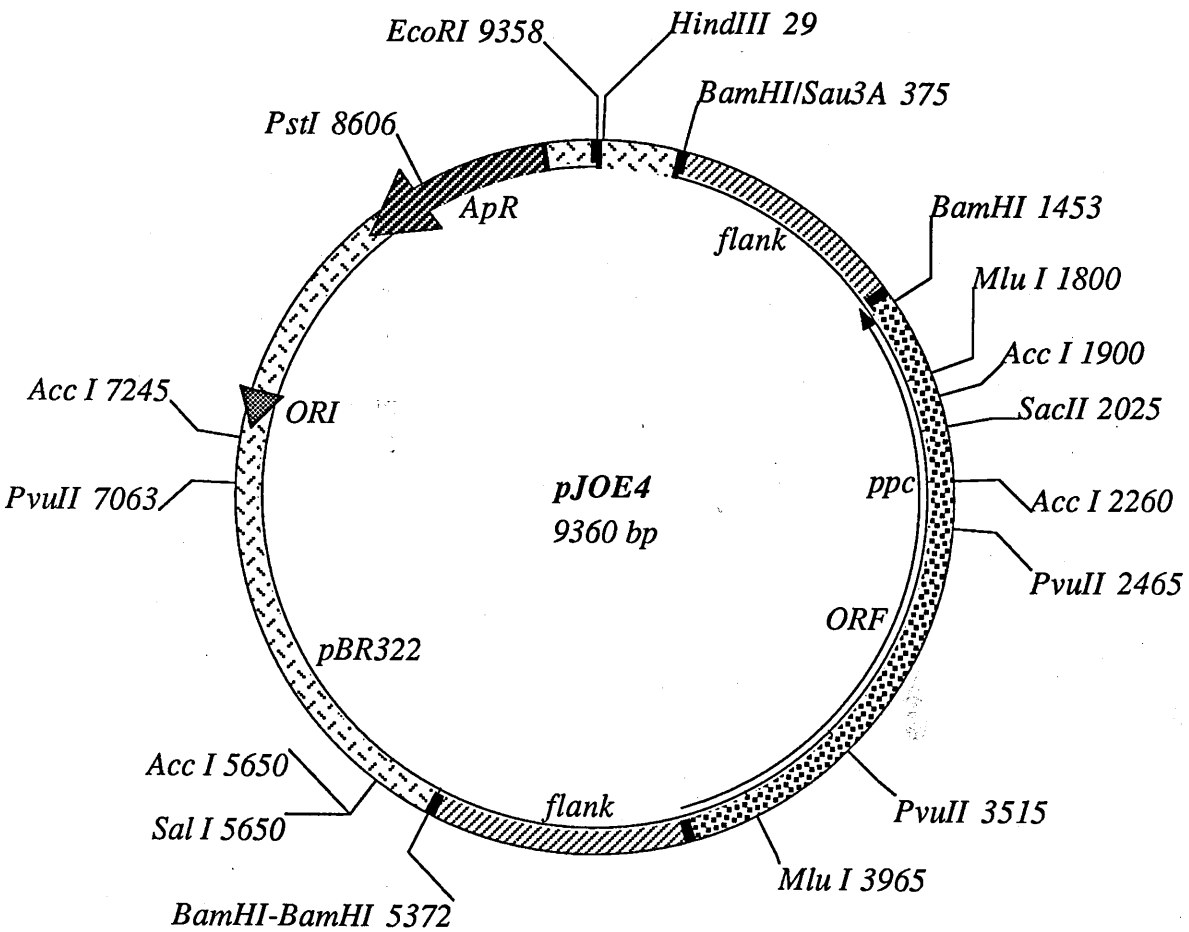
A working restriction map of hybrid plasmid pJOE4 was constructed as shown in figure 3.11 based on these deductions.

FIGURE 3.11

CIRCULAR RESTRICTION MAP OF RECOMBINANT PLASMID pJOE4 (pBR322-*ppc*$^+$)

The map was constructed from the restriction patterns shown in preceding sections. The numbering of the nucleotide bases was after the convention which began with the first thymine (T) and ended with the second adenine (A) of the unique *Eco*RI site (G↓AATTC) on cloning vector pBR322.

The genomic *Sau*3A–insert of *Escherichia coli* chromosome containing the *ppc* gene, interposed the unique *Bam*HI sequence of vector pBR322 (375/4363*bp*) at which the circular plasmid was linearized and ligated to the insert. Both restriction enzymes *Bam*HI (G↓GATCC) and *Sau*3A (↓GATC) generated compatible protruding termini. A *Bam*HI site was probably reconstituted at the hybrid end located about 5.4*kb* away from the zero point within the unique *Eco*RI recognition sequence located on the vector. The segment corresponding to the *ppc* gene (opposite) was consistent with the published sequence for the gene. The restriction sites also matched the published map. The presence and location of a third *Mlu*I site could not be supported from the patterns obtained during restriction analysis.


EcoRI 9358
HindIII 29
BamHI/Sau3A 375
PstI 8606
ApR
flank
BamHI 1453
Mlu I 1800
Acc I 1900
SacII 2025
Acc I 7245
ORI
PvuII 7063
pJOE4
9360 bp
ppc
Acc I 2260
PvuII 2465
ORF
pBR322
Acc I 5650
Sal I 5650
flank
PvuII 3515
Mlu I 3965
BamHI-BamHI 5372

3.3 Determination of Phospho*enol*pyruvate Carboxylase Enzyme Activities of Test Strains

E. coli PA342 and its derivative from transformation with hybrid plasmid pJOE4 were grown for 2 generations in glucose minimal salts medium. L-aspartate was necessary to support the growth of the parent strain while ampicillin was needed to maintain the plasmid in the recombinant. Ultrasonic cell extracts were assayed for phospho*enol* pyruvate carboxylase (PEPc) activity.

No activity was detected in cell extracts of *E.coli* PA342 or cells transformed with pBR322. Ultrasonic cell extracts of the recombinant strain showed significant PEPc activity of 3.536μ*mol.* mg. protein^{-1}. min^{-1}. Given that neither the parent nor its pBR322-transformant showed any PEPc activity (Table 3.5), the activity detected was attributable to the expression of the genomic insert in the hybrid plasmid pJOE4. The PEPc in the crude extract was relatively stable at 4°C in a buffer solution of pH 7.5.

3.3.1 Determination of Phospho*enol* pyruvate Carboxylase Activity of *E. coli* ATCC15224:

Phospho*enol* pyruvate carboxylase activity of the main test organism–*E.coli* ATCC 15224 (Table 2.1) was determined and compared with the levels of detectable enzyme activities in strains of *E. coli* PA342. *E.coli* ATCC15224 had a PEPc activity of 24.4*nmol. mg.* (protein)$^{-1}$. min^{-1}. This activity was considerably less than the level found in ultrasonic extracts of *E. coli* PA342-pJOE4 (Table 3.5).

E. coli Strain	Relevant Genotype	Carbon Source	PEPc Activity *(nmol. mg. protein.$^{-1}$. min.$^{-1}$)*
PA342 parent	(*ppc*)	**Glucose (+aspartate)**	**n-d-a***
PA342-pBR322	pBR322-*-ppc*)	**Glucose (+aspartate)**	**n-d-a***
PA342-pJOE4	pBR322-*-ppc* $^{+}$)	**Glucose (+aspartate)**	**2711**
PA342-pJOE4	pBR322-*-ppc* $^{+}$)	**Glucose**	**3536**

TABLE 3.5

PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITIES OF *E. coli* PA342 (PARENT, pBR322 & pJOE4 PLASMID-CONTAINING STRAINS)

Cell-free ultrasonic extracts of organisms grown in glucose minimal media were assayed for PEPc activities. The enzyme activities were monitored by the rate of oxidation of NADH (λ_{340}) coupled with the reduction of oxaloacetate formed to malate after adding exogenous PEP. Specific enzyme activity was expressed as *nmol. mg.* protein.$^{-1}$. hour.$^{-1}$

n-d-a* no detectable activity

E. coli Strain	RELEVANT GENOTYPE	CARBON SOURCE	PEPc ACTIVITY (*nmol. mg.protein^{-1}. min.$^{-1}$*)	RATIO
ATCC 15224	(*ppc* +)	Glucose	24.4	
PA342 parent	(*ppc*)	Glucose (+ aspartate)	n-d-a*	
PA342–pBR322	(*ppc*)	Glucose (+ aspartate)	n-d-a*	
PA342–pJOE4	(*ppc* +)	Glucose (+ aspartate)	2711	111
PA342–pJOE4	(*ppc* +)	Glucose	3536	144

TABLE 3.6

COMPARISON OF PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITIES OF *E. coli* ATCC 15224 AND PA342 (PARENT, pBR322 AND pJOE4 PLASMID-CONTAINING STRAINS)

Cell-free ultrasonic extracts of organisms grown in glucose minimal media were assayed for PEPc activities. The rate of NADH oxidation after addition of PEP was interpreted as enzyme activity. Specific enzyme activity was expressed as *nmol mg* (Protein)$^{-1}$hour^{-1}*

PEPc activity — Phospho*enol* pyruvate carboxylase activity

*n-d-a — no detectable PEPc activity

RATIO — represents PEPc activities of ultrasonic extracts relative to the activity of *E. coli* ATCC15224 as standard.

3.4 DISCUSSION

Phospho*enol* pyruvate carboxylase (PEPc) mutants required C_4-dicarboxylic acid intermediates of the TCA cycle or their immediate derivatives, to grow on glucose as sole carbon and energy source. This auxotrophy was characteristic of mutants which carried a lesion for the *ppc* gene.

A population of recombinant plasmids containing *Sau* 3A fragments of *Escherichia coli* genomic DNA on vector pBR322, was screened for pBR322-mediated transformation and complementation of the metabolic lesion due to PEPc mutation in *E. coli* PA342 (*ppc*). Recombinant plasmid pJOE4 was designated as pBR322-*ppc* $^+$ because of its ability to complement the relevant nutritional lesion of *E. coli* PA342 by transformation. The plasmid conferred very high phospho*enol* pyruvate carboxylase activity on the recombinant strains. The total size of the hybrid plasmid was estimated by restriction analysis at 9.36kb consisting of 4.363kb of the cloning vector and a genomic insert of nearly 5.kb. The size was within the range of *Sau* 3A genomic fragments generated for the construction of the gene bank (Clugston, 1986). Genetic and enzymatic evidence confirmed the presence of the *ppc* gene in plasmid pJOE4. The restriction map was consistent with the published nucleotide sequence of the *ppc* gene (Fujita *et al*, 1984).

PEPc is an important regulatory enzyme which was first elucidated by Bandurski and Greiner (1953). Its activity is subject to a multiplicity of controls (Sanwal *et al.*, 1966). Its relatively high *Km* for its specific substrate–PEP[11.2mM] considerably limits its activity in normal cell metabolism (Sanwal and Maeba, 1966). It is therefore strongly dependent on activators. Acetyl-CoA is a major allosteric activator of PEPc which enhances enzyme activity 30-fold. In addition, fructose-1,6-*bis* phosphate, long-chain fatty acids (and their CoA derivatives), nonpolar solvents and nucleotides (CDP, GTP and CMP) also activate the enzyme (Sanwal, 1970; Teraoka *et al* , 1974). These may act in synergism as shown by the significantly reduced *Km* of nucleotides for the enzyme in the presence of acetyl-CoA (Maeba and Sanwal,1966). The enzyme in ultrasonic extracts of *E. coli* PA342-pJOE4 was barely detectable when acetyl-CoenzymeA was absent from the assay cocktail, which was consistent with previous observations (Cánovas and Kornberg (1969). Acetyl-CoA is saturating for

maximal activity of PEPc at 1mM (Cánovas and Kornberg, 1966).

The activity of PEPc is also modulated by allosteric inhibitors, some of which are intermediates of the tricarboxylic acid cycle (e.g. L-malate) or their derivatives (e.g. L-aspartate which is reversibly convertible to oxaloacetate) (Izui *et al.* , 1981). Ultrasonic extracts from cells of the of *E. coli* PA342-pJOE4 grown in the presence of 250$\mu g.\ ml.^{-1}$ L-aspartate contained about 25% less PEPc activity than cells grown in unsupplemented glucose medium (Table 3.5). In *E. coli* AB1622–pLC20-10 (*ppc, thi,* str^R/colE1-ppc^+), plasmid-mediated PEPc activity was reduced by 49% in aspartate-supplemented medium. In the wild type strain W3110, the activity decreased by 20% (Izui *et al.*, 1981). At the early stages of this study, aspartate was a mandatory nutritional requirement for the growth of *E. coli* PA342 on glucose as sole carbon and energy source since the mutant could derive oxaloacetate from it in the absence of flux through PEPc. Only after transformation with hybrid plasmid pJOE4 did the cloning host grow on glucose without L-aspartate which is central to the divergent flux of oxaloacetate to biosynthesis (Fisher and Magasanik, 1984). Oxaloacetate and L-aspartate are interconvertible (Schlegel, 1988) and as an effector, L-aspartate is the most powerful single allosteric inhibitor of phospho*enol*pyruvate carboxylase activity reported in bacterial systems (Sanwal, 1970). The response of the enzyme obtained from ultrasonic extracts of *E. coli* ATCC15224-pJOE4 grown on glucose to acetyl-CoA and L-aspartate, supported the belief that the allosteric integrity of the enzyme-protein from the cloned *ppc* gene was still intact and was consistent with the mechanism by which PEPc activity was regulated to maintain a balance in its metabolic functions. The highly elevated activity of PEPc in the strain of *E. coli* ATCC15224 containing the hybrid plasmid–pJOE4 may therefore be explained by increased gene dosage effect and to a lesser extent, increased gene expression. The vector is a multicopy plasmid with an estimated copy number of 30 (±10) (Balbas, 1988). Fifty to 180-fold over-production of metabolic enzymes expressed from multicopy plasmids have been reported (Guest and Roberts,1983; Matsuyama *et al.*, 1989).

The estimated size of the insert (5 *kb.* , figure 3.11) was nearly 2 *kb* more than the published sequence of the *ppc* structural gene (3.1*kb.,* Izui *et al.,* 1981). In order to determine if accessory gene sequences contributed to the new properties attributed to plasmid pJOE4, a study was undertaken to inactivate the *ppc* gene in the hybrid plasmid with a view to expressing those other genes preferentially.

3.4.1 INACTIVATION OF THE *ppc* GENE

The mechanism of deletion was adopted for inactivation of the *ppc* gene. A *unique* recognition sequence for the restriction endonuclease, *Sst*II (*iso* schizomer, *Sac*II) on the open reading frame was selected as specific target for limited deletion of bases. The vector did not contain a *Sac*II site (*USB* , Manual 1990). Mung Bean and S1 Nucleases, DNA polymerase1 (*Klenow fragment*), T4 DNA ligase and restriction enzymes were used at various stages (figure 3.12).

3.4.1.1 PREPARATION AND DIGESTION OF DNA SAMPLES BY MUNG BEAN NUCLEASE

10$\mu g.$ of recombinant plasmid pJOE4 was digested with *Sac*II, precipitated with *iso*propanol and resuspended in 200$\mu l.$, single-strength Mung Bean Nuclease buffer (see methods). 2$\mu l.$ sample was withdrawn for electrophoresis before the rest was treated with Mung Bean Nuclease to a final concentration of 3*units. ug.* DNA^{-1} and incubated at 30°C for 30 minutes.

3.4.1.2 PRECIPITATION, LIGATION AND ANALYSIS OF LIGATE

30$\mu l.$, 8mM-NaCl, 4μl [20%, w/v] SDS solution and 250$\mu l.$ (TE-buffered, 1:1 chloroform:phenol) were mixed with the digest to inactivate the Mung Bean Nuclease and deproteinize the sample. The DNA was precipitated with *iso*propanol. The upper aqueous phase (200$\mu l.$) and 120$\mu l.$ (*iso* propanol; 20$\mu l.$, 5M potassium acetate; 15$\mu l.$, 30ng $\mu l.$ *t*-RNA^{-1}.were incubated at -20°C for 10 minutes, pelleted and dried under vacuum before taking in 25$\mu l.$ single strength *Klenow* buffer.

20$\mu l.$ DNA was incubated with 1$\mu l.$, 2mM nucleotide triphosphates (NTPs) and 1$\mu l.$ *Klenow* enzyme at 37°C for 30 minutes. Reaction was stopped at 70°C for 5 minutes before chloroform-phenol extraction precipitation with *iso*propanol .

The pellet was dissolved in 20$\mu l.$, single-strength ligation buffer and incubated overnight at 4°C with 4$\mu l.$ T4 DNA ligase. The ligation mixture was extracted once with chloroform:phenol and precipitated with *iso*propanol. The final pellet was dissolved in 40$\mu l.$ TE buffer.

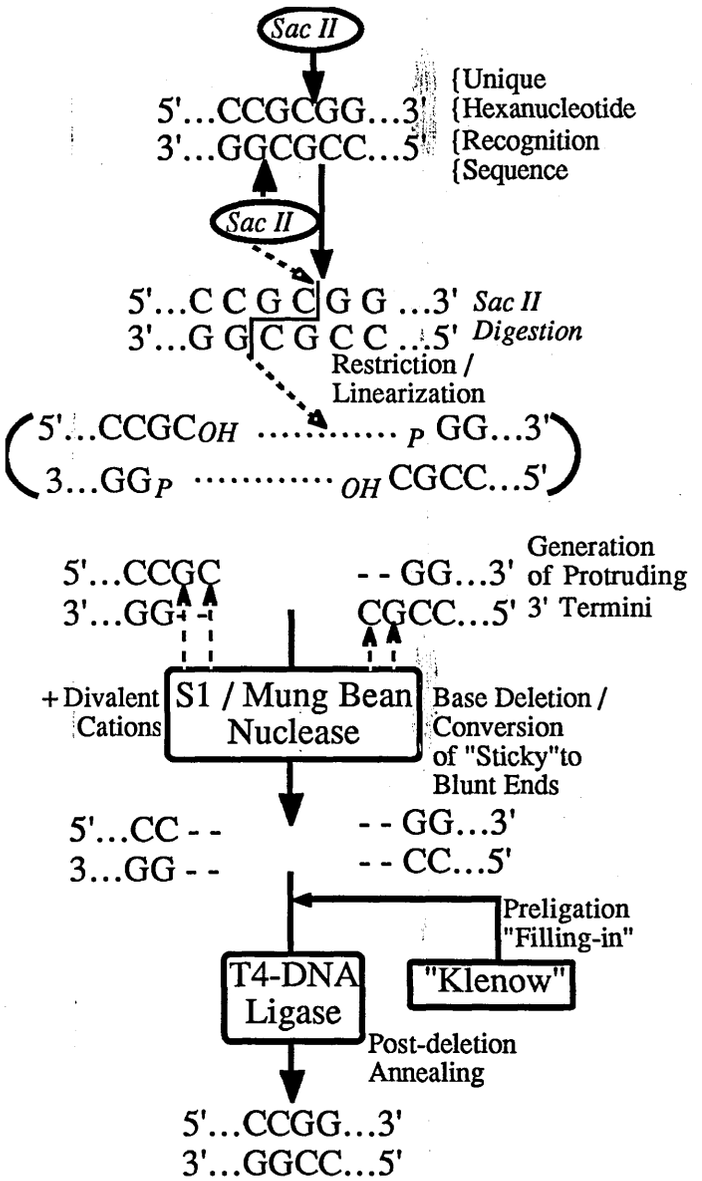


FIGURE 3.12

SCHEME FOR INACTIVATION OF THE *ppc* GENE IN PLASMID pJOE4 BY SITE-SPECIFIC BASE DELETION

The published nucleotide sequence of the *ppc* gene contained 3106 base pairs. The restriction enzyme *Sac*II (*Sst*II) recognized a unique hexanucleotide sequence from base 2242 to 2247 (inclusive), and specifically cleaved between 2245 and 2246 to create "GC" overhangs which can be digested with single-strand specific nucleases, such as S1 or Mung Bean Nucleases.

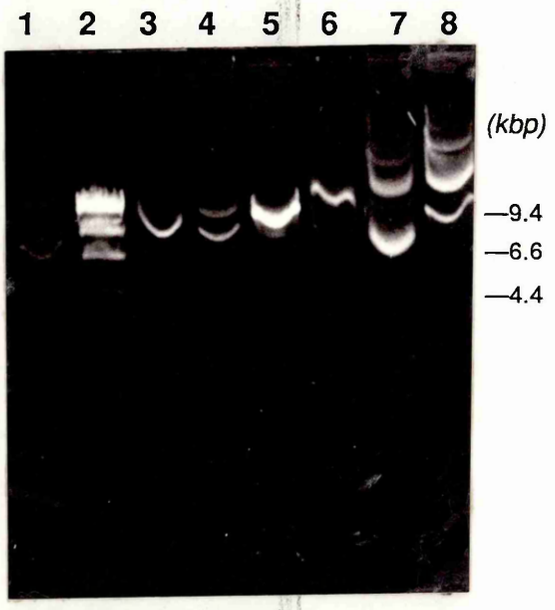


FIGURE 3.13

EFFECT OF MUNG BEAN NUCLEASE TREATMENT ON THE UNIQUE *SacII* RESTRICTION SITE OF THE *ppc* GENE IN RECOMBINANT PLASMID pJOE4

Samples were analysed by (0.8%) agarose gel electrophoresis in TBE buffer at 70v

LANES

1	Untreated plasmid pJOE4
2	Molecular weight marker (phage λ treated with *Hin*dIII)
3	Redigestion with *Sac*I after deletion treatment & ligation
4	Ligated plasmid after treatment for deletion
5	*Sac*I Digest treated with Mung Bean Nuclease
6	Primary *Sac*I digest of plasmid pJOE4
7	} Untreated samples
8	} of plasmid pJOE4

The ligate was tested for the disruption of the *Sac*II site by redigesting with *Sac*II. Secondary digestion was carried out as described for the primary process. Electrophoretic patterns showed linearization by primary *Sac*II digestion. However, ligation was only partially successful and plasmids in the ligation mixture failed to transform competent cells (figure 3.13). A number of subsequent treatments produced completely ligated samples which were subjected to secondary *Sac*II treatment. Secondary digestion with *Sac*II suggested that the treatment failed to delete the target-bases and more importantly, that the recombinant plasmid remained intact. An alternative strategy was considered involving the use of S1 nuclease in place of Mung Bean Nuclease.

3.4.2 S1 NUCLEASE TREATMENT

S1 nuclease treatment was not initially considered for producing deletions because of its more severe action and higher intrinsic activity on nicks in duplex structures, *vis-a-vis* Mung Bean Nuclease (Kroeker *et al.*, 1976; USB Manual, 1990).

3.4.2.1 CALIBRATION OF S1 NUCLEASE ACTIVITY

500ng, plasmid pJOE4 treated with *Sac*II restriction enzyme, was exposed to varying concentrations of S1 nuclease in single strength S1-nuclease buffer at 25°C for 30 minutes, then repaired with *Klenow* fragment before ligation. Each stage of the calibration was sampled for analysis. Secondary post-ligation cleavage with *Sac*II was analysed by agarose gel electrophoresis to determine the effectiveness of S1 treatment (figure 3.14).

3.4.2.2 EFFECT OF TREATMENT WITH S1 NUCLEASE

All samples treated with 1 to 10*units* of S1 nuclease retained unique sequences for *Sac*II. In view of this, the standard methods were customized to obtain deletion mutation on the *ppc* gene.

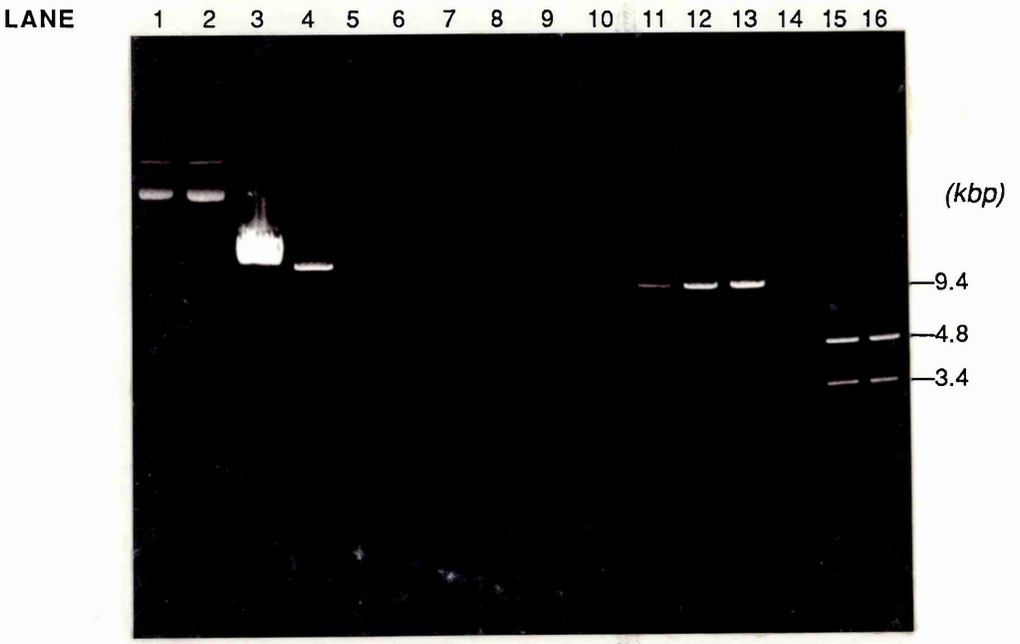


FIGURE 3.14

EFFECT OF S1 NUCLEASE TREATMENT ON THE UNIQUE *Sac*II RESTRICTION SITE OF THE *ppc* GENE IN RECOMBINANT PLASMID pJOE4

Samples were analysed by agarose gel electrophoresis in TBE buffer

LIGATES OF S1 NUCLEASE-TREATED SAMPLES

1 Untreated Plasmid pJOE4, sample 1
2 Untreated Plasmid pJOE4, sample 2
3 *Sac*I-treated plasmid pJOE4 (bulk)
4 *Sac*I-treated plasmid pJOE4 (control)
5 1.0 unit S1 Nuclease
6 2.5 units S1 Nuclease
7 5.0 units S1 Nuclease
8 7.5 units S1 Nuclease
9 10 units S1 Nuclease

POST-LIGATION *Sac*II DIGESTS OF S1 NUCLEASE-TREATED SAMPLES

10 1.0 unit S1 Nuclease
11 2.5 units S1 Nuclease
12 5.0 units S1 Nuclease
13 7.5 units S1 Nuclease
14 10 units S1 Nuclease
15 Plasmid pJOE4 treated with *Pvu*II (Sample 1)
16 Plasmid pJOE4 treated with *Pvu*II (Sample 2)

3.4.3 CUSTOMIZED METHOD FOR GENERATING SITE-SPECIFIC DELETION OF BASES AND FRAMESHIFT MUTATION

Ligates (Samples 6-10, figure 3.14) were combined into a single volume and linearized by redigesting with *Sac*II. By this method, plasmids which contained deletions from previous S1 treatment but were masked, could be detected by default. Such plasmids should not be susceptible to subsequent treatment with *Sac*II. They should also remain circularized and transform competent cells. Ligates of S1-treated samples were redigested with *Sac*II. Reaction was stopped after 60 minutes by heating at 70°C for 7 minutes and cooling on ice.

3.4.3.1 TRANSFORMATION OF *E. coli* PA342 AND ANALYSIS OF TRANSFORMANTS

Competent cells of *E. coli* PA342 were transformed with the *Sac*II digest. Transformants were selected for ampicillin resistance on solid medium after 24 hours of incubation. The plasmids isolated from overnight cultures of four transformants were analysed by restriction and agarose gel electrophoresis relative to hybrid plasmid pJOE4. Two of these (pJOE 9 & pJOE 10) were digestible by *Sac*II; the other two (pJOE11 & pJOE12) were not (figure 3.15). *E. coli* PA342 transformants of pJOE11, pJOE12 did not grow on glucose as sole carbon source except after supplementation with L-aspartate (Table 3.7).

3.4.4 COMPARISON OF ENZYME ACTIVITIES

Cells transformed with intact pJOE4 (pBR322-*ppc*$^+$ containing unique *Sac*II site) and pJOE12 (pBR322-*ppc*$^+$ without unique *Sac*II site) were grown in 100ml, glucose-aspartate-ampicillin minimal media. Comparative analysis of phospho*enol* pyruvate carboxylase (PEPc) activities of ultrasonic cell extracts were carried out using the *E. coli* PA342-pBR322 sample as negative control.

Transformants of recombinant plasmid pJOE4 retained amplified PEPc activity. No PEPc activity was detected in *E. coli* PA342-pBR322. More significantly, PEPc activities of pJOE12-transformants were barely detectable (Table 3.7).

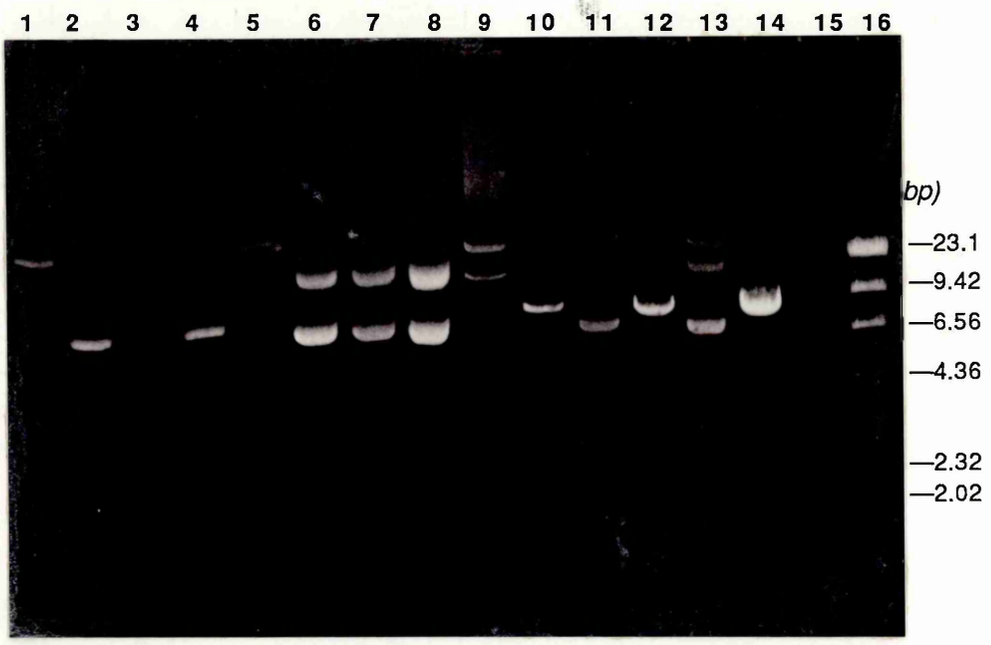


FIGURE 3.15

COMPARATIVE ANALYSIS OF RECOMBINANT PLASMIDS pJOE4, pJOE9, pJOE10, pJOE11 and pJOE12.

E. coli PA342 was transformed with hybrid plasmid pJOE4 which had been digested with *Sac*II and ligated after treatment with S1-nuclease. Plasmids isolated from transformants were redigested with *Sac*II and checked by agarose gel electrophoresis in TBE buffer.

LANES

1 Control (Undigested hybrid plasmid pJOE4)
2 Untreated hybrid plasmid pJOE11 (≠ *Sac*II site)
3 Untreated hybrid plasmid pJOE9
4 Untreated hybrid plasmid pJOE12 (≠ *Sac*II site)
5 Untreated hybrid plasmid pJOE10
6 } *Sac*II-digests of
7 }Recombinant plasmids isolated
8 } from pJOE12-transformants
9 } of *E. coli* PA342 Isolates 1- 4.
10 *Sac*II-treated plasmid pJOE4
11 *Sac*II-treated hybrid plasmid pJOE11
12 *Sac*II-treated hybrid plasmid pJOE10
13 *Sac*II-treated hybrid plasmid pJOE12
14 *Sac*II-treated hybrid plasmid pJOE9
15 Original combined ligate (untreated)
16 Molecular weight marker (λ*Hin* dIII digest)

Plasmid in *E. coli* PA342	Relevant Genotype	GROWTH Glucose	GROWTH Glucose+asp	PEPc ACTIVITY (*nmol* $mg^{-1}min^{-1}$)
nil(Parent)	(*ppc*)	–	++	**0**
pJOE4	(pBR322-*ppc*$^+$)	+	++	**2636(±257)**
pJOE9	(pBR322-*ppc*$^+$)	+	++	**2159(±104)**
pJOE10	(pBR322-*ppc*$^+$)	+	++	**n.d**
pJOE11	(pBR322; truncated *ppc*$^+$)	–	++	**n.d**
pJOE12	(pBR322; truncated ppc$^+$)	–	++	**0.7(±0.8)**
pBR322	(pBR322)	–	++	**0**
(control) K10J	(*ppc*$^+$; pBR322-*ppc*$^+$)	++	+++	**10.2**

TABLE 3.7

COMPARISON OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE ACTIVITIES AND GROWTH ON GLUCOSE AS SOLE CARBON AND ENERGY SOURCE BY *E. COLI* PA342 BEFORE AND AFTER TRANSFORMATION WITH INTACT AND DISRUPTED PLASMID pJOE4

Constitution of the replica-plates are listed in 2.5.2. *E. coli* K10 and *E. coli* PA342-pJOE4 were used as positive controls while *E. coli* PA342(parent) and transformant with pBR322 were negative controls. Glucose plates supplemented with L-aspartate (Glucose-asp) were complete media for PEPc-negative strains

n.d. not determined (-) no appreciable growth after 48 hours; (+, ++, +++) Varying degrees of positive growth after 48hours

3.4.5. Effect of Deletion Mutation on the Expression of Phospho*enol* pyruvate carboxylase Activity by *E. coli* PA342-pJOE11 and *E. coli* PA342-pJOE12

A major way of establishing the relationship between a cloned gene and newly observed phenotypes was deletion and/or insertion mutation of the gene (Eisenstadt, 1987; Sambrook *et al.*, 1989). The underlying principle of the technique was the use of dysfunction as a clue to function (Weitzman,1981; Rodriguez and Denhardt, 1988).

Results presented showed the effects of disruption of the *ppc* gene of the hybrid plasmid, pJOE4 by limited, site-directed deletion resulting in frameshift mutation of the cloned gene (figure 3.16). The resulting plasmids were code-named pJOE11 and pJOE12. Their transformants were easily differentiated from pJOE4 transformants by loss of ability to grow on glucose in the absence of C_4-dicarboxylic acid supplements or their derivatives. Enzyme assay also demonstrated the loss of phospho*enol* pyruvate carboxylase (PEPc) activities, previously demonstrated in *E. coli* PA342-pJOE4.

The observations above provided additional circumstantial evidence that the genomic insert of hybrid plasmid pJOE4 contained the *ppc* gene and that the high activity of PEPc in *E. coli* PA342-pJOE4 as well as its ability to grow on glucose as sole carbon and energy source, were due to complementation of the PEPc lesion of the parent by the gene.

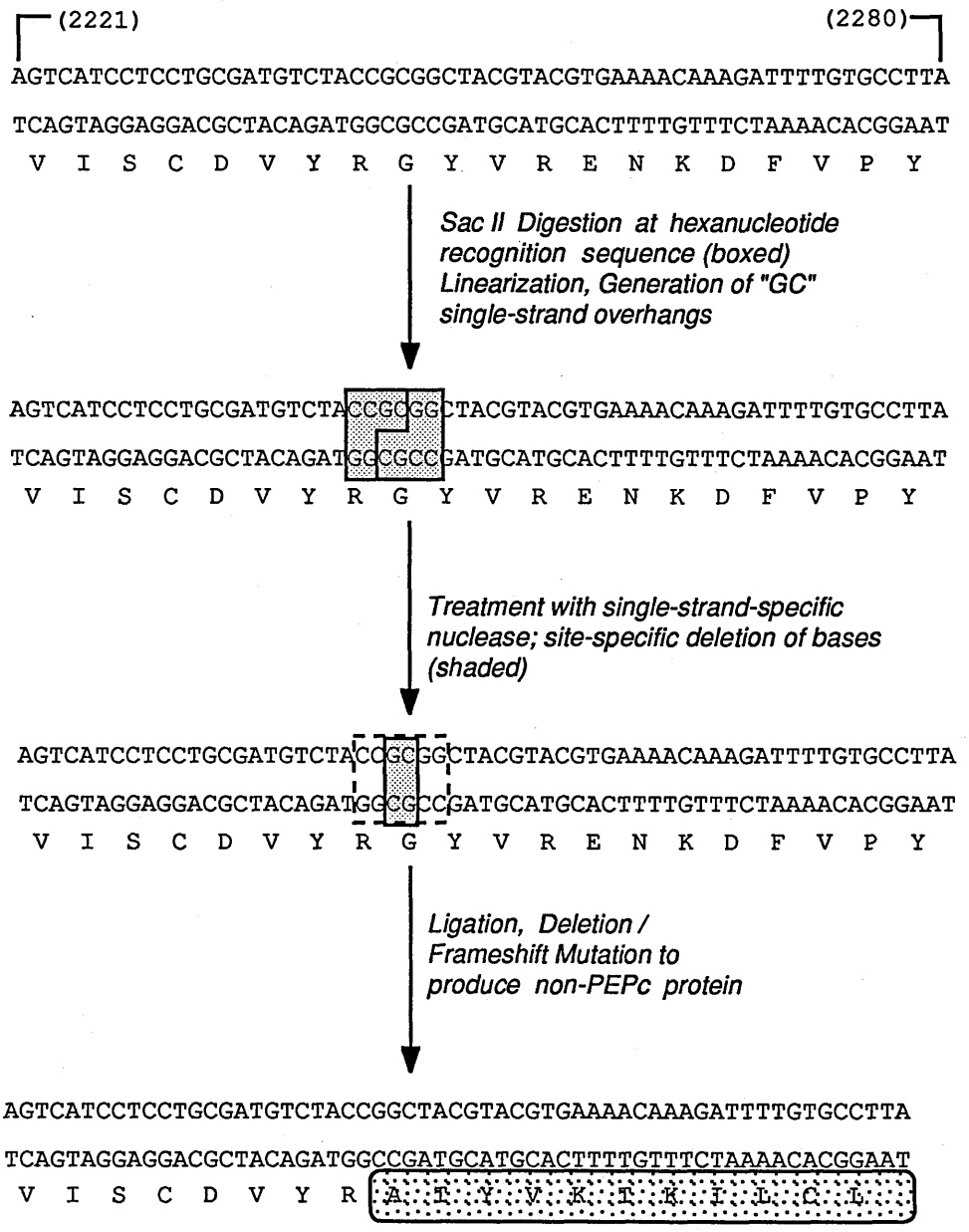


FIGURE 3.16

POSSIBLE EFFECT OF LIMITED SITE-DIRECTED BASE DELETION FROM THE *ppc* -GENE AND NEW PRODUCT RESULTING FROM FRAMESHIFT MUTATION

(Amino acid symbols are standard single -alphabetic abbreviations)

Shaded amino acid sequence represents a shift in the reading frame and sequence for a new gene product different from phospho *enol*pyruvate carboxylase

CHAPTER 4

EVALUATION OF THE EFFECTS OF OVEREXPRESSION OF PHOSPHO*ENOL*-PYRUVATE CARBOXYLASE ACTIVITY ON GLUCOSE METABOLISM BY *Escherichia coli* ATCC15224

4.1 INTRODUCTION

In the preceding chapter, the cloning of the *ppc* gene was reported. This was undertaken with a view to increasing the level and activity of the enzyme phospho*enol*pyruvate carboxylase (PEPc) in the main test organism, *Escherichia coli* ATCC15224. Like typical strains of *Escherichia coli,* it utilizes glucose by the conventional route of glycolysis which is coupled to the tricarboxylic acid (TCA) cycle under aerobic conditions (Krebs, 1937; 1940a; b; 1942; Spencer and Guest, 1987). Analysis of the basic framework for operating its metabolic fluxes under aerobic conditions, took account of non-biomass/non-energy outputs as additional indices for the determination of the efficiency of substrate utilization in the organism (Holms, 1986a). During aerobic metabolism, acetate was the most important of these (Britten, 1954) and its excretion is a symptom of an inefficiently controlled bio-flux system (Holms, 1986a). Pan *et al.* (1987) found that the excretion of acetate was a principal constraint to high biomass yield in *Escherichia coli* . In batch cultures, increasing acidity due to accumulation of acetate and other acidic byproducts inhibited general cell metabolism, productivity (Luli and Strohl, 1990) and in extreme cases, resulted in high mortality of the cells (Namdari and Cabelli, 1990).

Quantitatively, carbon fluxes to pyruvate contributed most to the output of acetate (Koepsell and Johnson, 1942; vanUrk *et al.*, 1989). Since all catabolic fluxes converged at pyruvate, it was highly likely that acetate excretion could at least be controlled if throughputs to pyruvate from PEP was reduced. This chapter reports the various effects of manipulation of carbon flux at phospho*enol* pyruvate (PEP) through selective amplification of the activity of the enzyme phospho*enol* pyruvate carboxylase (PEPc). Increased PEPc activity was achieved by transforming *E. coli* ATCC15224 with recombinant plasmid pJOE4, while the effects were determined during aerobic metabolism of glucose. To evaluate the magnitude of the metabolic effects of overexpression of PEPc on *E. coli* ATCC15224, preliminary analysis of its metabolism of glucose was undertaken in batch culture. The derivation of a PEPc-overproducing strain of *E. coli* ATCC15224 by transformation, is also reported; its metabolism of glucose was compared with the parent using a transformant containing the vector as the control strain. The strains used have been designated as follows:

1) Parent — *E. coli* ATCC15224 (test organism)
2) Recombinant — strain ATCC15224-pJOE4 (pBR322-*ppc* $^{+}$)
3) Vector-Control — strain ATCC15224-pBR322

4.2 STUDY OF GLUCOSE METABOLISM

Cells were grown in aerated batch culture containing 4mM glucose in 800ml minimal medium for 12 hours during which substrate utilization, biomass production and other detectable products of metabolism were monitored (2.8).

4.2.1 GROWTH AND BIOMASS PRODUCTION

The method for preparation of the inoculum (2.8.1) eliminated the lag phase in batch culture. The growth curve reflected an exponential increase in the density of viable cells which was sustained by a mass-doubling time of 46 minutes, equivalent to a specific growth rate (μ) of $0.90h^{-1}$ (figure 4.1). The log phase was succeeded by a phase of gradual increase in mass doubling time which is typical of glucose-limited cultures of *E. coli* approaching the stationary growth phase. The cells entered the stationary phase after 3 hours 45 (±5) minutes.

4.2.2 SUBSTRATE UTILIZATION & GROWTH YIELD

The sole carbon and energy source was glucose. Each *milliMolar* glucose supplied 72*ppm* (parts per million) of organic carbon to a final initial concentration of 288*ppm* in the growth medium containing 4*mM*-glucose. The addition of $100\mu g.\ ml.^{-1}$ ampicillin to the media for strains carrying plasmids was reflected in the total organic carbon but did not contribute to the bioavailable carbon for metabolism. The amount of substrate in culture decreased steadily as reflected in depletion of residual glucose (figure 4.2). Complete depletion of glucose occurred after 3 hours 45 minutes which corresponded to the transitional zone between the late log phase and early stationary phase. At an initial substrate concentration of 4.0mM, 5 generations were sustained with a net cell density(apparent OD_{420}) of 1.76±0.03 and biomass yield of 362 (± 6)*mg.* dry weight. $litre^{-1}$. Molar growth yield ($Y_{GLUCOSE}$) was therefore 90.5*g.* dry weight. *mole* $glucose.^{-1}$ (figure 4.3), representing an input-equivalent of 11.05*mmol.* glucose (-6-phosphate). *g.* bacterial dry biomass.$^{-1}$.

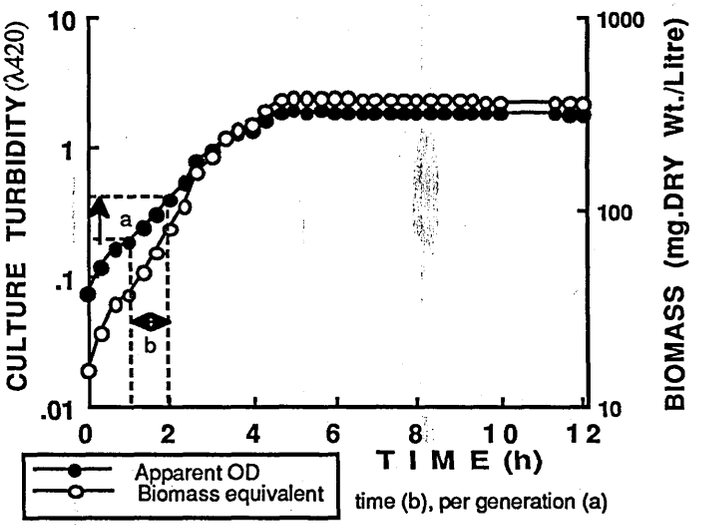


FIGURE 4.1 GROWTH OF *Escherichia coli* ATCC 15224 IN AEROBIC GLUCOSE-LIMITED BATCH CULTURE

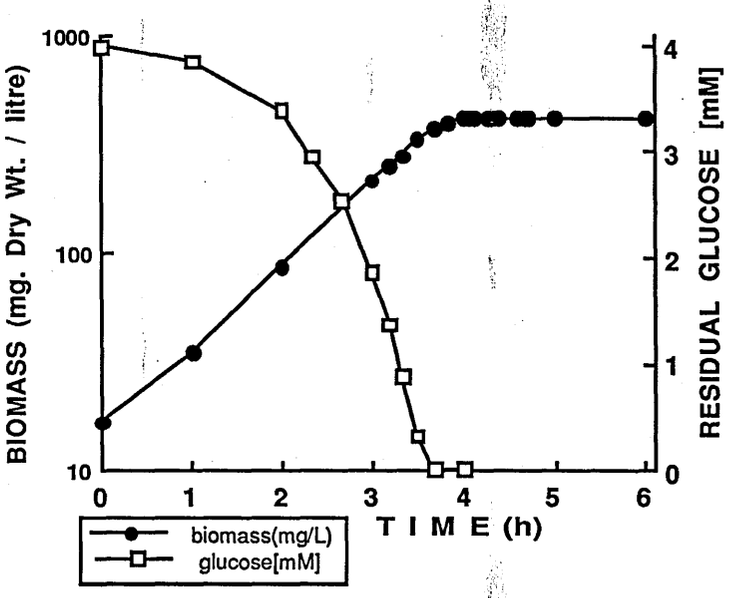


FIGURE 4.2: SUBSTRATE UTILIZATION DURING AEROBIC GROWTH ON GLUCOSE BY *E. coli* ATCC15224

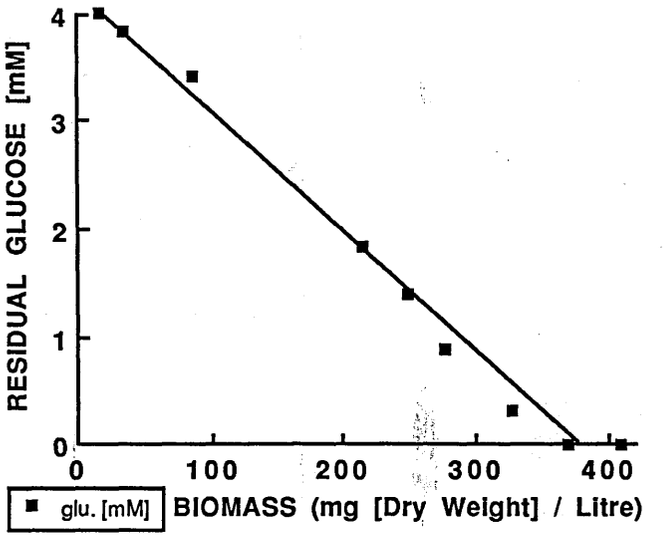


FIGURE 4.3 ESTIMATION OF YIELD COEFFICIENT OF *E. coli* ATCC15224 GROWING ON GLUCOSE

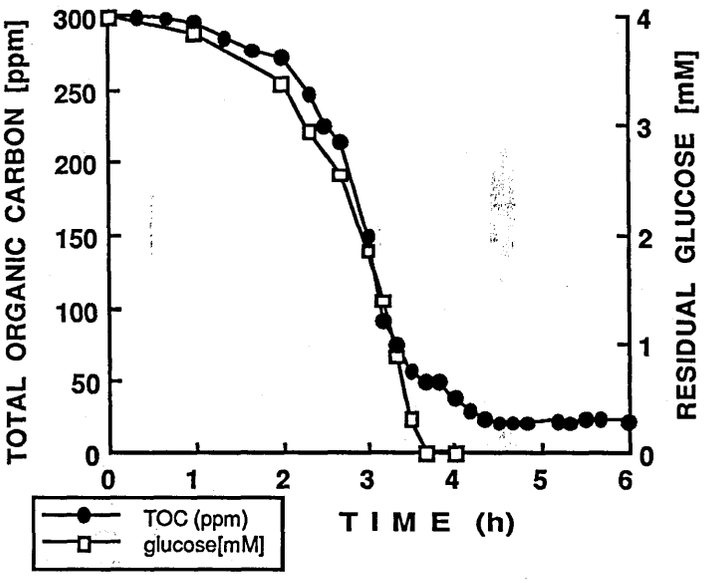


FIGURE 4.4 ANALYSIS OF THE TOTAL ORGANIC CARBON IN GLUCOSE CULTURE OF *E. coli* ATCC15224

4.2.3 OTHER METABOLIC OUTPUTS

Residual glucose did not account for the total organic carbon (TOC) in the culture supernatant. Up to 24ppm TOC was still detectable, 2 hours after glucose was completely depleted (figure 4.4). Much of this was subsequently reflected in excreted outputs. Besides substrate utilization and biomass production, the next most important metabolic activity was the excretion of acetate which occurred throughout the entire growth cycle, but especially as the cells passed into the stationary phase of growth. Qualitative and quantitative analyses of acetate were performed by HPLC. Acetate excretion was evident from the first generation and increased with time and biomass production in exponentially growing cells (figure 4.5). The relationship between acetate excretion and glucose concentration was reciprocal. Maximum acetate excretion coincided with the complete depletion of glucose and cessation of growth. Thereafter, the concentration decreased steadily in the absence of glucose. There was however no remarkable change in biomass production over the period of decreasing concentration of acetate (figure 4.6). The major results of aerobic metabolism of glucose by *E. coli* ATCC15224 have been summarized in Table 4.1.

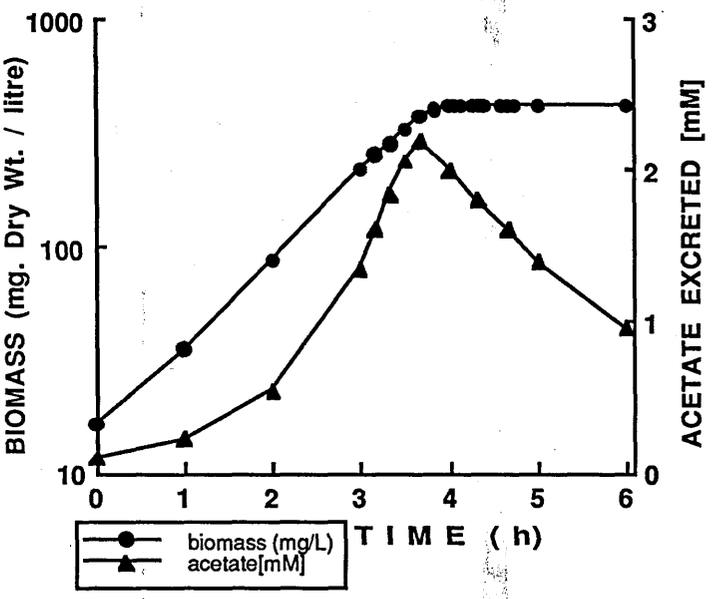


FIGURE 4.5 EXCRETION OF ACETATE IN AEROBIC BATCH CULTURE OF *E. coli* ATCC15224 ON GLUCOSE

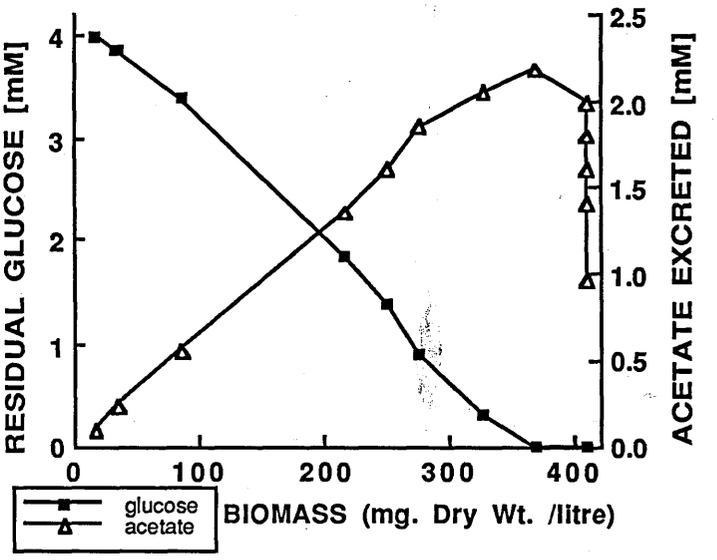


FIGURE 4.6 RELATIONSHIP BETWEEN SUBSTRATE CONSUMPTION AND ACETATE EXCRETION DURING BIOMASS PRODUCTION BY *E. coli* ATCC15224

PARAMETERS	VALUES
Glucose supplied (mM)	4.0
Complete Depletion of Glucose (h)	3.75
Mean generation time (min)	46
Growth rate (μ; hour^{-1})	0.90
Net Acetate excreted (mM)	2.06
Net Biomass produced (*mg.* dry wt. L^{-1})	362
Molar Growth yield ($Y_{GLUCOSE}$) (*g.* dry. wt. *mole.* glucose.$^{-1}$)	90.5
Input Equivalent (*mmol.* g dry wt.$^{-1}$)	11.05
PEPc Activity (*nmol. mg.* protein^{-1}. min^{-1})	24.4

TABLE 4.1

SUMMARY OF THE GROWTH, METABOLISM AND PEPC ACTIVITY OF *E. coli* ATCC15224 GROWING AEROBICALLY ON GLUCOSE

4.3 OVEREXPRESSION OF PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITY IN *E.coli* ATCC15224

4.3.1 ESTABLISHMENT OF COMPETENCE

Attempts to induce competence in *E. coli* ATCC15224 benefited from the experiments in *E. coli* PA342 (section 3.2). Cells in the early log phase were made competent as described previously for *E. coli* PA342. Transformation efficiencies of batches of cells were determined relative to plasmid vector pBR322.

Several initial attempts to transform the test strain were unsuccessful; in many cases, significantly high mortality of treated cells resulted. *E. coli* ATCC15224 was therefore subjected to considerable experimentation to make cells competent. Results presented reflect the mean of the most efficient transformations obtained (Table 4.2).

The main obstacle to the transformation of *E. coli* ATCC15224 was the ease with which the cells lysed after treatment with $CaCl_2$. Organisms could only tolerate mild changes in environmental factors. The calcium chloride treatment was carried out at a concentration of 25mM. Buffering with Tris was unfavourable to the viability of cells. Since cells were relatively intolerant to Tris-HCl or EDTA, plasmid preparations were suspended in distilled water only. To minimize the difference between the growth medium and $CaCl_2$ treatment, L-broth was supplemented with 10mM-$CaCl_2$ during inoculum preparation and main growth. Cells were best harvested during the third generation (μ=1.39.hour^{-1}) after an initial apparent OD_{600} < 0.05. Although the final apparent OD was useful for determination of the density of cells, the duration of growth appeared equally important. After treatment with $CaCl_2$, cells became even more fragile than most other strains. It was observed that complete removal of cells from the transformation medium by short centrifugation (10,000x*g* , 30 seconds, 4°C) before suspension in expression medium (L-broth), prevented complete lysis of competent cells. Cells were then allowed to warm up at room temperature before incubation at 37°C without shaking. Both shaking and heat-shock at 42°C lysed cells completely.

4.3.2 TRANSFORMATION WITH HYBRID PLASMID pJOE4 AND ANALYSIS OF CLONES

Competent cells of *E. coli* ATCC 15224 (apparent OD_{600} 0.261) were transformed with 1μg. of recombinant plasmid pJOE4 DNA and expressed in 2ml L-broth. The earliest transformants which formed colonies on selective medium after 16 hours of incubation were immediately streaked on fresh *ampicillin* plates of L-agar and incubated for another 24 hours to proliferate.

4.3.2.1 ISOLATION, IDENTIFICATION AND COMPARATIVE ANALYSIS OF PLASMIDS

Plasmids were isolated from six transformants and characterized by restriction analysis and agarose gel electrophoresis using hybrid plasmid pJOE4 as authenticated reference. Results confirmed that the transformants carried plasmid pJOE4 (figure 4.7, 4.8) together with a cryptic plasmid (figure 4.9, sample well 6). The phenotype of the endogenous plasmid and its restriction pattern were not immediately obvious. Its role in the transformation of *E. coli* ATCC15224 by other plasmids could not be determined. However, it was apparent that the plasmid was compatible with hybrid plasmid pJOE4 and contained a *Hin*dIII site (5th lane; figure 4.8ii).

CULTURE TURBIDITY (OD_{600})		TIME (min)	TRANSFOR-MANTS (cft) 10^0		VIABLE COUNT (cfu)	TRANSFOR-MATION EFFICIENCY (cft µg
Initial	Final		pBR322	pAT153	10^{-5}	$pBR322^{-1}$
0.034	0.133	70	29	111	313	$1.16x10^4$
0.035	0.134	70	27	55	352	$1.08x10^4$
0.049	0.250	80	nd	—	610	—
0.057	0.261	81	50	nd	635	$2.0x10^4$

TABLE 4.2

TRANSFORMATION OF *E. coli* ATCC15224:

Reference plasmids used included pBR322 (4.3 *kb*) and pAT153 (3.6 *kb*). Transformants were isolated on plates of L-agar, made selective with 25µg *ampicillin* per plate, after incubation for 24 hours. Transformation efficiency was determined relative to pBR322 transformation only

cft	Colony-forming transformants
cfu	colony-forming units (total viable count)
µg	microgram
10^0	undiluted expression mix
nd	not determined
OD_{600}	Apparent optical density at λ_{600}

4.3.3 ASSAY AND COMPARISON OF PHOSPHO*ENOL* - PYRUVATE CARBOXYLASE ENZYME ACTIVITIES

Ultrasonic extracts of cells from glucose cultures of *E. coli* ATCC15224 containing hybrid plasmid pJOE4, were assayed for PEPc activities. Extracts were also obtained from parent & pBR322-transformed strains of *E. coli* ATCC15224 and corresponding strains of PA342 for comparison. Activities were expressed as *nmol. mg. protein*$^{-1}$*. min.*$^{-1}$ (Table 4.3). PEPc activity was overexpressed 75-fold in *E. coli* ATCC15224-pJOE4 but only 1.1 in *E. coli* ATCC15224-pBR322, relative to 24.4 *nmol. mg.*$^{-1}$*. min.*$^{-1}$. in the parent.

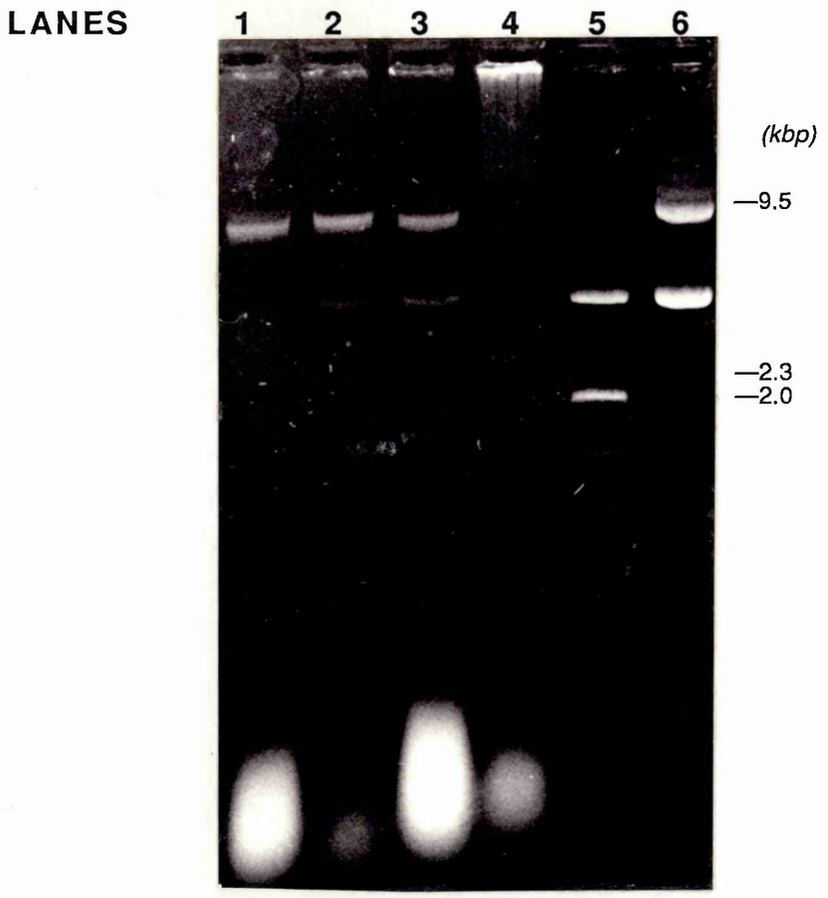


FIGURE 4.7

AGAROSE GEL ELECTROPHORESIS OF PLASMID PREPARATION FROM *E. coli* ATCC15224 AFTER TRANSFORMATION WITH HYBRID PLASMID pJOE4

Microscale plasmid preparations were made from ampicillin-resistant transformants of *E.coli* ATCC15224 derived from hybrid plasmid pJOE4. Samples were analysed by (0.8%) agarose minigel electrophoresis in TBE buffer

LANE 1	PLASMID PREP Isolate 1	LANE 4	Sample from Untransformed Parent
LANE 2	PLASMID PREP Isolate 2	LANE 5	Bulk PLASMID PREP Isolate 1
LANE 3	PLASMID PREP Isolate 3	LANE 6	PLASMID pJOE4 (*E. coli* PA342)

FIGURE 4.8 (i):

COMPARATIVE RESTRICTION ANALYSIS OF PURE PLASMID ISOLATES FROM TRANSFORMANTS OF *E. coli* ATCC15224 WITH pJOE4

Restriction patterns of Purified Bulk plasmid preparation from pJOE4-transformant of *E. coli* ATCC15224 were compared with hybrid-plasmid pJOE4.from *E. coli* PA342 whose fragments had been calibrated previously by comparison with authenticated size-markers.

LANE 1	Untreated	**}PURIFIED**
LANE 2	treated with *Bam*H1	**}PLASMID**
LANE 3	treated with *Pvu*II	**}FROM Isolate 1,**
LANE 4	treated with *Pst*I	**}*E. coli* ATCC15224**
LANE 5	Phage (λ*Hin* dIII) (size-marker)	
LANE 6	+*Pst*I	**}HYBRID PLASMID**
LANE 7	treated with *Pvu*II	**}pJOE4 PURIFIED**
LANE 8	treated with *Bam*H1	**}FROM *E. coli* PA342**

FIGURE 4.8 (ii)

LANE 1	Untreated (from *E. coli* PA342)
LANE 2	Untreated (from *E. coli* ATCC15224)
LANE 3	treated with *Hin*dIII (from *E. coli* PA342)
LANE 4	treated with *Hin*dIII (from *E. coli* ATCC15224)
LANE 5	treated with *Eco*RI (from *E. coli* PA342)
LANE 6	treated with *Eco*RI (from *E. coli* ATCC15224)

FIGURE 4.8 (i)

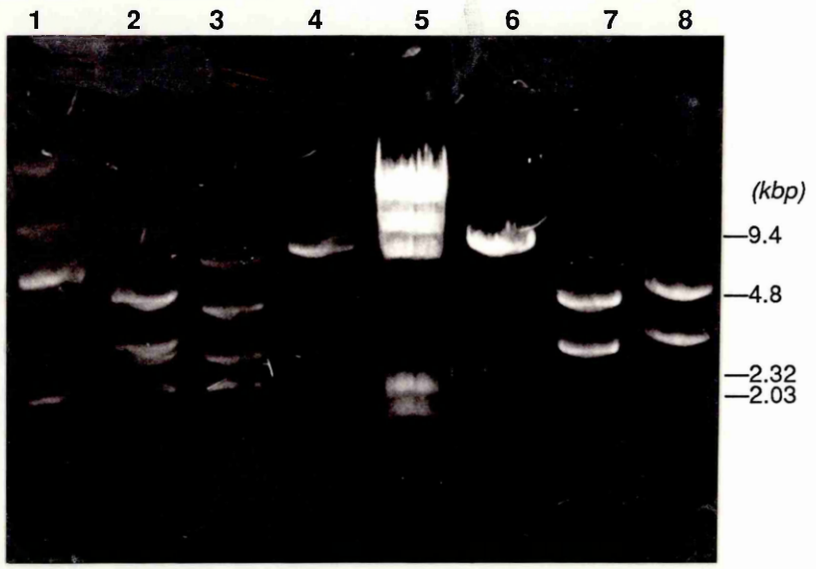
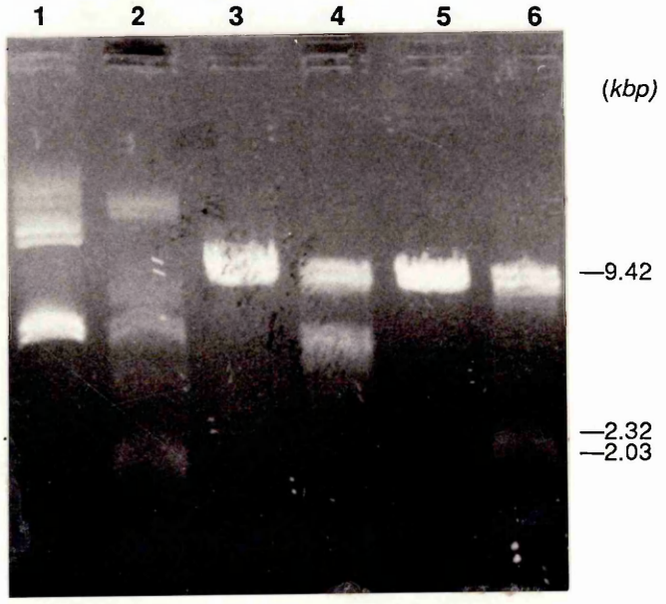


FIGURE 4.8 (ii)



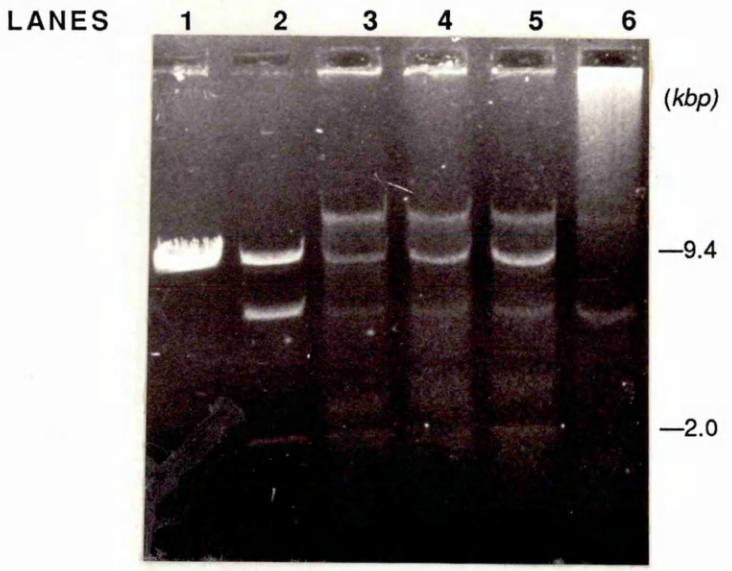


FIGURE 4.9

DEMONSTRATION OF A CRYPTIC PLASMID IN *E. coli* ATCC15224

Comparative agarose gel electrophoresis of plasmids isolated from *E. coli* ATCC15224, before and after transformation with Hybrid plasmid pJOE4. Plasmid preparation from *E. coli* ATCC15224 prior to transformation with plasmid pJOE4 is shown in lane 6. Samples 4 to 6 (lanes 3 to 5) and the bulk prep. (lane 2) were recovered from independently-arising isolates after transformation with recombinant plasmid pJOE4. Lane 1 contained bulk plasmid prep from *E. coli* PA342-pJOE4.

LANE 1	PLASMID pJOE4 (from PA342)	LANE 4	PLASMID PREP (Isolate 5)
LANE 2	Bulk PLASMID PREP (ATCC15224)	LANE 5	PLASMID PREP (Isolate 6)
LANE 3	PLASMID PREP (Isolate 4)	LANE 6	PREP (parent ATCC15224)

STRAIN OF *E. coli*	RELEVANT GENOTYPE	SPECIFIC§ ACTIVITY
	***Escherichia coli* PA342**	
parent	*ppc*	0
+ pBR322	pBR322; *ppc*	0
+ pJOE4	pBR322-*ppc*+	3536
	***Escherichia coli* ATCC15224**	
parent	*ppc* +	24.4
+ pBR322	*ppc* + (pBR322)	27.6
***Escherichia coli* ATCC15224-pJOE4 Transformants**		
Isolate 1	*ppc* + (pBR322-*ppc* +)	1819
Isolate 2	*ppc* + (pBR322-*ppc* +)	1597
Isolate 3	*ppc* + (pBR322-*ppc* +)	1256

TABLE 4.3

COMPARISON OF PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITIES OF *Escherichia coli* STRAINS PA342, ATCC15224 AND pBR322 | pJOE4 DERIVATIVES

PEPc Activities of parents and transformants of *E. coli* PA342 including three distinct transformants of ATCC15224 with pJOE4 were assayed and compared after growing on glucose. §Activity was expressed in *nmol.* (NADH oxidized). *mg.*$^{-1}$. protein. *min.*$^{-1}$.

4.4 Comparison of Glucose Metabolism by Parent and Recombinant Strains of *E. coli* ATCC15224

4.4.1 GROWTH

The growth curves for all strains were characteristic of substrate-limited batch cultures. Specific growth rates (μ) for parent, control and recombinant strains were 0.90, 0.92 h^{-1} and 0.87 h^{-1} respectively (figure 4.10).

4.4.2 DETERMINATION OF AVAILABLE SUBSTRATE CARBON

The total organic carbon (TOC) detected in all cultures reflected both residual glucose, excreted products and ampicillin in culture samples from strains ATCC15224-pBR322 and ATCC15224-pJOE4 (figure.4.11).

There was significant difference in glucose utilization by the parent and recombinant strains (figure 4.13), but not between the parent and control strain ATCC15224-pBR322 (not shown). The molar yield coefficient on glucose ($Y_{GLUCOSE}$) was 90.5*g.* dry weight.*mol.* glucose^{-1} in the parent strain. but 114.4*g. mol.*$^{-1}$ in strain 15224-pJOE4 (figure 4.14). This represented 26.4% improvement in growth yield. The percentage difference between the parent and control strains was only 1.7.

Evaluation of the differences between the physiology and metabolism of the *E. coli* ATCC15224 & *E. coli* ATCC15224-pBR322 on the one hand (table 4.4), and *E. coli* ATCC15224 & *E. coli* ATCC15224-pJOE4 on the other, made the differences between the latter systems (table 4.5) prominent by comparison. It was therefore possible to attribute most differences between *E. coli* ATCC15224 and *E. coli* ATCC15224-pJOE4 to the effect of the genomic insert in hybrid plasmid pJOE4 which contained the *ppc* gene.

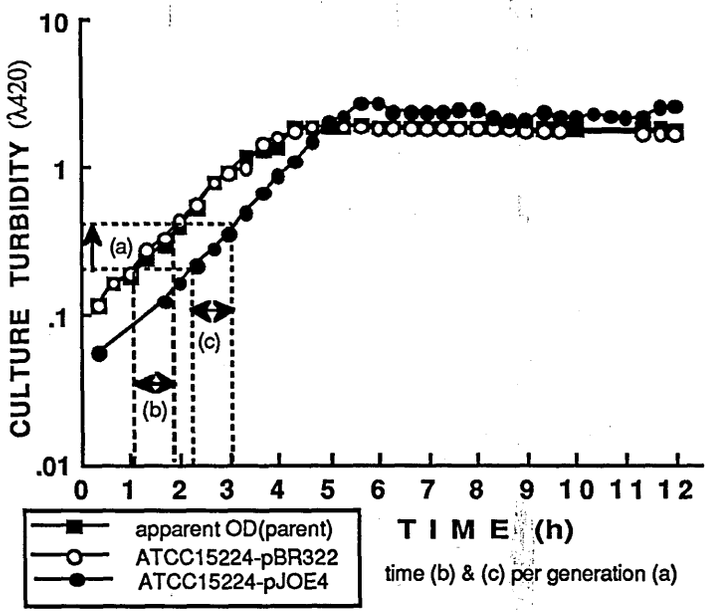


FIGURE 4.10
COMPARISON OF GROWTH OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4 ON GLUCOSE

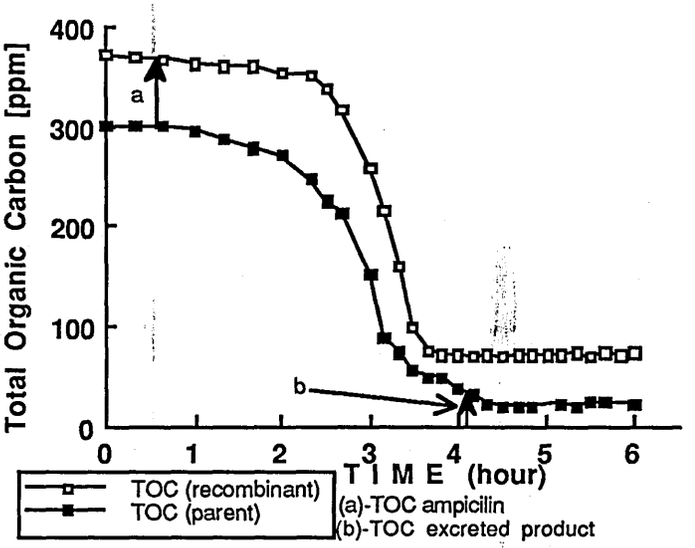


FIGURE 4.11 ANALYSIS OF TOTAL ORGANIC CARBON IN GLUCOSE CULTURES OF TEST STRAINS

4.4.3 EXCRETION OF ORGANIC PRODUCTS

Cultures were analysed by HPLC to determine non-biomass organic outputs which were reflected in the TOC especially after exhaustion of the carbon source. In all cultures, acetate formed the major excreted product. A maximum of 2.06 (±0.05) mM acetate was excreted by both parent and control strains growing on glucose (figure 4.12a). This was consistent with the levels predicted by TOC analysis (figure 4.11), and represented 0.515*mol.* acetate. *mol.* glucose^{-1}.

Although strain ATCC15224-pJOE4 also excreted acetate, the concentration was nearly half of the amount detected in cultures of the parent and pBR322-transformant. The maximum concentration was 1.07 (±0.075) mM. This was equivalent to 0.268*mol. mol.* glucose^{-1} and represented 48% reduction in acetate excretion, *vis-a-vis* parent and control strains growing on glucose (figure 4.12b).

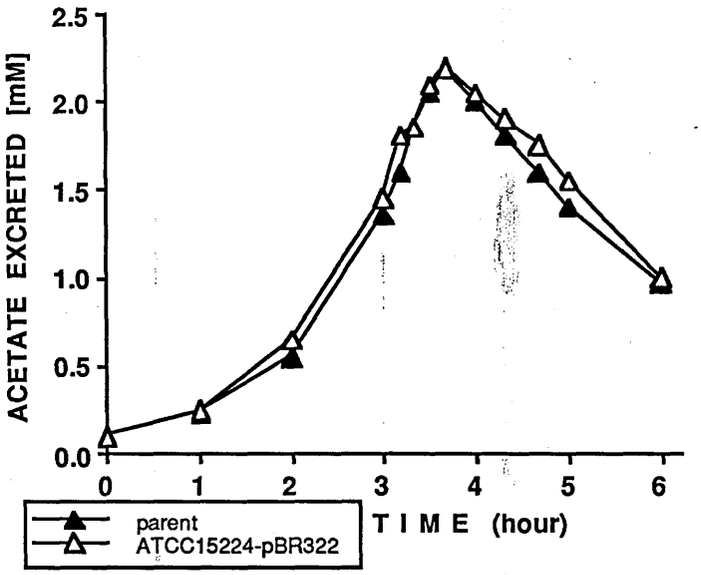


FIGURE 4.12 (a) EXCRETION OF ACETATE BY PARENT AND CONTROL STRAINS IN AEROBIC GLUCOSE CULTURES

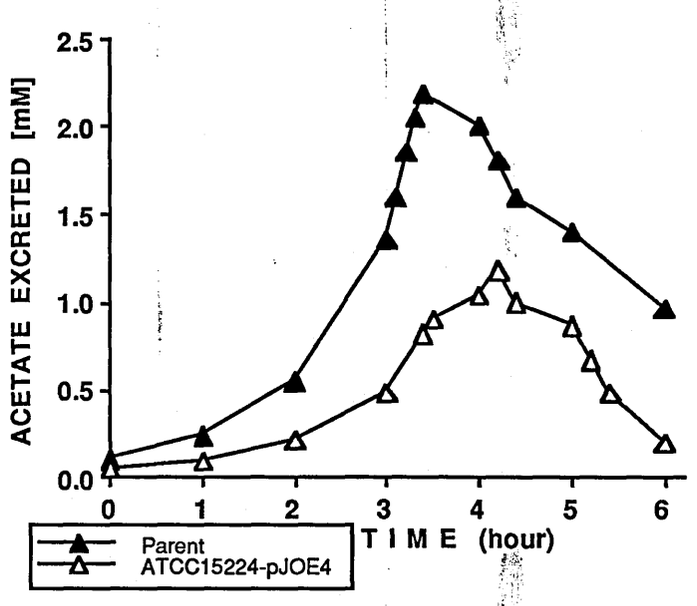


FIGURE 4.12 (b) COMPARISON OF ACETATE EXCRETION BY PARENT AND PEPC-OVERPRODUCING STRAINS ON GLUCOSE

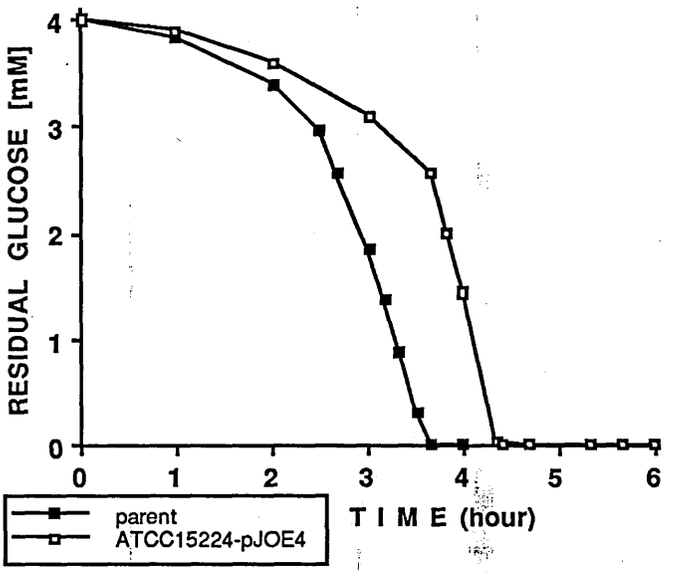


FIGURE 4.13 DEPLETION OF GLUCOSE IN CULTURES OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4

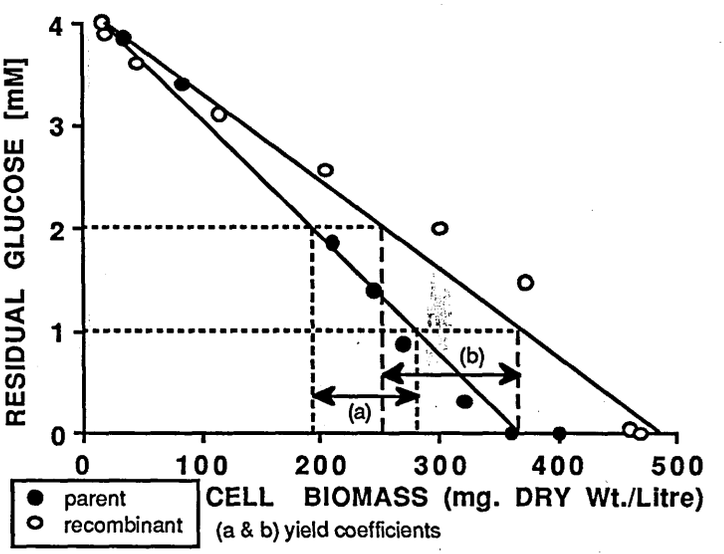


FIGURE 4.14 BIOMASS PRODUCTION AS FUNCTION OF SUBSTRATE UTILIZATION BY PARENT AND RECOMBINANT STRAINS

PARAMETER	PARENT (a)	VECTOR (b)	DIFF (b-a)	RATIO
PEPc ACTIVITY (*nmol*. mg. protein^{-1}. min^{-1})	24.4	27.6	3.2	1.13
Glucose Supplied [mM]	4.0	4.0	0	1.0
Rate of Glucose Uptake (*mol.* h^{-1})	1.07	1.07	0	1.0
Growth Rate (μ; h^{-1})	0.90	0.92	0.02	1.02
Acetate Excreted [mM]	2.06	2.08	0.02	1.01
Net Biomass Produced (mg. dry wt.L^{-1})	362	368	6.0	1.02
Molar Growth Yield (*g.* d. Wt. *mol.* glucose^{-1})	90.5	92	1.5	1.02
Input Equivalent (*mmol. g.* d. wt.$^{-1}$)	11.05	10.9	0.2	-0.99

TABLE 4.4

COMPARATIVE GLUCOSE METABOLISM AND PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITIES OF PARENT AND pBR322-DERIVED STRAINS OF *E. COLI* ATCC15224

RATIO = (b|a) Ratio of parameter in strain ATCC15224-pBR322, relative to parent
Negative signs represent values that have been reduced due to transformation by the vector

PARAMETER	PARENT (*a*)	RECOMB. (*c*)	RATIO (*c*/*a*)
PEPc ACTIVITY (*nmol.* mg. protein^{-1}. min^{-1})	24.4	1819	74.5
Glucose Supplied (mM)	4.0	4.0	1.0
Rate of Glucose Uptake (*mol.* h^{-1})	1.07	0.92	-0.86
Growth Rate (μ; h^{-1})	0.90	0.87	-0.97
Acetate Excreted [mM]	2.06	1.02	-0.49
Net Biomass Produced (mg. dry. wt. L^{-1})	362	457.6	1.27
Molar Growth Yield (*g.* d. wt. *mol.* glucose.$^{-1}$)	90.5	114.4	1.27
Input Equivalent (*mmol. g.* d. wt.$^{-1}$)	11.05	8.74	-0.79

TABLE 4.5

COMPARATIVE METABOLISM AND PHOSPHO*ENOL*PYRUVATE CARBOXYLASE ACTIVITIES OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4

(c|a) Ratio of parameter in Recombinant relative to parent
Negative signs represent values that have been reduced in strain ATCC15224-pJOE4

4.5 DISCUSSION

Results reported in this study, show the effects of the manipulation of carbon & metabolic fluxes at the crossroads of diverging fluxes from phospho*enol*pyruvate (PEP). During aerobic glucose metabolism by a bacterial system which overexpressed phospho*enol*pyruvate carboxylase (PEPc) activity, the fluxes of input-carbon were modified in favour of diminished fluxes to acetate excretion and enhanced throughputs to biosynthesis. Carbon flux through each enzyme of the central metabolic pathways in parent and recombinant strains can be computed *a priori,* by applying the specific growth rates (table 4.5) to the derived throughputs (figures 4.15; 4.16).

The PEPc-overproducing strain, required a lower input of substrate to generate an equivalent amount of biomass to the parent strain. In addition, it sustained a lower flux of input carbon to acetate excretion (figure 4.17). Its rate of glucose consumption was also lower. These observations are similar to findings in *E. coli* JM105, in which a combination of reduced rates of glucose consumption and growth, as well as lower acetate excretion, resulted in higher cell density (Luli and Strohl, 1990). In the case cited however, biphasic growth was also observed. *Escherichia coli* strain-B, which also showed similar effects, failed to produce high biomass, suggesting that increased growth yield did not depend solely on those extrinsic effects reported.

Typical molar growth yields reported for strains of *Escherichia coli* on glucose ($Y_{GLUCOSE}$), usually varied from 85 to 95*g.* dry weight. *mol.* $^{-1}$ during aerobic growth (Harrison, 1978; Ratledge, 1987). Neither additional supplement of inorganic salts, nor higher substrate concentration altered $Y_{GLUCOSE}$ (Hollywood and Doelle, 1976).

It has been suggested that growth yield in aerobic glucose cultures, may be limited by the accumulation of organic acid-byproducts (Doelle *et al.,* 1974), or respiration (Andersen and vonMeyenburg, 1980), signalling the onset of the *Crabtree* effect (vanUrk *et al.,* 1990). Continuous removal of by-products by physical processes, was found to improve growth yield on glucose (Landwall and Holme, 1977b; Anderson *et al.*, 1984).

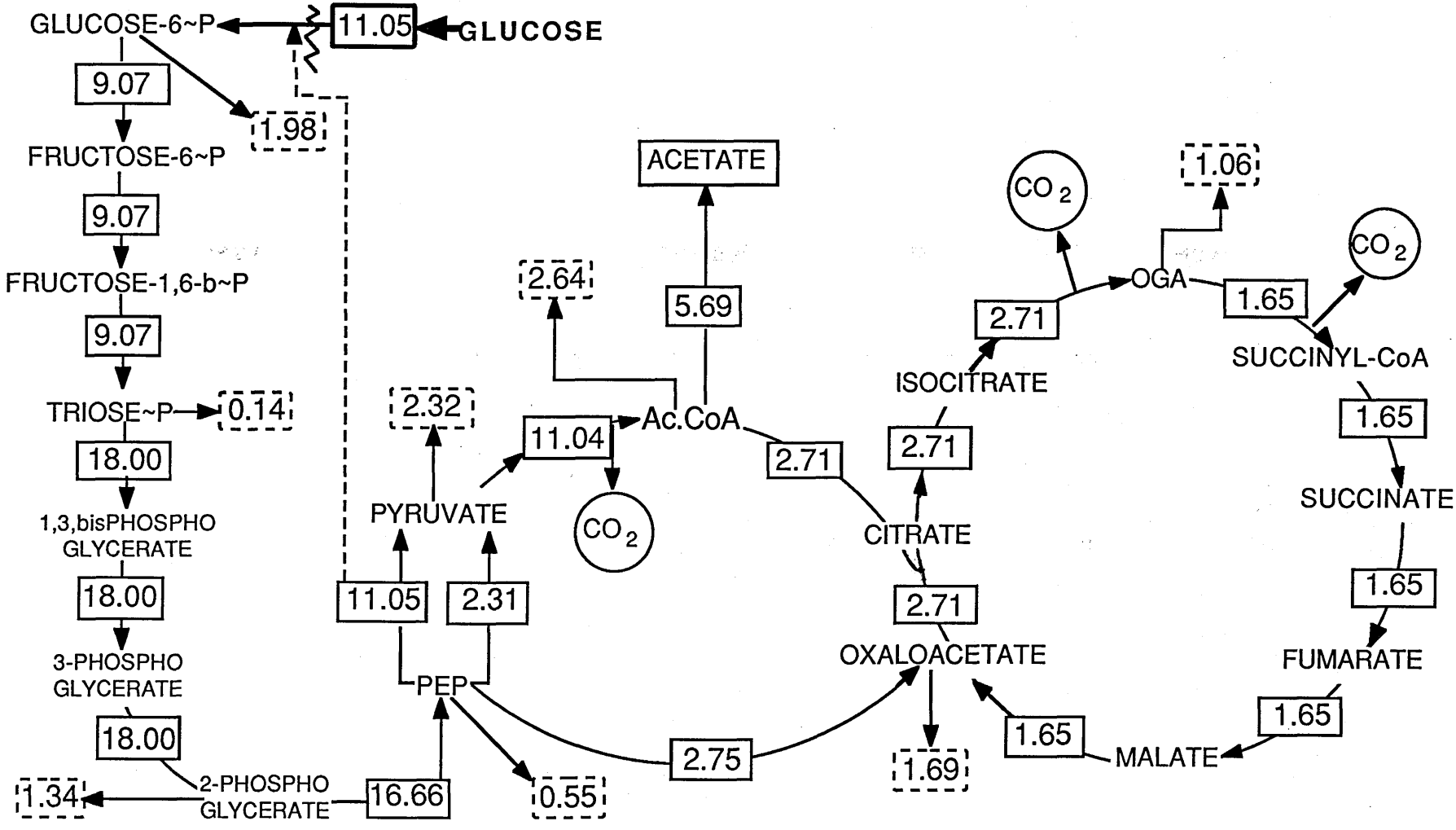
FIGURE 4.15

THROUGHPUTS IN THE CENTRAL METABOLIC PATHWAYS OF *Escherichia coli* ATCC 15224 DURING AEROBIC GROWTH ON GLUCOSE

The pathways shown are glycolysis and the TCA cycle. Throughputs to biosynthesis are highlighted by dashed boxes and were based on the estimated drainage of specific intermediate metabolites to the synthesis of amino acids & nucleotides shown in figures 1.2 & 1.3. Input was calculated from $Y_{GLUCOSE}$ during aerobic growth (table 4.1; figure 4.14) while throughput to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced. Arrows point to the direction of fluxes which effect net throughputs at a specific growth rate of 0.90 h^{-1}.

VALUES SHOWN WERE EXPRESSED IN

mmol. (input, output or intermediate metabolite)
g. (dry bacterial cell biomass).$^{-1}$


GLUCOSE-6~P
11.05
GLUCOSE
9.07
1.98
FRUCTOSE-6~P
9.07
FRUCTOSE-1,6-b~P
9.07
TRIOSE~P
0.14
18.00
1,3,bisPHOSPHO GLYCERATE
18.00
3-PHOSPHO GLYCERATE
18.00
2-PHOSPHO GLYCERATE
1.34
16.66
PEP
0.55
11.05
2.31
PYRUVATE
2.32
11.04
CO2
2.64
ACETATE
5.69
Ac.CoA
2.71
CITRATE
2.71
OXALOACETATE
2.75
1.69
2.71
ISOCITRATE
CO2
OGA
1.06
1.65
CO2
SUCCINYL-CoA
1.65
SUCCINATE
1.65
FUMARATE
1.65
MALATE

Pan *et al.* (1987) observed that accumulation of excreted products was an inherent property of *Escherichia coli* . Growth rate in the exponential phase also reflected an inherent characteristic (Andersen and vonMeyenburg, 1980; Koch, 1988). Thus, the regulatory mechanisms of the cell tended to allow fluxes to acetate excretion when growth and respiratory mechanisms operated at maximum fluxes. For enterobacteria like *E.coli*, which do not accumulate storage products or energy reserves (Wanner and Egli, 1990), the rapid catabolism of glucose which is universally-metabolizable, to produce acetate which is not, must be akin to extracellular hoarding of carbon and energy source, by its conversion to a transitional resource which is less bioavailable to potential competitors. For the organism, although the central pathways operated at maximum capacity during aerobic glucose metabolism, this did not necessarily correlate with optimal efficiency, nor was it so intended. The present study suggests that their efficiency may be limited by disproportionate throughputs of carbon to key catabolites, and reduced stringency in coupling of catabolic and anabolic fluxes of amphibolic pathways.

The operation, partition and control of fluxes in *Escherichia coli,* must be viewed against the background of adaptation to its natural ecological niche in the still-aerobic part of the ileum (Koch, 1988) where the nutrient status is copiotrophic (Owens and Legan, 1987) but cells are in constant motion. For instance, when *Escherichia coli* utilizes glucose under aerobic conditions, it operates carbon fluxes through the glucose-phosphotransferase (PTS) system, glycolysis and the Krebs cycle respectively (Meadow *et al.*, 1986; Spencer and Guest, 1987; Wanner and Egli, 1990). The partition of carbon flux at PEP determines the eventual course and fate of input carbon (figure 4.18). Throughput of PEP to biosynthesis has been estimated at 0.55*mmol. g.* (dry biomass)$^{-1}$ irrespective of carbon source but relative to other biosynthetic precursors, in precise relative ratios (Holms, 1986a). Thus flux to biosynthesis is a simple function of the growth rates unique to specific carbon sources and culture. Flux through the carboxylating route of PEP is also relatively constant, possibly to guarantee the supply of oxaloacetate and 2-*oxo*glutarate under unfavourable conditions of growth. By contrast, throughputs to pyruvate via the PTS and pyruvate kinase, are more adaptive to changes in carbon sources apart from the specific growth rate. The ensuing discussion attempts to highlight the relative sensitivities of these fluxes to various outputs from PEP and each other.

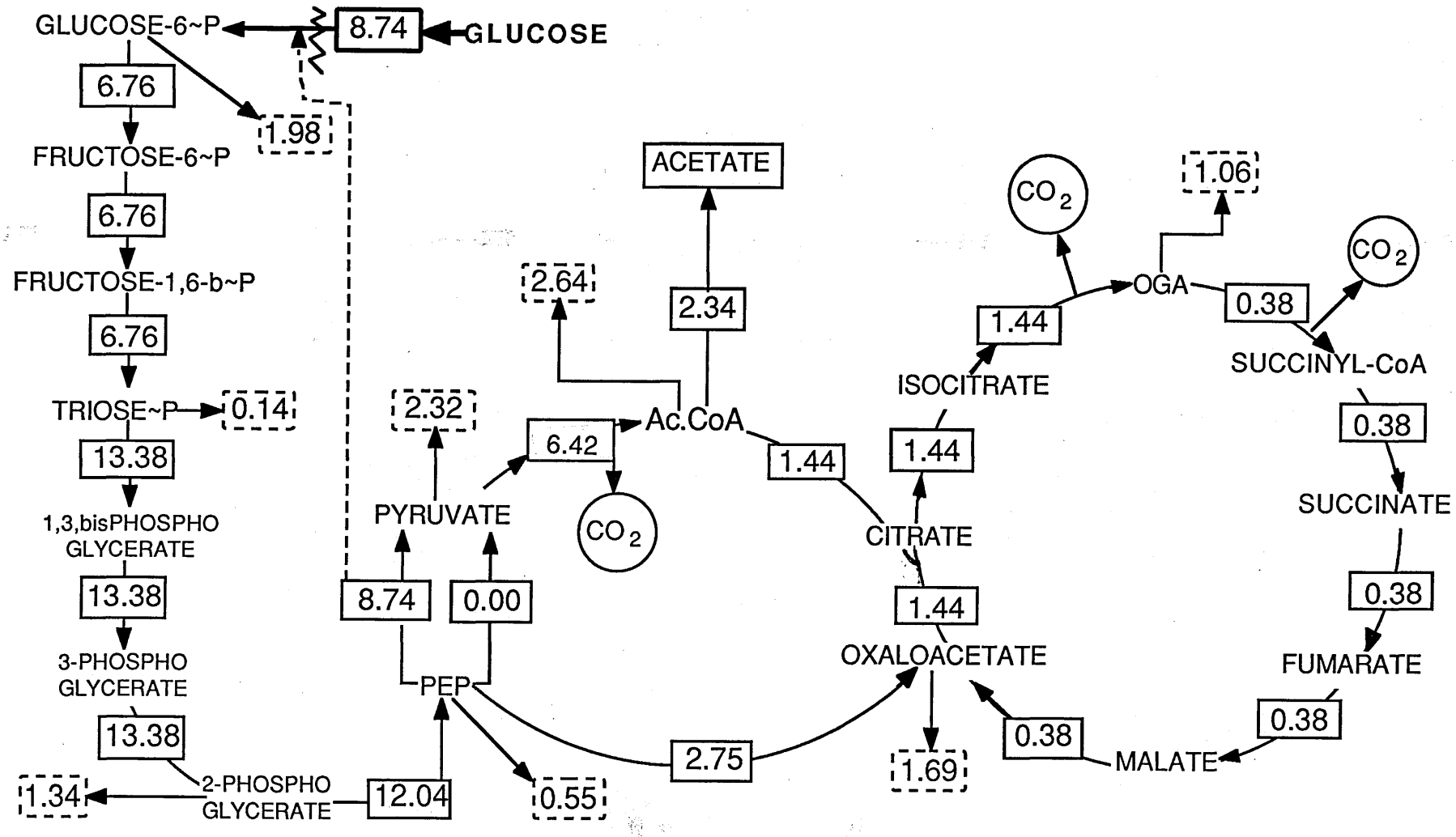
FIGURE 4.16

THROUGHPUTS IN THE CENTRAL METABOLIC PATHWAYS OF *Escherichia coli* ATCC 15224-pJOE4 (WITH AMPLIFIED PEP CARBOXYLASE ACTIVITY) DURING AEROBIC GROWTH ON GLUCOSE

The pathways shown are glycolysis and the TCA cycle. Throughputs to biosynthesis are highlighted by dashed boxes and were based on the estimated drainage of specific intermediate metabolites to the synthesis of amino acids & nucleotides shown in figures 1.2 & 1.3. Primary input was derived from $Y_{GLUCOSE}$ during aerobic growth (table 4.5; figure 4.14) while throughput to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced. Arrows point to the direction of fluxes which effect net throughputs at a specific growth rate of 0.87 h^{-1}.

VALUES SHOWN WERE EXPRESSED IN

mmol. (input, output or intermediate metabolite)
g. (dry bacterial cell biomass).$^{-1}$


GLUCOSE-6~P
8.74
GLUCOSE
6.76
1.98
FRUCTOSE-6~P
6.76
FRUCTOSE-1,6-b~P
6.76
TRIOSE~P
0.14
13.38
1,3,bisPHOSPHO GLYCERATE
13.38
3-PHOSPHO GLYCERATE
13.38
2-PHOSPHO GLYCERATE
1.34
12.04
2.32
PYRUVATE
8.74
0.00
PEP
0.55
2.64
6.42
CO2
ACETATE
2.34
Ac.CoA
1.44
2.75
CITRATE
1.44
1.44
OXALOACETATE
1.69
CO2
1.06
OGA
1.44
ISOCITRATE
CO2
0.38
SUCCINYL-CoA
0.38
SUCCINATE
0.38
FUMARATE
0.38
MALATE

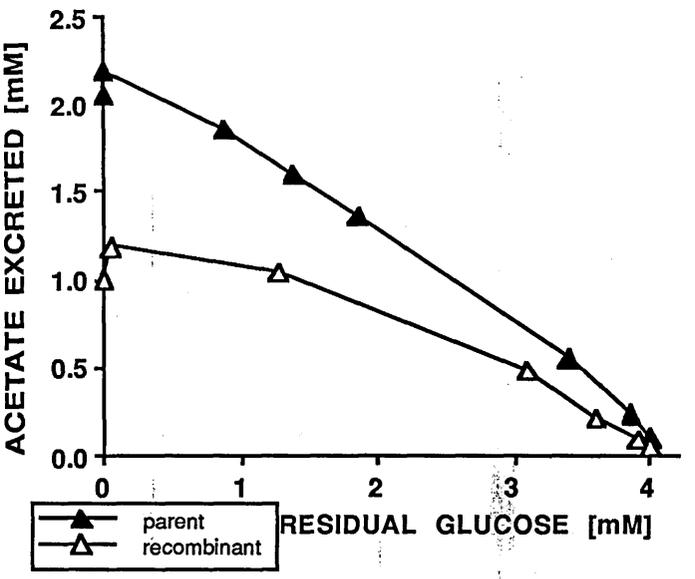


FIGURE 4.17

RELATIONSHIP BETWEEN GLUCOSE DEPLETION AND THE EXCRETION OF ACETATE IN BATCH CULTURES OF PARENT AND RECOMBINANT STRAINS OF *E. coli* ATCC15224

Phosphotransfer resulted in the dephosphorylation of phospho*enol* pyruvate (PEP) into pyruvate and corresponding phosphorylation of glucose (Roseman, 1990), which represented the initial flux of glycolysis. The relative K_m values of PEP and pyruvate for "*enzyme1*" of the PTS system are 0.4mM and 2.0mM respectively (Saier *et al.*, 1980). These values are 5 times in favour of the dephosphorylation of PEP into pyruvate and translocation of substrate into the cell provided other reactants are present in saturating amounts. Moreover, the K_m [PEP] of *"enzyme1"* is considerably less than the K_m for other enzymes which compete at the divergent, quadrilateral flux from PEP (figure 4.18). For *pyruvate kinase* and *PEP carboxylase* , the values are 3.5mM (Sanwal, 1970) and 12mM (Cánovas and Kornberg, 1966) respectively. The relative demand of the PTS system for PEP as motive force is stronger than other competing fluxes from PEP, such that 1*mol* (PEP).*mol* (substrate)$^{-1}$ is invariably invested in the translocation of glucose into the cell (Kornberg, 1981). Eventually, PEP is regenerated during glycolysis (2*mol.* PEP *per mol.* glucose-6-phosphate) and other regulatory mechanisms of the cell [e.g,K_i *enzyme 1* for PEP=2.5mM; pyruvate=20mM, other reactants not limiting] (Saier *et al.,* 1980) enable other competing and essential routes, like the glycolytic flux through pyruvate kinase to function at a higher rate (Thompson, 1987; Goodridge, 1990) (K_m[PEP] for FBP-activated pyruvate kinaseF=3.5mM at saturation; Sanwal 1970). The equilibrium of the pyruvate kinase flux is substantially in favour of the forward reaction (Malcovati and Kornberg, 1969) and carbon flux through the pyruvate dehydrogenase multienzyme complex (Severin *et al.*, 1985) with acetyl-CoA as the principal output (figure 4.19). Like the pyruvate kinase reaction, this step is also irreversible under physiological conditions, and is pivotal to the complete oxidation of glucose in aerobic cultures (Patel and Roche, 1990). Acetyl-CoA can potentiate the activity of PEP carboxylase as much as 30-fold (Cánovas and Kornberg, 1966). In so doing, it effectively activates the flux which produces its co-reactant in the first reaction of the TCA cycle. At saturation [0.5-1mM], acetyl-CoA reduces the K_m of PEPc for PEP at least ten-fold to 1mM, or below the K_m [3.5mM] of pyruvate kinase but between the K_m [300μM] and K_i[2.5mM] of *enzyme1* of the PTS system (Saier *et al* , 1980). Even when the concentration of acetyl-CoA is sub-optimal, a synergistic arrangement involving FBP and acetyl-CoA maintains the activity of PEPc (figure 4.20) and throughput to oxaloacetate (Sanwal, 1970).

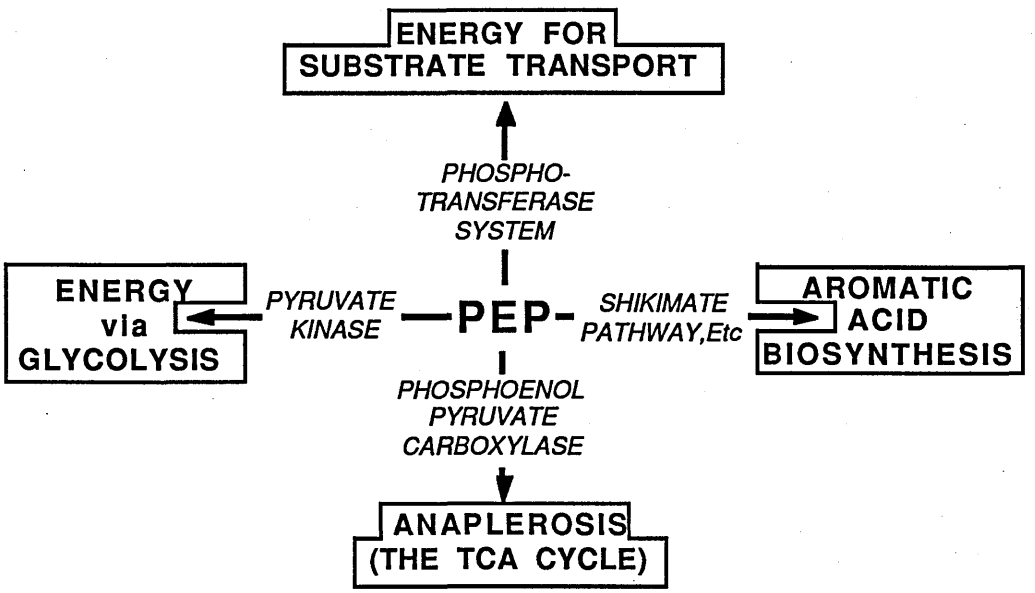


FIGURE 4.18 QUADRILATERAL PARTITION OF CARBON FLUX AT PHOSPHO*ENOL* PYRUVATE ON PTS-SUGARS

The flux which yields acetyl-CoA from pyruvate, also produces some carbon dioxide (1*mol.* CO_2. *mol.* PEP^{-1}); (figure 4.19) which the cells can always resort to particularly in the unlikely event that exogenous availability of CO_2 became limiting (Godley *et al.,* 1990) for the parallel carboxylation of PEP into oxaloacetate. To ensure that this scenario is streamlined to produce acetyl-CoA and oxaloacetate sequentially, throughput of PEP to its phosphorylated and carboxylated routes are coordinately and sequentially controlled (figure 4.21). Fluxes of PEP through PEPc and pyruvate kinase are unidirectional and their respective outputs also feed into physiologically irreversible fluxes through citrate synthase or the pyruvate dehydrogenase multi-enzyme complex. Furthermore, PEP positively modulates pyruvate dehydrogenase (Sanwal, 1970) presumably because of the demand for its acetyl-CoA and CO_2 outputs for the parallel flux of PEP to oxaloacetate. Acetyl-CoA, the main output of the pyruvate dehydrogenase flux, activates

the carboxylation of PEP, while also auto-regulating its own output in the phosphorylated route by feedback inhibition (figure 4.21). The carboxylated flux of PEP yields oxaloacetate which is the carrier-molecule for the terminal oxidation of its phosphorylated route. The alternative energy-generating route which results (figure 4.22), increases throughput to precursors from the TCA cycle and enables more C_3 intermediates of amphibolic glycolysis to fulfil biosynthetic functions at higher relative ratios of the precise amounts of biosynthetic precursors (Krebs, 1940b; Gest, 1987; Holms, 1987).

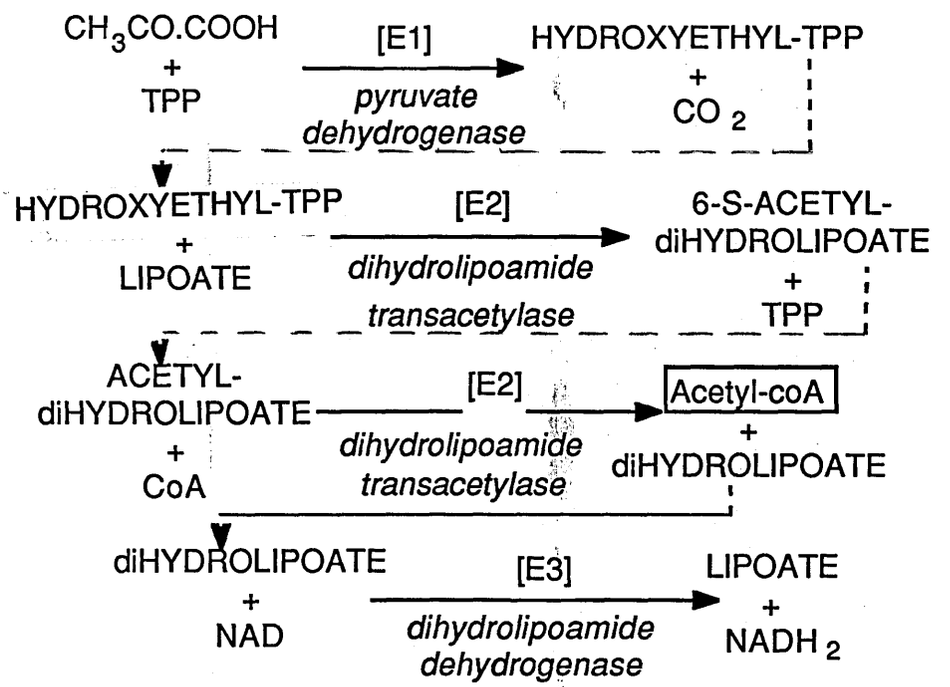


FIGURE 4.19

REACTIONS EFFECTING THE PIVOTAL DECARBOXYLATION OF PYRUVATE TO ACETYL-*COENZYME* A VIA THE PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX

Alternatively, this arrangement makes more precursors available for biosynthesis through the TCA cycle, at the expense of the energy of glycolysis (Sanwal, 1970; Spencer and Guest, 1987). Since majority of fluxes through both glycolysis and the TCA cycle are amphibolic, only the relative availability of their respective biosynthetic precursors (figures 1.2 & 1.3), would determine where the main burden of energy-generation or procurement of biosynthetic precursors would lie. Such dual roles demand sensitive, coordinate control operations in fluxes through the TCA cycle which is generally sensitive to the nature of substrate-input (Krebs, 1981; Ratledge, 1987), and changes in the energy state of the cell (Sanwal, 1970; Spencer and Guest, 1987; Hellingwerf and Konings, 1985). In particular, its operation reflects the unique regulation of each of its component fluxes (Beeckmans and Kanarek, 1987; Crabtree and Newsholme, 1987). The initial reaction of the cycle, has been identified as the limiting step in its operation (Weitzman, 1987). The inputs of this key flux, are **acetyl-CoA** and **oxaloacetate**. It is important to this discussion that both of these are outputs of divergent unidirectional fluxes from PEP. Furthermore, the former activates the flux which produces the latter, powerfully (Cánovas and Kornberg, 1969), while the latter exerts a feedback control on the flux which consumes the former (Krebs, 1981); (figure 4.21). Their joint-output (citrate), also exerts negative control on the phospho-fructokinase (PFK) flux (Dawes, 1986) which eventually supplies PEP. Fructose-1,6-*bis* phosphate, the immediate output of the PFK flux, positively modulates the output of pyruvate (hence, acetyl-CoA) (figure 4.20) and cooperatively activates phospho*enol* pyruvate carboxylase which yields oxaloacetate.

Since the availability of oxaloacetate, is central to the operation and regulation of the TCA cycle, and acetyl-CoA *potentiates* throughput to oxaloacetate while participating in flux from it (Krebs and Eggleston, 1940;Krebs, 1981; Gest, 1987), it is predictable that flux through *citrate synthase* would be rate-limiting for the cycle (Wilde and Guest, 1986; Weitzman, 1987). Furthermore, the enzyme was a powerful activator of dihydrolipoamide transacetylase of the pyruvate dehydrogenase complex (Srere *et al.*, 1987) which yielded acetyl-CoA (figure 4.19).

In addition to the *catalytic* role of oxaloacetate in the oxidation of acetyl-CoA (Krebs, 1981), it is also a dual biosynthetic precursor, upon which throughputs to four nucleotides, L-aspartate, 2-oxoglutarate and their derivative-amino acids, depend (figures 1.2; 1.3; 4.21). The constant throughput of PEP to oxaloacetate *via* PEPc (Holms, 1986a) regardless of

the carbon source and culture conditions, must have been an adaptive arrangement for ensuring the production of oxaloacetate and 2-oxoglutarate for biosynthesis, even when nutrient supply and growth conditions became more austere.

A major advantage of the transition from anaerobic to aerobic modes of nutrition and growth, was the ability to regenerate oxaloacetate through the TCA cycle, as well as increased throughputs to 2-oxoglutarate, fumarate, malate, aspartate and nucleotides (Sanwal *et al.*, 1970; Krebs, 1981; Gest, 1987). However, these outputs do not favour the PEPc flux; indeed, together they constitute its most significant allosteric inhibitors (Sanwal, 1970; Teraoka *et al.*, 1974; Yoshinaga *et al.*, 1974; Izui, Fujita *et al.*, 1984). It is apparent that the regulatory mechanisms for the partition of carbon flux between the phosphorylated and carboxylated routes at PEP during aerobic metabolism by *E. coli* ATCC15224, is a vestige of the anaerobic scenario. Both phospho*enol*pyruvate carboxylase and pyruvate kinaseF are jointly activated by FBP (figure 4.20) to take advantage of increase in available substrate which the concentration of FBP frequently indicates. However, while this single activator is sufficient for maximal activity of pyruvate kinaseF, PEPc must in addition rely on synergistic activation by acetyl-CoA (the output of the second step in the phosphorylated route). The conversion of PEP to pyruvate *via* pyruvate kinase is not sufficiently reversible to convert pyruvate back to PEP when the latter is present in excess of its metabolic requirements (Sanwal and Maeba, 1968). Indeed, the conversion of pyruvate to acetyl-CoA through the pyruvate dehydrogenase complex actually benefits from activation by PEP (Sanwal *et al.*, 1971). Under this arrangement, PEPc is only activated to a reasonable level for carboxylation of PEP after acetyl-CoA has been formed from pyruvate (Ashworth and Kornberg, 1966).

Clearly these regulatory sequences are more suited for anaerobiosis where two divergent fluxes arise from oxaloacetate (Miles and Guest, 1987) purely for throughputs to 2-oxoglutarate and succinyl-CoA as biosynthetic precursors (Gest, 1987). In all probability, the control structure was designed to ensure that the right-hand reactions which relied on condensation of oxaloacetate with acetyl-CoA to produce 2-oxoglutarate, was not competitively undermined in the partition of flux at oxaloacetate. There is a case for this, if oxaloacetate was produced before acetyl-CoA, since this would be prejudicial to the C_6-tricarboxylic acid half of the anaerobic metabolic system.

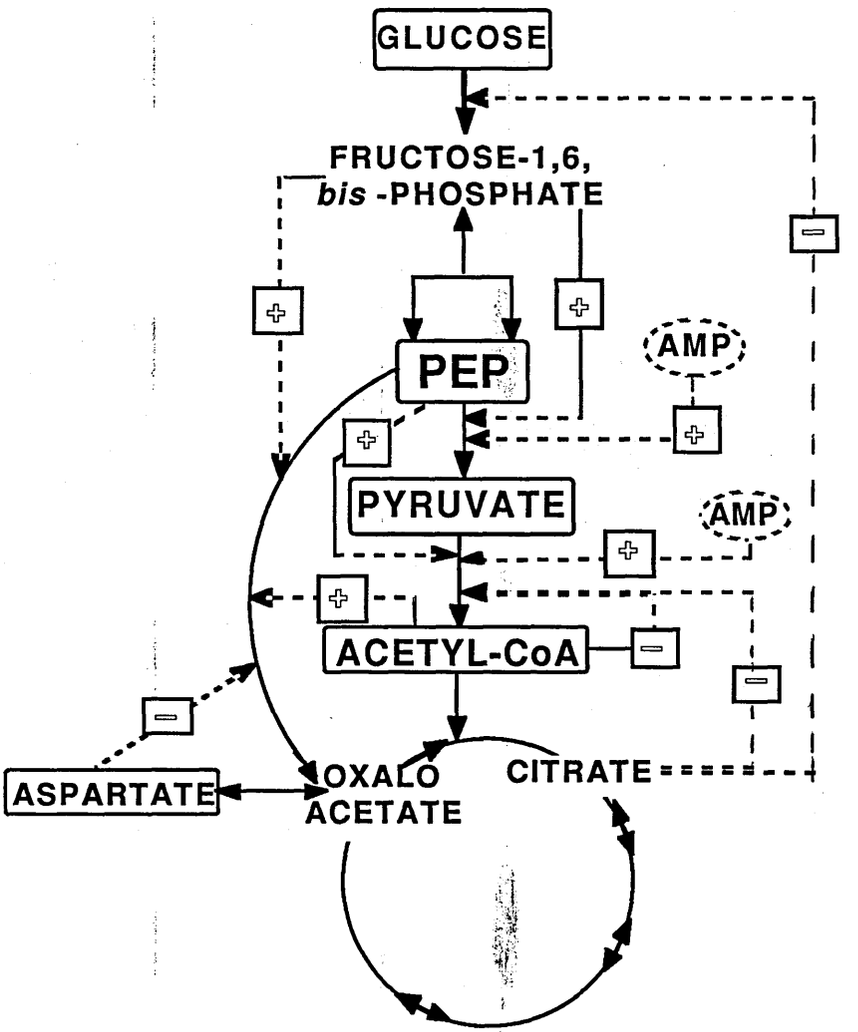


FIGURE 4.20

REPRESENTATIVE NETWORK OF CONTROL OF RELEVANT FLUXES IN THE CENTRAL METABOLIC PATHWAYS OF GLYCOLYSIS AND THE *KREBS* CYCLE BY EFFECTORS

Dotted lines/arrows represent the direction of modulation of fluxes indicated.

+--- positive modulators (ACTIVATORS OF FLUXES)

---- negative modulators (INHIBITORS OF FLUXES)

AMP Adenosine monophosphate. Index of state of cellular energy supply / requirement

It is interesting that all the powerful allosteric inhibitors of PEPc (table 3.1) are outputs of the C_4-dicarboxylic half to which acetyl-CoA does not feed in anaerobes. Under anaerobic growth or austere conditions, these regulatory mechanisms are perfectly in order, but appear to be major handicaps to the maximization of the unique potentials inherent in the regeneration of oxaloacetate by a cyclic TCA sequence during aerobic metabolism. The regeneration of oxaloacetate and increased throughputs to the C_4-dicarboxylic acids or their derivatives from the cycle, only inhibited PEPc.activity, thereby limiting increased flux through PEPc solely to increase in growth rate (Holms, 1986a), but not increased throughput, even when nutrient status was copiotrophic. The most evident outcome of this, was the excretion of acetate. During this study however, it was possible to diminish flux of acetyl-CoA to acetate excretion substantially during the aerobic metabolism of glucose by *E. coli* ATCC15224-pJOE4 (figure 4.17). Given the multiplicity of allosteric effectors, amplification of PEPc activity probably resulted more from increased gene dosage effect, than enhanced expression. It is also proposed that any relaxation of the constraints imposed on net throughput of carbon to oxaloacetate would tend to result in increased growth yield without necessarily resulting in a concomitant increase in growth rate. The aggregation of the precise cocktail of biosynthetic precursors would occur in higher quantitative ratios as throughputs to oxaloacetate and 2-*oxo*glutarate increased and the burden for generation of biosynthetic energy tended to rest on glycolysis. The biosynthetic function of oxaloacetate predates its role in energy generation (Gest, 1987) and is perhaps more important than its more celebrated status as the output of anaplerosis. Strictly speaking, phospho*enol*pyruvate carboxylase may be an anabolic rather than an *anaplerotic* enzyme, as this study, and a critical inspection of certain mutant systems in the next section would tend to suggest.

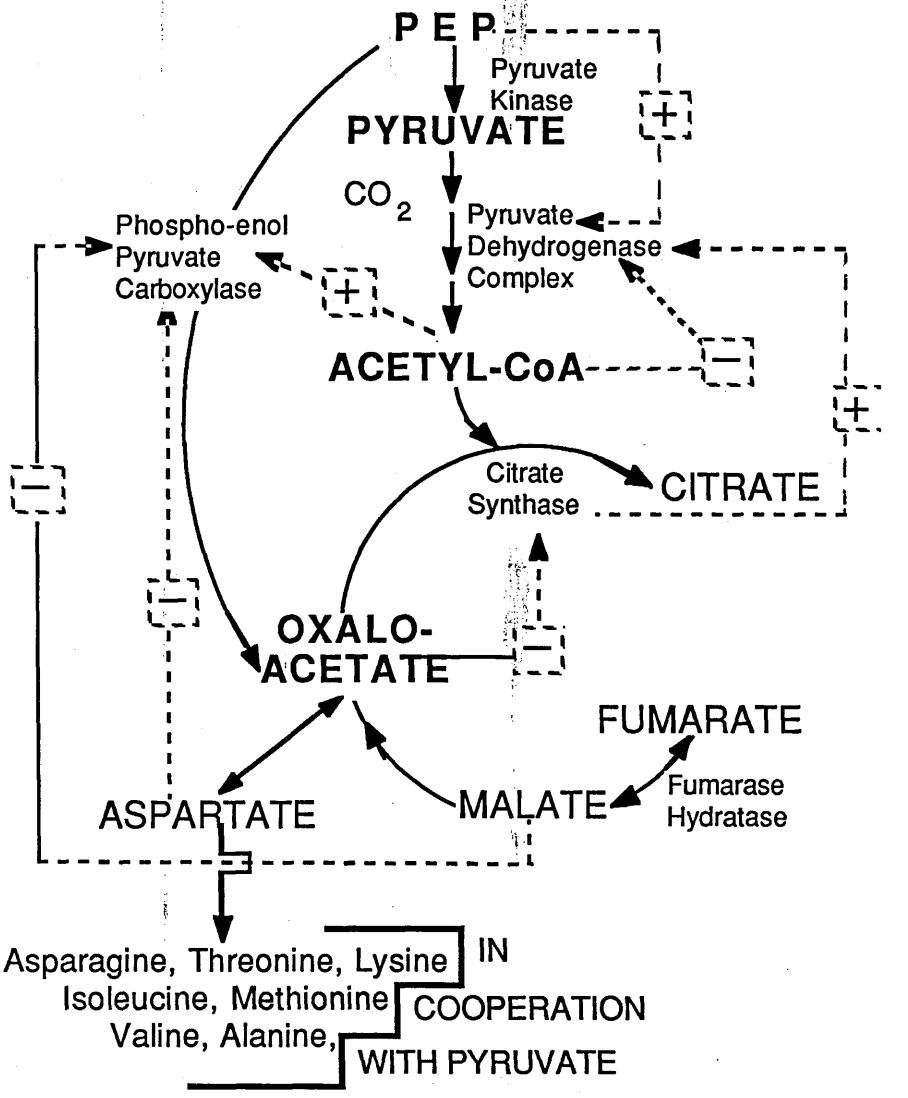


FIGURE 4.21

COORDINATION OF FLUXES OF PEP TO OXALOACETATE, ACETYL-*CoA* AND CITRATE

Arrows indicate direction of fluxes Enzymes are located by the reactions they catalyse. Dashed arrows indicate control of fluxes by intermediate metabolites

\+ positive modulation (Activation)
– negative modulators (Inhibition)

4.5.1 EVIDENCE IN SUPPORT OF THE PRIMARY BIOSYNTHETIC ROLE OF THE PEPc FLUX

Isotopic studies demonstrated that the role of the TCA cycle during exponential growth of *Escherichia coli* , was almost exclusively to furnish biosynthetic precursors (Dawes and Large, 1982). The cells subsisted on energy from glycolysis (Miles and Guest, 1987) and the operation of the cycle depended very much on the availability of oxaloacetate (Krebs, 1940b; Krebs, 1981) from PEP *via* PEPc flux (Kornberg, 1966; Sanwal, 1970). PEPc-negative mutants used in this study, did not grow on glucose as sole carbon source. This defect was total, even though they possessed all TCA cycle enzymes in functional states. *E. coli* PA342 (*ppc*) regained full growth on glucose after complementation with the *ppc* gene in plasmid pJOE4, or supplementation with L-aspartate, an oxaloacetate-derivative. Other C_4-dicarboxylic acid intermediates of the TCA cycle would have provided similar relief (Ashworth and Kornberg, 1966). Although it remained deficient in the *anaplerotic* function before complementation, supplementation was mainly necessary to initiate and establish full metabolism. Thereafter, the TCA cycle became self-perpetuating. By contrast, an active PEPc flux was indispensable in PEPc-negative mutants of the cyanobacteria, *Synechococcus sp.* (Luinenburg and Coleman, 1990). The nutritional lesion could not be circumvented by supplementation with TCA cycle intermediates. This was predictable, since reactions of the TCA cycle, are not cyclic in anaerobic systems (Gest, 1987), and oxaloacetate is not regenerated

A strain of *Serratia marcescens* which overproduced PEPc (and presumably, oxaloacetate), synthesized 25 (±5)% more L-threonine and 10 (±5)% more biomass, without any change in its growth rate (Sugita and Komatsubara, 1989). This is suggestive of a selective increase of the biosynthetic fluxes of general metabolism when PEPc activity was raised, without prejudice to overall metabolism. Under such conditions, the energy requirements of the cell would tend to rely on glycolysis (Sanwal, 1970).

Citrate synthase-negative mutants of *Escherichia coli* also failed to grow on glucose as sole carbon & energy sources. They resumed full growth after supplementation with 2-*oxo*glutarate derivatives (Guest, 1981b), even though their TCA cycles remained truncated, and hence, non-functional.

Mutants like the citrate synthase-negative strain operated the TCA cycle almost like anaerobes. Wilde and Guest (1986), observed that energy-output from glycolysis was sufficient to support their growth on glucose, provided PEPc fluxes were intact and functional. If the reversibility of all fluxes from oxaloacetate were possible thermodynamically, a citrate synthase-negative mutant would synthesize 2-oxoglutarate, which is a biosynthetic precursor, thereby circumventing the severe effect of its lesion. Clearly, flux through PEPc could not be *anaplerotic* in these mutants where a cycle of TCA reactions was non-existent.

Reports of experiments with *E. coli* AB1622 (*ppc*) have also been considered, in the light of the critical role of oxaloacetate in biosynthesis and glucose metabolism. Washed cells of the mutant were metabolically active on unsupplemented pyruvate and accumulated nine times more 'acetate' (acetyl-*Co*A?) than the wild-type, in proportion to the degree of oxidation of primary substrate (Ashworth and Kornberg, 1966). Like citrate synthase mutants, they derived adequate metabolic energy from glycolysis. However, a major difference between them, was the possession of a full complement of TCA cycle enzymes by PEPc mutants. While, supplementation with C_4-dicarboxylic acids or derivatives, did not restore PEPc activity, it furnished the output of the flux through a functional TCA cycle/malate dehydrogenase flux, thus making the oxidation of acetyl-*Co*A and provision of 2-oxoglutarate and biosynthetic precursors, possible. The *ppc* mutant grew without an intrinsic *anaplerotic* enzyme.

Another mutant which elucidated the need for steady primary supply of oxaloacetate through PEPc for biosynthesis, carried a lesion for fumarate hydratase. In contrast to the mutant-systems discussed above, the growth of a fumarate hydratase-negative mutant was only minimally impaired on glucose (Guest and Roberts, 1983). Like the citrate synthase-negative mutant, it possessed a functional PEPc enzyme/flux, but unlike the PEPc-negative mutants, it contained a degenerate TCA cycle. It is proposed that unsupplemented glucose medium supported the growth of the fumarate hydratase-negative mutant, because it was neither impaired in the synthesis of phospho*enol* pyruvate carboxylase, nor throughput to oxaloacetate. Since it could generate biosynthetic precursors and 2-oxoglutarate from oxaloacetate, its mutation was not critical to biosynthesis or growth on glucose.

By contrast, the citrate synthase-negative mutant sustained throughput to oxaloacetate, which it was incapable of transforming into other biosynthetic precursors of the TCA cycle (2-*oxo*glutarate and succinyl-CoA where applicable). It must therefore derive the essential precursors exogenously. In effect, its mutation was critical to biosynthesis. Although PEP was carboxylated in both systems, *"anaplerosis"* would be an inappropriate terminology to describe their PEPc fluxes, since a complete and functional TCA cycle did not need to be replenished, as it did not exist in the first instance.

In all the mutant-systems cited, a requirement for the primary generation of oxaloacetate through PEPc, or its supply and utilization were the major issues: PEPc-negative mutants were impaired in its production; citrate synthase-negative mutants were impaired in its utilization and both failed to grow on glucose. Fumarate hydratase negative mutants grew on glucose because they were competent, both in production and utilization of oxaloacetate, although they lacked complete TCA cycles. The metabolic lesions of these mutant systems, provided some insight into two major observations during glucose metabolism by *E. coli* ATCC15224-pJOE4. These were: (a)increased growth yield, without a corresponding increase in growth rate; (b) the possibility that PEPc is not an *anaplerotic* enzyme in this context. It is proposed that the primary effect of PEPc overexpression, was a selective increase of the biosynthetic precursors originating from the TCA cycle. This is supported by the increased output of threonine in the PEPc-overproducing strain of *S. marcescens* (Sugita and Komatsubara, 1989; see also figures 1.2; 1.3). In *E. coli* ATCC15224-pJOE4, increased throughputs to oxaloacetate and 2-oxoglutarate were apparently achieved at the expense of zero flux through pyruvate kinase (figure 4.16). Potentially, diminished flux through pyruvate kinase was sufficient to affect further increase in growth rate in the recombinant strain, by a net reduction of carbon flux through glycolysis (Sanwal, 1970; Garrido-Pertierra and Cooper, 1977; Goodridge, 1990). Carbon flux through pyruvate kinase was an early casualty of adaptive processes during starvation survival, when cells were forced by nutrient deprivation to grow very slowly (Thompson, 1987; Chesbro, 1988; Morita, 1988).

Since carbon flux through the TCA cycle is generally small (Holms, 1986a), any increase in flux through the cycle in *E. coli* ATCC15224 - pJOE4 was likely to be overwhelmed by zero flux through pyruvate kinase; a net increase in growth rate would therefore not be evident. Comparison of the effects of the metabolic lesions of the fumarase-negative strain, which operated flux through pyruvate kinase (Guest and Roberts, 1983), and a slow-growing pyruvate kinase-negative strain, appear to support this view (Garrido-Pertierra and Cooper, 1977). Studies conducted with *E. coli* AB1622 (*ppc*), suggested that the PEPc lesion was more severe on the anabolic branch of the amphibolic pathways than the catabolic, since accumulation of acetate was demonstrated in a *Warburg* manometer flask experiment during the utilization of pyruvate(Ashworth and Kornberg, 1966).

Results from the present study on the other hand, showed that amplified PEPc activity increased anabolism, reduced acetate accumulation without necessarily improving growth rate. Since acetate excretion suggested that precursors from glycolysis were already available in substantial amounts for biosynthesis (Holms and Mansi, 1989), it stands to reason that improvement of growth yield only required selective augmentation of oxaloacetate and 2-*oxo*glutarate (figure 1.2). The metabolic lesion of the citrate synthase mutant (Wilde and Guest, 1987), encouraged this speculation. In *E. coli* ATCC15224-pJOE4 amplification of PEPc already guaranteed increased throughput to oxaloacetate, other factors being equal. Evidence from reduced throughput to acetate excretion and increased growth yield, also suggested that throughput to 2-oxoglutarate must have increased from a higher ratio of condensation between oxaloacetate and acetyl-CoA.

From the foregoing, it is proposed, that the primary role of the flux of PEP through PEPc, is the initiation of the TCA cycle to generate biosynthetic precursors and regenerate oxaloacetate. Occurring concomitantly, is an output of considerable amounts of free energy (Morowitz, 1978), which is conserved as usable high-energy molecules (figure 4.22). It is further suggested, that a major explanation for overflow reactions from glycolysis to acetate excretion, and the limitation of growth yield associated with the bacterial *Crabtree* effect, is the diminished supply of biosynthetic precursors from the TCA cycle, below their predicted optimal ratios, *vis-a-vis* other biosynthetic precursors.

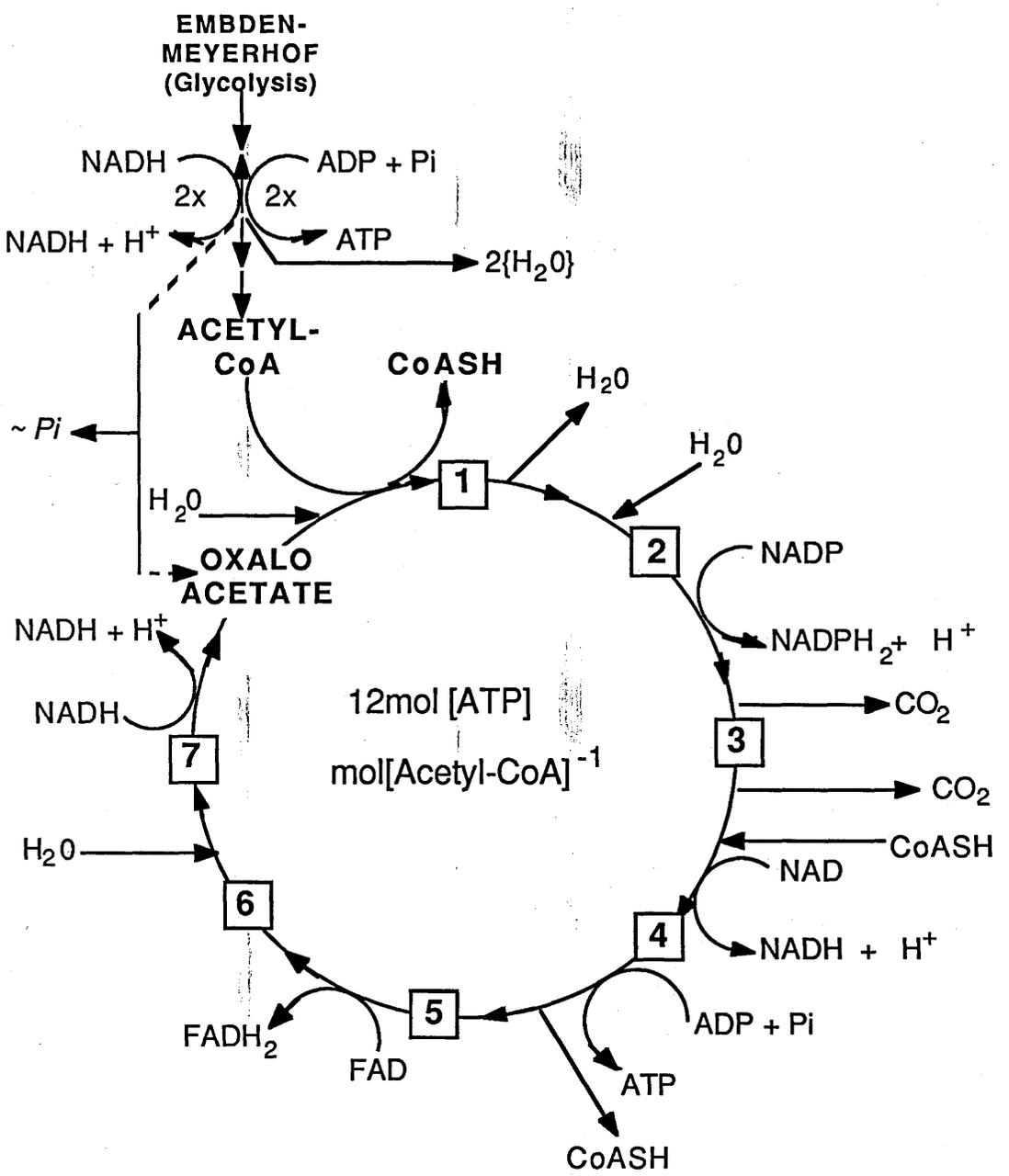


FIGURE 4.22
GENERATION OF ENERGY-MOLECULES FROM FREE ENERGY OF AEROBIC GLUCOSE METABOLISM BY *Escherichia coli*

Arrows are shown for forward reactions only as a chain of fluxes between intermediates

2x (Double sequence of the relevant reaction); Pi (Inorganic Phosphate;
1 (Citrate) 2 (*Iso* citrate) 3 (2–*oxo*glutarate) 4 (Succinyl-CoenzymeA)
5 (Succinate) 6 (Fumarate) 7 (L-malate) 8 (Oxaloacetate)

CHAPTER 5

COMPARATIVE STUDY OF THE METABOLISM OF FRUCTOSE GLYCEROL AND PYRUVATE BY PARENT AND PEPc-OVERPRODUCING STRAINS OF *Escherichia coli* ATCC15224

5.1 INTRODUCTION

5.1.1 FRUCTOSE

Fructose is an isomer of glucose with which it shares a number of common physico-chemical properties, including qualitative input of elements into central metabolism. Although initial reactions differ in both compounds, their metabolic routes converge at fructose-1,6-*bis* phosphate (Gottschalk, 1986). Fructose is rarely found in the free state *in vivo* (Jensen, 1990); usually, it enters the cell after phosphorylation into fructose-1-phosphate (Hollmann, 1964). Fructose-6-phosphate is however, a ubiquitous key intermediate of a vast number of metabolizable substrates (Wöhrl *et al.*, 1990).

Fructose is translocated via an inducible (Kornberg and Elvin, 1987), substrate-customized, PEP-driven phosphotransferase system (fru-PTS) (Postma and Lengeler, 1985) which is believed to antedate other PTS systems (Chin *et al.*, 1987). Like other PTS systems, the fru-PTS consists of non-specific cytoplasmic phosphoproteins (Lengeler *et al.*, 1982) as well as membrane-bound components which are exclusive to fructose transport (Bramley and Kornberg, 1987). In addition to the unique phosphotransferases, a specific *fructose-1–phosphate kinase* is also required in fructose metabolism (figure 5.1); (Kornberg and Elvin, 1987). This enzyme is distinct from the hexokinase equivalent for glucose utilization (Kornberg and Smith, 1970). Bacterial systems which lack the enzyme experience fructose intolerance because its specific substrate, fructose-1-phosphate which accumulates as a consequence, is cytotoxic (Ferenci and Kornberg, 1973).

The genes of the fructose PTS and fructose-1-phosphate kinase enzymes form a clustered *fru* operon (Geerse *et al.*, 1989a) which is regulated by a repressor-protein, **fru R** (Geerse *et al.*, 1989b).

Fructose is utilized sequentially after glucose in mixed substrate cultures (Clark and Holms, 1976). The repressor protein is thought to inhibit fructose uptake by mimicking the glucose-effect (Chin *et al.*, 1989).

Evaluation of the effects of PEPc amplification on aerobic metabolism of fructose was expected to elucidate the results obtained earlier on glucose (chapter 4).

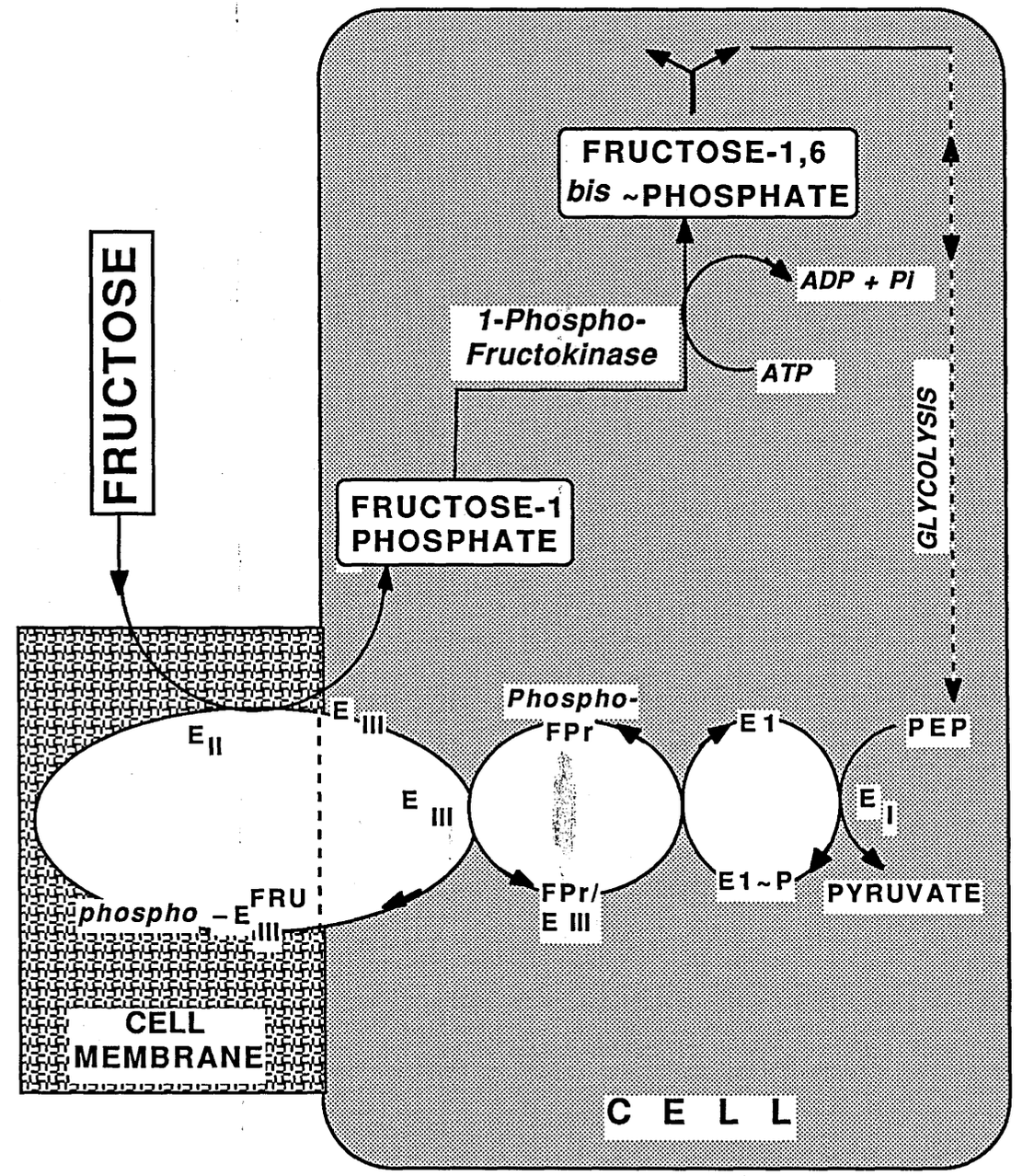


FIGURE 5.1

DIAGRAMMATIC REPRESENTATION OF THE PEP-DRIVEN FRUCTOSE-PHOSPHOTRANSFERASE UPTAKE SYSTEM

EI, II, III	The phosphotransferase group of enzymes
EI	Non-specific cytoplasmic component (a fructose-specific variant (EI^{FRU}), is described)
EII; EIII	Fructose-specific components (EII / III^{FRU})
FPr	Fructose-inducible carrier protein with both pseudo-HPr and EnzymeIII capabilities

5.1.2 GLYCEROL

The pathway of glycerol metabolism is highly substrate-inducible (Moat and Foster, 1988) and merges with the central pathways of *E. coli* at dihydroxyl acetone phosphate (DHAP) (Hollmann, 1964) after a two-step sequence involving a kinase and a dehydrogenase (figure 5.2). DHAP is then metabolized via glycolysis (Pettigrew *et al.,* 1988). This series of reactions contains convergent fluxes with lipid and carbohydrate metabolism (Dawes and Large, 1982).

The transportation of glycerol into the cell does not involve a phosphotransferase system. Hypertonic solutions of glycerol do not plasmolyze cells of *Escherichia coli* ; rather concentrations on both sides of the cell membrane soon become equilibrated, suggesting that glycerol accumulation is effected by facilitated diffusion (Dills *et al.*, 1980). The process uniquely employs a catalytic *facilitator protein.* The rapid rate of conversion of glycerol to *sn*-glycerol-3-phosphate (figure 5.2) ensures that the concentration gradient favours steady diffusion of glycerol into the cell (Sweet *et al.*, 1990).

The first reaction of glycerol metabolism is the most important in its regulation (Novotny *et al.*, 1985). The activity of *glycerol kinase* (and hence glycerol metabolism) is controlled through negative feedback inhibition by fructose-1,6-*bis* phosphate. In multiple-substrate cultures, the uptake and metabolism of glycerol, a non-PTS sugar, is inhibited by *enzymeIII* of the glucose-PTS system through *inducer exclusion* (Nelson and Postma, 1984). The $K_i[III^{GLC}]$ for glycerol kinase is about 50 μM *in vitro* (Saier, 1989).

Glycerol enters the central pathways at a junction which will allow all C_3, and C_4 biosynthetic precursors to be produced without resort to gluconeogenesis (Kornberg, 1966). However, synthesis of the C_6 intermediates from glycerol relies on the reversibility of most glycolytic fluxes (Gottschalk, 1986). Three of the ten steps of glycolysis are highly exergonic and therefore irreversible for most practical purposes in living systems (Cooper and Kornberg, 1967). Two of these are essential for glycerol-related gluconeogenesis. Mutants of *Escherichia coli* which cannot synthesize fructose-6-phosphate from FBP during growth on glycerol, carry a lesion for fructose-1,6 *bis*- phosphatase (Dawes and Large, 1982). Glycerol-grown cells also required flux through glucose-6-phosphatase and throughput to glucose by dephosphorylation of glucose-6-phosphate (Schlegel, 1988). Finally, complete metabolism of glycerol requires flux through the enzyme phospho*enol* pyruvate carboxylase (PEPc) which is considered *anaplerotic* for *E. coli* (Ashworth and Kornberg, 1966).

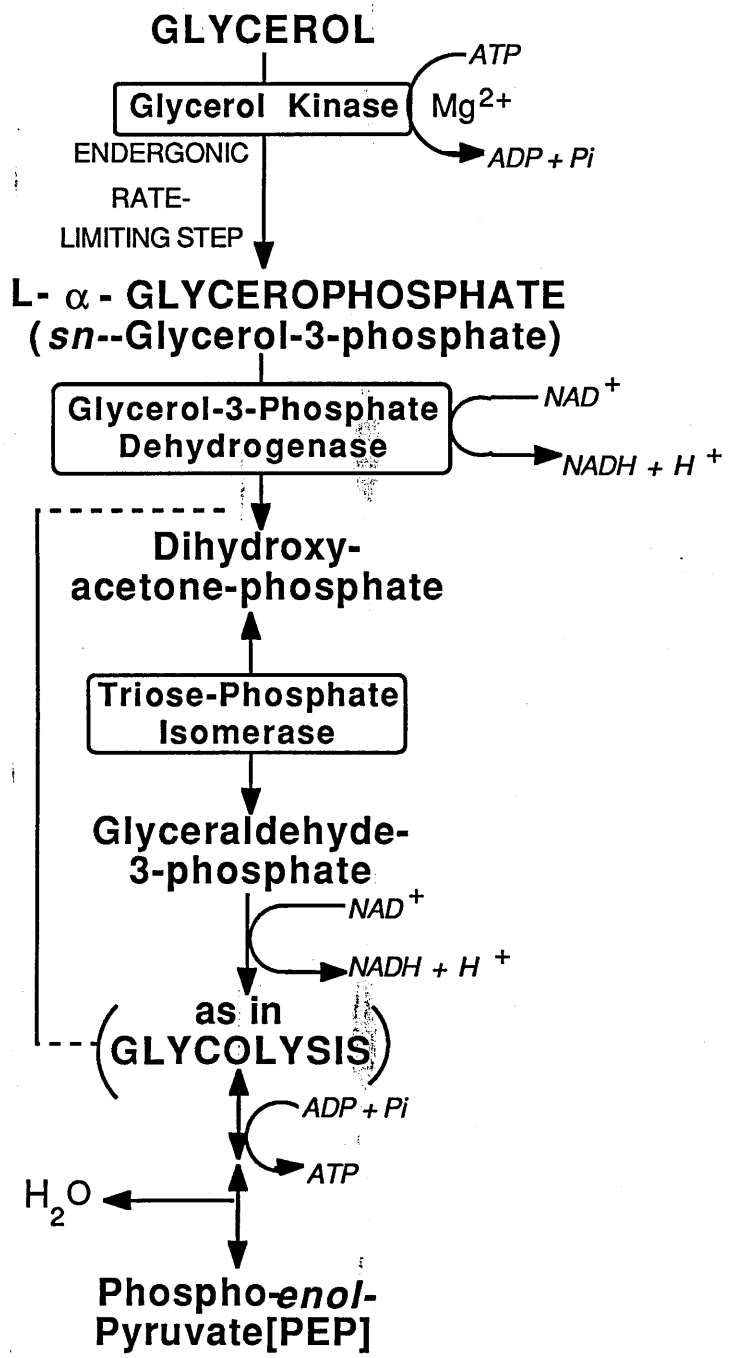


FIGURE 5.2

PIVOTAL REACTIONS FOR THE ENTRY OF GLYCEROL INTO THE GLYCOLYTIC PATHWAYS

Dotted lines show common reactions with glycolysis. Enzymes are boxed

5.1.3 PYRUVATE

Pyruvate is a ubiquitous central metabolite of carbohydrate and general metabolism, as well as one of the products of the PTS system (Roseman, 1990). A plethora of anabolic and catabolic intermediate routes diverge from here (Snœp *et al.*, 1990) depending on the intrinsic biochemistry of organisms. The outputs of these fluxes which include L-alanine, L-valine, and L-leucine, are often of diagnostic importance in determinative bacteriology (Moat and Foster, 1988). Pyruvate also produces other amino acids in association with oxaloacetate (figure 1.2).

5.1.3.1 METABOLISM OF PYRUVATE FROM EXOGENOUS SOURCE

The flux of carbon from exogenous pyruvate differs from the endogenously-generated intermediate. Like glycerol, C_6, C_3 and TCA cycle intermediates must be synthesized from exogenous pyruvate and furnished for biosynthesis (Dawes and Large, 1982). These demands result in :

(a) mandatory requirement for gluconeogenesis;
(b) mandatory operation of flux through the TCA cycle:
(c) obligatory synthesis of PEP to fulfil its central metabolic and distributive roles

(Cooper and Kornberg, 1967; Geerse *et al.*, 1989; Saier and Chin, 1990).

Gluconeogenesis is essentially a reversal of the glycolytic flux at pyruvate. Most of the mediating reactions are thankfully reversible (Gottschalk, 1986). Unlike glycerol however, the formation of phospho*enol* pyruvate occurs in the gluconeogenic direction of pyruvate metabolism. The reversal of the *pyruvate kinase* reaction is energetically unfavourable (Sanwal, 1970) and is therefore substituted by flux through *phosphoenol pyruvate synthase* (PEP synthase) (Cooper and Kornberg, 1965) to initiate the metabolism of pyruvate (Saier and Chin, 1990).

The fructose PTS appears to exert a negative control on gluconeogenesis during pyruvate metabolism (Chin *et al.*, 1987). This is partly because the repressor protein of the fructose regulon (*fru*R) (Kornberg and Elvin, 1987) is a mandatory requirement for activation of PEP synthase (Saier and Chin, 1990). *FruR* mutants of *Salmonella typhimurium* were also deficient in those gluconeogenic enzymes which complemented the three relatively irreversible steps of glycolysis (Chin *et al.*, 1987a). The repressor protein (fruR) was necessary for the transcription of *pps*, the structural gene for phospho*enol* pyruvate synthase (Geerse *et al.*, 1989).

RELATIVE FLUXES OF CARBON TO & FROM PEP AND PYRUVATE DURING AEROBIC METABOLISM OF GLUCOSE BY *Escherichia coli* ATCC 15224

Values represent distribution of carbon fluxes from each metabolite (*mmol. g.* dry bacterial cell biomass^{-1}.*hour*$^{-1}$) and are expressed as percentage of total fluxes of input- carbon to each metabolite, given that net flux of substrate-carbon to each metabolite for each gram of dry biomass produced from glucose-6-phosphate, was as follows :

Flux from glucose-6- phosphate	10 *mmol.* $g^{-1}.h^{-1}$
Flux to phospho*enol* pyruvate (PEP)	15.3 *m mol.* $g^{-1}.h^{-1}$
Flux to pyruvate	12.33 *mmol.* $g^{-1}.h^{-1}$

(Specific growth rate (μ) = 0.90 *hour*$^{-1}$)

FBPa	Fructose-1,6-*bis* phosphate aldolase
Pdh	Pyruvate dehydrogenase multienzyme complex
PEPc	Phospho*enol* pyruvate carboxylase
PEPs	Phospho*enol* pyruvate synthase
PTS	Phosphotransferase multienzyme system

(all values based on figure 4.15)

FIGURE 5. 3 (a)
PARTITION OF FLUXES AT PHOSPHO*ENOL* PYRUVATE.

Flux to PEP (15.3 *m mol.* $g^{-1}.h^{-1}$) = 100%

FIGURE 5.3 (b)
PARTITION OF FLUXES AT PYRUVATE

Flux to pyruvate (12.33 *m mol.* $g^{-1}.h^{-1}$) = 100%

FIGURE 5.3 (a)

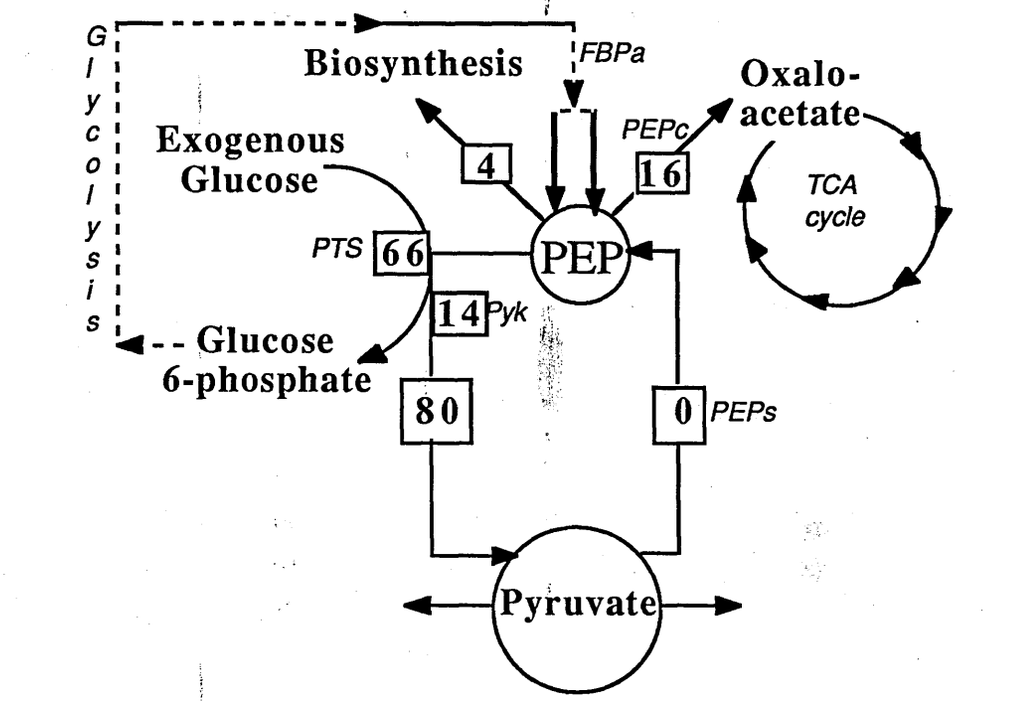
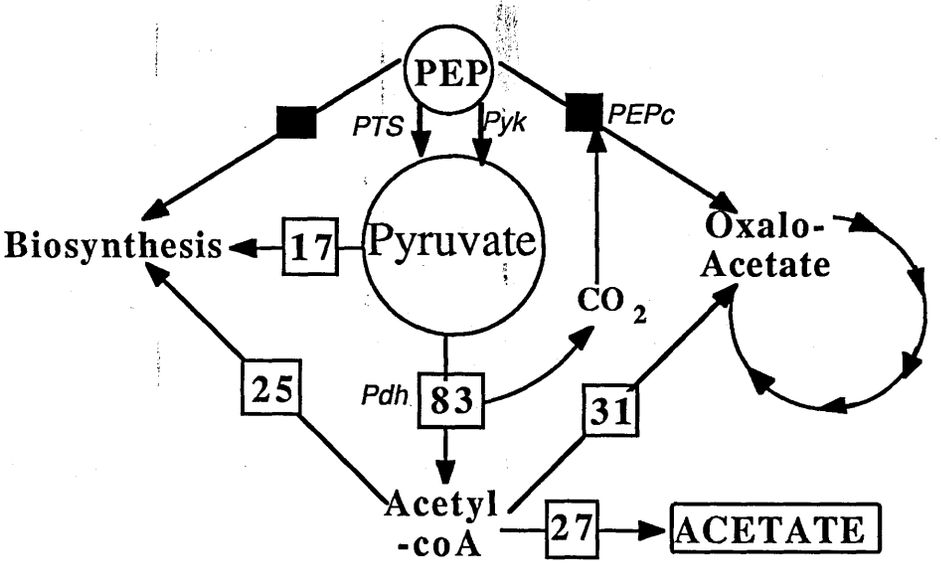


FIGURE 5.3 (b)



5.1.3.2 CHOICE OF PYRUVATE AS SUBSTRATE

Investigation of growth on pyruvate as sole carbon and energy source was considered in these studies after it was demonstrated that overproduction of phospho*enol* pyruvate carboxylase significantly reduced the throughput of glucose-carbon to acetate (section 4.4.3; figure 4.16). During aerobic glucose metabolism, nearly 80% of the partitioned carbon flux at PEP generates pyruvate (figure 5.3). This throughput is superfluous to biosynthesis (El-Mansi and Holms, 1989) and thus, makes pyruvate the single most important intermediate in acetate excretion (vanUrk *et al.*, 1989). A study of the metabolism of pyruvate as sole carbon and energy source was expected to elucidate the mechanisms governing diminished acetate excretion in *E. coli* ATCC15224-pJOE4.

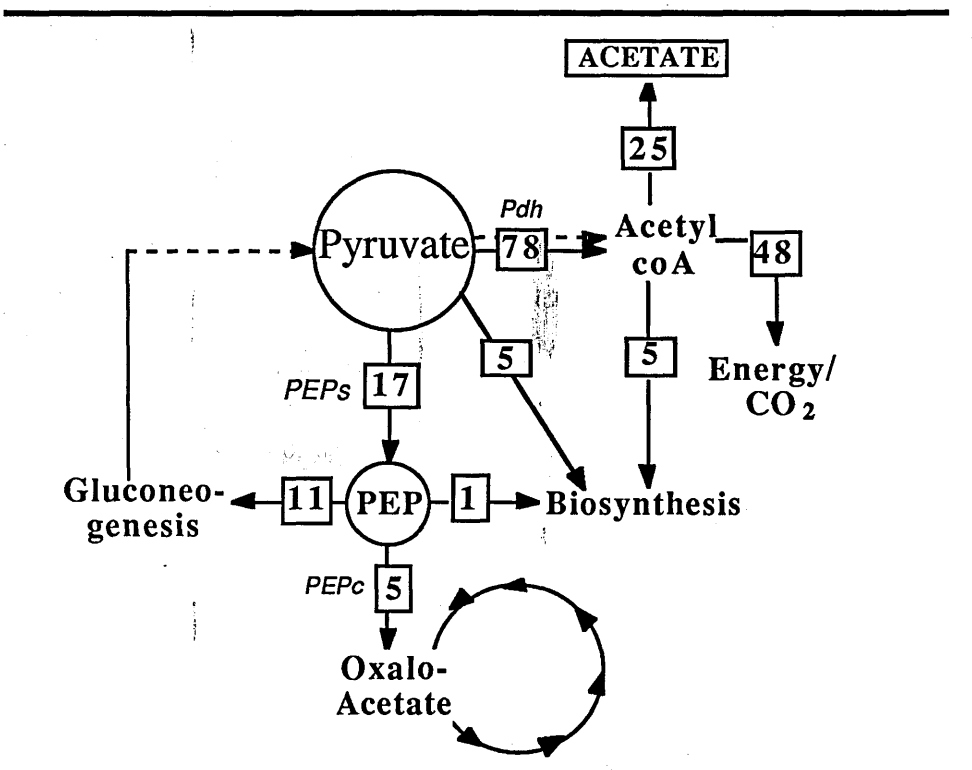


FIGURE 5.3 (c)

CARBON FLUXES THROUGH THE RELEVANT PATHWAYS OF AEROBIC PYRUVATE METABOLISM

Pathways include gluconeogenesis and flux through *phosphoenol pyruvate synthase* which is inoperative during glucose metabolism. Values were derived as described in figure 5.3 a & b

Dotted arrows represent post-gluconeogenic fluxes.Initial input flux = 35 $mmol.g^{-1}.h^{-1}$ (El-Mansi and Holms, 1989).

5.2 GROWTH AND GROWTH RATES ON FRUCTOSE, GLYCEROL AND PYRUVATE

E. coli ATCC15224 (parent) and *E. coli* ATCC15224-pJOE4 (recombinant) strains were first adapted to growth on D-fructose, glycerol or pyruvate, then grown as described previously (2.8.1). Results in the preceding chapter had demonstrated that the metabolism of *E. coli* ATCC15224-pBR322 did not differ significantly from the parent strain. The studies reported in this chapter were therefore carried out without a control culture for the plasmid effect. Concentration of substrates in the main batch cultures were 4mM-fructose, 5mM-glycerol and 7.5mM-pyruvate, respectively. Media for the recombinant strain were supplemented with 100$\mu g.$ ml.$^{-1}$ ampicillin. Cultures were monitored throughout the logarithmic and stationary phases for:

a) Growth/Cell density
b) Residual substrate/Substrate utilization (assayed according to Heyrovsky, 1956)
c) Total organic carbon (TOC)
d) Acetate and other organic acids

The mean generation times for parent and recombinant strains growing on fructose, were 54 and 57 minutes respectively. These represented specific growth rates of 0.77 and 0.72 h^{-1} respectively, or a 6.5% reduction in growth rate in the recombinant strain (figure 5.4). The growth rate for the parent was comparable with previous rates obtained in *E. coli* ATCC15224 (Mandelstam, 1962; Clark and Holms, 1976; Hunter, 1977; Holms, 1986a). Both strains grew at identical rates on glycerol. Mean generation time was 58 minutes in each test organism, representing a growth rate of 0.72 h^{-1} (figure 5.5). There was no major difference between these and the rates observed on fructose (figure 5.4). During pyruvate metabolism, the parent and recombinant strains doubled their biomass in the exponential growth phase every 74 and 71 minutes respectively (figure 5.6). These represented individual specific growth rates of 0.56 and 0.59 h^{-1} and showed that overproduction of PEPc enhanced growth rate on pyruvate by 6%.

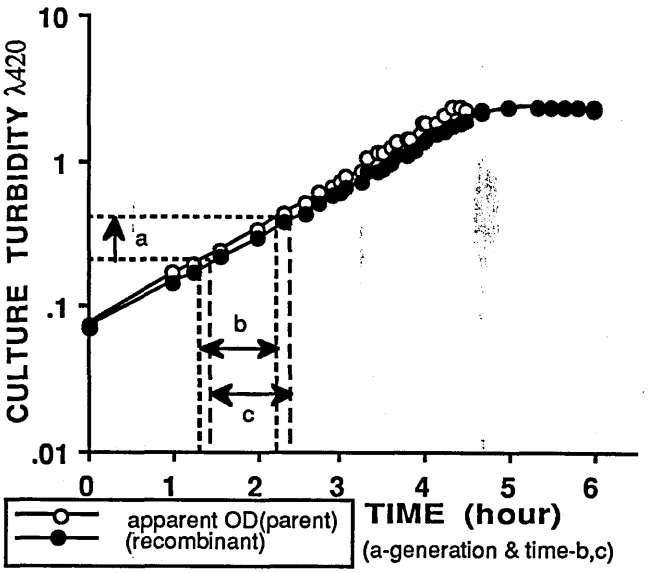


FIGURE 5.4
GROWTH CURVES DURING AEROBIC METABOLISM OF FRUCTOSE BY *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4

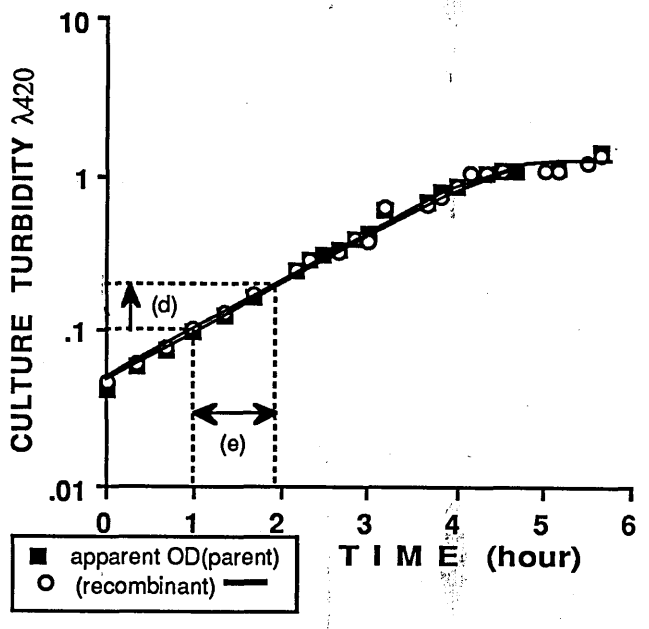


FIGURE 5.5
GROWTH CURVES OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4 DURING AEROBIC GLYCEROL METABOLISM

5.2.1 BIOMASS PRODUCTION AND GROWTH YIELDS

Table 5.1 summarizes biomass production and yields on fructose, glycerol and pyruvate in both test strains. Maximum biomass production occurred at the transition between the exponential and stationary phases of growth, which also coincided with complete exhaustion of each substrate. Molar growth yields ($Y_{\text{CARBON SOURCE}}$) varied within strains growing on different substrates, and between the parent and recombinant strains.

5.2.2. ANALYSIS OF ACETATE IN CULTURES

Analysis of samples from batch cultures of parent or recombinant strains growing on fructose or glycerol by HPLC, showed that no acetate was excreted. Other peaks represented products which were quantitatively insignificant. Both parent and recombinant strains excreted different amounts of acetate on pyruvate. In the parent culture, a maximum of 0.33 *mol |mol.* (pyruvate) was demonstrated. Overexpression of the *ppc* gene diminished this output by 30.3% to 0.23*mol. |mol* (pyruvate); (figure 5.7).

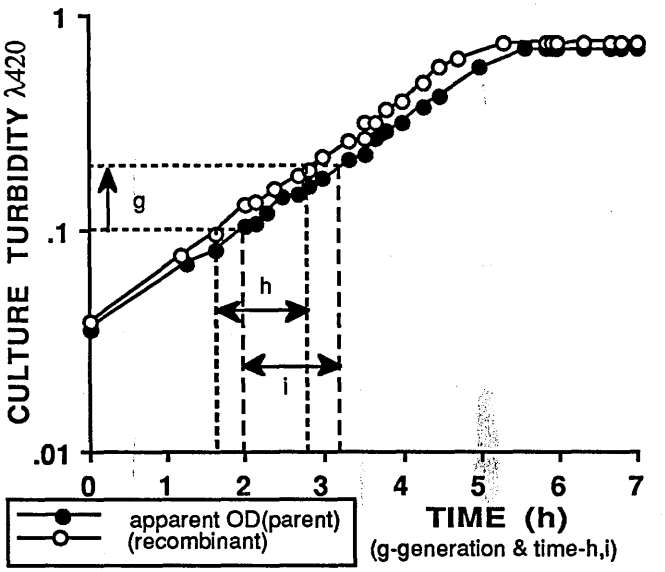


FIGURE 5.6
GROWTH CURVES OF *E. coli* ATCC15224 (PARENT AND STRAIN ATCC15224-pJOE4) DURING AEROBIC PYRUVATE METABOLISM

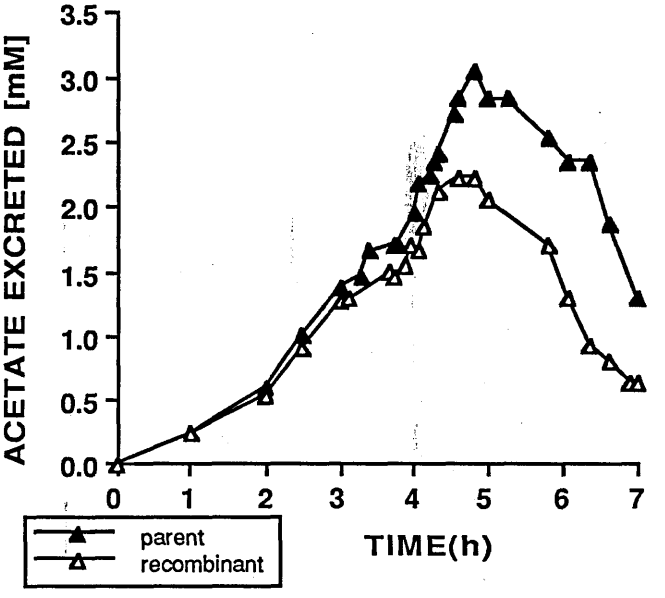


FIGURE 5.7
EXCRETION OF ACETATE BY PARENT AND RECOMBINANT STRAINS OF *E. coli* ATCC15224 DURING AEROBIC GROWTH ON PYRUVATE

5.3 Comparison of Growth, Biomass Yields and Acetate Excretion on Selected Substrates and Glucose

Fructose was utilized more slowly than glucose by 17 to 20% in both test strains. Growth yield on fructose was generally higher. The margins between $Y_{GLUCOSE}$ and $Y_{FRUCTOSE}$ in recombinant cultures were however reduced when compared to the same margin in the parent. Compared to glucose, the difference in molar growth yields between *E. coli* ATCC15224 and *E. coli* ATCC15224-pJOE growing aerobically on pyruvate was minimal and practically inconsequential on glycerol. The effect of PEPc overexpression was therefore most pronounced during glucose metabolism. Since both fructose and glycerol did not sustain fluxes to acetate excretion, only acetate excretion on pyruvate and glucose could be compared directly. Net output of acetate during the metabolism of glucose by *E. coli* ATCC15224 was 5.69 *mmol. g.* (dry weight)$^{-1}$ (figure 4.15), but 18.6 *mmol. g.* (dry weight)$^{-1}$ on pyruvate. Amplification of PEPc activity reduced the excretion of acetate on both substrates by 48 and 30% respectively (Table 5.1).

Carbon/ Energy Source	Growth Rate(μ) (h^{-1})	Carbon Input (*mmol.* C. *g.* dry weight.$^{-1}$)	Acetate Output (*mmol.* *g.* dry weight.$^{-1}$)	$Y_{(SUBSTRATE)}$ (*g.* dry weight *mol.* substrate^{-1})	PEPc Activity (*nmol. mg* protein^{-1}. *min* $^{-1}$)
		Escherichia coli ATCC15224 (*i*$^-$, *z*$^+$, *y*$^+$, *a*$^+$)			
GLUCOSE	**0.90**	**66.3**	**5.7**	**90.5**	**24.4**
FRUCTOSE	**0.77**	**56.2**	**0**	**106.8**	**nd**
GLYCEROL	**0.72**	**58.5**	**0**	**51.3**	**nd**
PYRUVATE	**0.56**	**168.7**	**32.5**	**17.8**	**nd**
		Escherichia coli ATCC15224-pJOE4 (*i*$^-$, *z*$^+$, *y*$^+$, *a*$^+$; pBR322-*ppc*$^+$)			
GLUCOSE	**0.87**	**52.44**	**2.3**	**114.4**	**1820**
FRUCTOSE	**0.72**	**51.37**	**0**	**116.8**	**nd**
GLYCEROL	**0.72**	**58.05**	**0**	**51.7**	**nd**
PYRUVATE	**0.59**	**157.8**	**21.6**	**19.0**	**nd**

TABLE 5.1

COMPARISON OF THE GROWTH CHARACTERISTICS OF PARENT AND PEPC-OVERPRODUCING STRAINS OF *E. COLI* ATCC15224 DURING THE AEROBIC METABOLISM OF SELECTED CARBON AND ENERGY SOURCES

nd - not determined

5.4 DISCUSSION

The effects of amplified phospho*enol* pyruvate carboxylase (PEPc) activity on aerobic metabolism of glucose, have been demonstrated in a PEPc-overproducing strain of *Escherichia coli* ATCC15224. Acetate excretion diminished concomitant with an increase in molar growth yield (chapter 4). Although glucose was the preferred substrate for most bacteria, its metabolism was not typical of other metabolizable carbon and energy sources. In the present study, the effects of PEPc overexpression on the metabolism of three other selected substrates were investigated.

5.4.1 EFFECT OF THE AMPLIFICATION OF PEPc ACTIVITY ON THE METABOLISM OF FRUCTOSE

During fructose metabolism, amplification of PEPc activity improved growth yield by 9% without improvement in growth rate. The results were also consistent with a zero-flux of carbon to acetate excretion as in the parent strain. The possibility of other excreted products could not be justified from the analysis of the TOC of culture samples.

The pathway of aerobic fructose metabolism involved its entry into the central pathways at fructose-1,6-*bis* phosphate (FBP) after translocation and concomitant phosphorylation into fructose-1-phosphate (figure 5.1). However, the biosynthetic demand for glucose-6-phosphate (figures 1.2; 1.3) necessitated throughput from FBP to fructose-6-phosphate (F-6-P). Since the flux through phosphofructokinase was unidirectional, this was achieved through fructose-6-phosphatase which is analogous to gluconeogenesis and believed to be a possible site for *a futile cycle* (Holms, 1986a). In support of the existence of a *futile cycle,* the efficiency of the central pathways during fructose metabolism (the output of CO_2 being an index of efficiency) was lower even when net throughput to biosynthesis was actually higher on fructose than glucose, and there was no flux to acetate excretion during aerobic fructose metabolism (Table 5.1). Initially, it could not be predicted that overexpression of PEPc activity would improve yield on fructose. Nevertheless, a net improvement of 9% in growth yield was recorded during aerobic utilization of fructose by *E. coli* ATCC15224-pJOE4. Since the parent strain did not sustain fluxes to acetate excretion (section 5.2.3), rationalization for the result must lie elsewhere.

In addition to the unique features of fructose uptake (Kornberg and Prior, 1989) and metabolism (Wöhrl *et al.,* 1990), the likelihood of a *futile* cycle following uptake by the fructose-PTS, offers a potential point for adjusting fluxes to accommodate the increased demand by overexpressed PEPc activity. One major effect of the *futile* cycle on fructose was a reduced carbon

conversion coefficient (Holms, 1986a). Factors which diminished carbon flux around the cycle could potentially enhance substrate utilization efficiency.

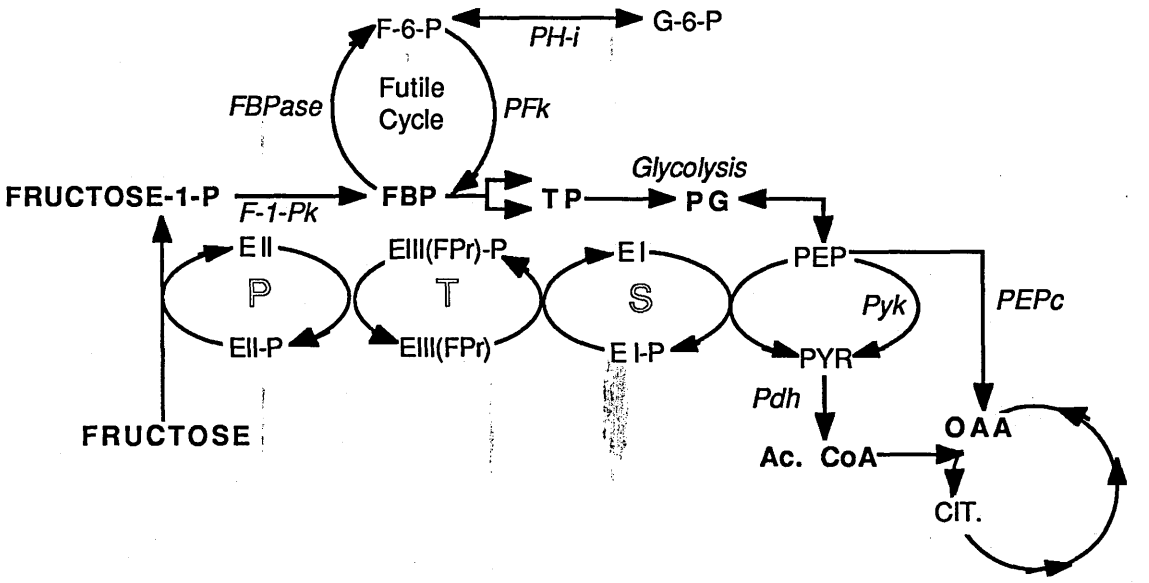


FIGURE 5.8 AEROBIC BREAKDOWN OF FRUCTOSE AND THE OPERATION OF A POSSIBLE FUTILE CYCLE

FBPase Fructose-1,6-bisphosphatase *PFk Phosphofructokinase*
F-1-Pk Fructose-1-phosphate kinase *PH-i Phosphohexose isomerase*

Opportunities for such a favourable scenario are not far-fetched in the recombinant strain. Changes in the *energy charge* of the cell had contrasting effects on both halves of the *futile* cycle. The arm operating in the direction of glycolysis was regulated positively by AMP but negatively by PEP (Dawes and Sutherland, 1976). Conversely, the half operating parallel to gluconeogenesis was inhibited by AMP (Roger *et al.*, 1988), but probably enhanced by the consumption of PEP by the fructose-PTS. When the activity of PEPc was amplified, increased flux of PEP to oxaloacetate was effected at the expense of diminished flux through pyruvate kinase (Thompson, 1987) — the K_m of PEP for *enzyme1* is about 300µM (Saier *et al.*, 1980), which is significantly lower than K_m of 1mM-PEP for maximally-activated PEPc (Sanwal, 1970); hence, it was not likely that amplification of PEPc activity would significantly reduce the initial flux of fructose. A lower flux through pyruvate kinase increased the AMP:ATP ratio and favoured the activities of

phosphofructokinase, PEPc and citrate synthase, which evacuated the output of the PEPc flux (Dawes and Large, 1982). On the other hand, a low energy charge of the cell reduced flux around the futile cycle quantitatively; indeed, there is a strong possibility of abolition of the cycle by AMP, altogether (Rogers *et al.*, 1988) such that only the essential *gluconeogenic* flux is sustained. This would be consistent with the first flux-model proposed for the cycle by Holms (1986a).

It is therefore proposed that the improved growth yield observed during aerobic metabolism of fructose by the PEPc-overproducing strain of *E. coli* ATCC15224, resulted from the reallocation of carbon at PEP and the attendant effects which contributed to diminished flux around the futile cycle. It is suggested further, that a more stringent control which diminished the futile cycle, may not necessarily guarantee a higher growth rate, as was previously demonstrated in this study (table 4.5).

5.4.1.1 Potential Effects of the Repressor Protein "FruR"

Apart from the known stringency imposed on fructose metabolism by the fructose-PTS, its role in the PEPc-overproducing strain could not be immediately ascertained. FruR is specified by the *fruR* gene and negatively regulates the transcription of the *fru* operon (Geerse *et al.*, 1989). Its main effect on fructose metabolism is more pronounced in cultures containing multiple substrates. The mechanism of its action may involve sensitivity to intracellular concentration of PEP and the energy charge of the cell (Postma *et al.*, 1989). In a single-substrate culture of fructose, it is repressed in favour of the transcription of enzymes of the *fru* operon (Kornberg and Elvin, 1987).

It has however been demonstrated that *fruR* mutation had contrasting effects on glycolytic and gluconeogenic fluxes in *Salmonella typhimurium* (Chin *et al.*, 1989) and possibly, *Escherichia coli* (Table 5.2). A functional fruR protein may therefore exert a pleiotropic effect in general metabolism and a dynamic balance between fluxes of FBP to glycolysis and gluconeogenesis during fructose metabolism (Saier and Chin, 1990). It is most likely that overexpression of PEPc activity reduced net throughput of carbon to pyruvate kinase in *E. coli* ATCC15224-pJOE4. It is interesting that a lesion in the *fruR* gene reduced the activities of pyruvate kinase and fructose-1,6-*bis*-phosphatase by equal amounts (Table 5.2). It is therefore apparent that both enzymes showed similar responses to the same effector. Equally interesting, was the observation that phosphofructokinase activity was elevated in the *fruR* mutant (Chin *et al.*, 1989). It is proposed that the activity of the fruR repressor protein coupled with enhanced PEPc activity, favoured the synthesis of glucose-6-phosphate without a futile cycle in the recombinant strain.

ENZYME IN METABOLISM	METABOLIC FLUX SERVICED	ACTIVITY* (RELATIVE TO WILD-TYPE)
PHOSPHO*ENOL*-PYRUVATE SYNTHASE	GLUCONEO-GENESIS	0.06
PHOSPHO*ENOL*-PYRUVATE CARBOXYKINASE	GLUCONEO-GENESIS	0.27
FRUCTOSE-1,6-*BIS*-PHOSPHATASE	GLUCONEO-GENESIS	0.52
ENZYME I	PEP : PTS (GLYCOLYSIS)	2.5
PHOSPHO-FRUCTOKINASE	E. M. P. (GLYCOLYSIS)	2.7
PYRUVATE KINASE	GLYCOLYSIS	0.51
ICDH	TCA CYCLE	0.28

* Values are relative to the activities found in the Parent strains with intact and functional *fruR* gene (based on *Saier and Chin,* 1990)

TABLE 5.2:

RELATIONSHIP BETWEEN *FRUR* MUTATION AND THE ACTIVITIES OF GLYCOLYTIC AND GLUCONEOGENIC ENZYMES

PEP:PTS Phospho*enol* pyruvate:Sugar phosphotransferase System
E. M. P. Embden-Meyerhof-Parnas Pathway
ICDH Isocitrate Dehydrogenase

5.4.2 EFFECT OF THE AMPLIFICATION OF PEPc ACTIVITY ON THE METABOLISM OF GLYCEROL

E. coli ATCC15224 possesses a glycerol-facilitator protein which catalyzes the transport of exogenous glycerol into the cell (Sweet *et al.,* 1990). No PEP is expended in the process yet, the half-time for the process is under 2.5 seconds per M-glycerol) (Sanno *et al.*, 1968). In order to meet requirements for C_6 biosynthetic precursors, fluxes through gluconeogenesis are mandatory. However, the output of PEP from glycerol is glycolytic as in fructose, but not in the gluconeogenic direction as in pyruvate.

During aerobic growth on glycerol, both parent and PEPc-overproducing strains of *E. coli* ATCC15224 did not excrete acetate. Their growth rates were also identical, while the difference in their growth yields was minimal. The results suggested that overexpression of PEPc activity did not significantly alter the metabolism of glycerol.

The aerobic metabolism of glycerol like fructose, is stringently controlled at very early stages. The first catabolic flux is the rate-limiting step (Lin, 1976). The step is catalyzed by the tetrameric enzyme, glycerol kinase (*Mr,* 220,000), which retains catalytic activity in the dimeric state. The K_m of glycerol kinase for glycerol is 7μM (Saier, 1989). The tetramer is inhibited allosterically by fructose-1,6-*bis* -phosphate[K_i=500μM] (Postma *et al.,* 1984). Neither 1mM, PEP, 0.3U ml^{-1} Enzymel /HPr nor phosphorylated EnzymeIII^{GLUCOSE}, affected glycerol kinase activity (Novotny *et al.,* 1985). At a concentration of 50μM however, nonphosphorylated EnzymeIII^{GLUCOSE} formed a complex with the cytoplasmic kinase (Postma *et al.,* 1989), preventing further accumulation and metabolism of glycerol as well as expression of the enzymes of the glycerol (*glp*) regulon (Postma *et al.,* 1984). The effects of the undissociated complex were not reversed by increasing the concentration of glycerol kinase a thousand-fold (Novotny *et al.,* 1985), but only by a combination of PEP, Enzymel and HPr (Postma *et al.,* 1984). The high stringency in the mechanism of glycerol metabolism may account in part, for non-excretion of acetate in *E. coli* ATCC15224. Since the effect of PEP on the inhibitory mechanism was minimal, it is conceivable that the effects of PEPc overproduction on the concentration of PEP would not influence glycerol metabolism. Results obtained in the present study are consistent with this speculation.

Additional controls might also exist at the triose phosphates where fluxes are partitioned between glycolysis and gluconeogenesis (figure 5.2). The region also unifies fluxes of glucose, fructose, and fat metabolism with glycerol (Dawes and Large, 1982). Since no acetate was excreted during glycerol metabolism by *E. coli* ATCC15224, it can be assumed that fluxes to pyruvate were not superfluous, while the gluconeogenic throughput to fructose-1,6-*bis* phosphate was likely to be highly regulated. Hence, the activity and quantitative expression of PEPc *in vivo*, during the aerobic metabolism of glycerol by *E. coli* ATCC15224-pJOE4 was probably at the normal physiological level. Results obtained suggest that the principal function of flux through PEPc was the initiation of the TCA cycle to generate essential precursors for biosynthesis. This function was sufficiently vital to impair growth on glycerol by PEPc-negative mutants such as *E. coli* PA342 (chapter 3) and *E. coli* AB1622 (Ashworth and Kornberg, 1966).

5.4.3 EFFECT OF THE AMPLIFICATION OF PEPc ACTIVITY ON THE METABOLISM OF PYRUVATE

Escherichia coli utilizes different pathways in the metabolism of pyruvate, depending on whether it is an intermediate metabolite of other carbon sources or a sole carbon and energy source itself. The results presented have been obtained from the growth of test organisms on pyruvate in single-substrate aerobic cultures. An amplified PEPc activity in *E. coli* ATCC15224-pJOE4 resulted in 30% diminished flux to acetate excretion and 7% improvement in growth yield *vis-a-vis* the parent (Table 5.1). The metabolism of pyruvate involves carbon fluxes through glycolysis, gluconeogenesis, and the TCA cycle (figure 5.9). Acetate is an output of the partition of flux from pyruvate to acetyl-CoA. Although pyruvate dehydrogenase (pdh) activity is believed to diminish in conditions akin to gluconeogenesis (Patel and Roche, 1990), the substantial output of acetate from pyruvate indicates a non-stringent control.

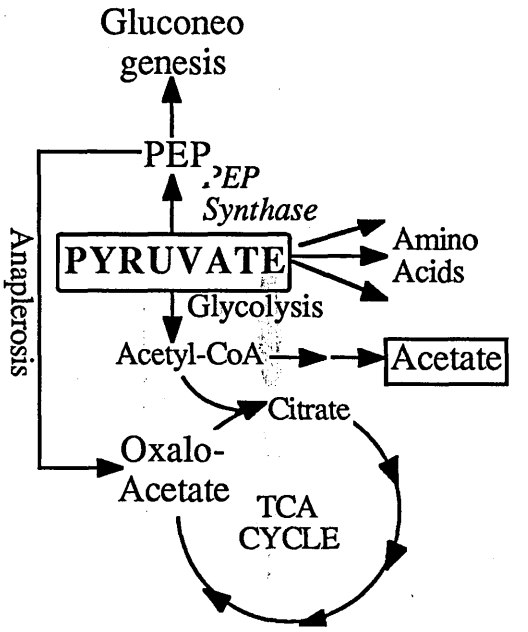


FIGURE 5.9

PRINCIPAL ROUTES OF AEROBIC METABOLISM OF PYRUVATE AS SOLE CARBON AND ENERGY SOURCE

In *E. coli* ATCC15224, it was estimated that 80% of carbon flux from pyruvate resulted in the output of acetyl-CoA (Holms, 1986a). Some of this became direct components of new cell biomass (figure 5.3c), but nearly 75% was partitioned between acetate excretion and oxidation in the TCA cycle. This allocation was clearly superfluous, resulting in 40% flux to acetate excretion from acetyl-CoA.

5.4.3.1 The Role of Phospho*enol* pyruvate Carboxylase

The substrate for phospho*enol* pyruvate carboxylase (PEPc) must first be synthesized from pyruvate. Carbon flux through phospho*enol* pyruvate synthase (PEP synthase) was estimated at 16% of the total fluxes from pyruvate and represented a major difference between gluconeogenesis on pyruvate and glycerol. It is also an important regulatory step during growth on pyruvate (Saier and Chin, 1990). Mutants in the *pps* gene could not utilize pyruvate as sole carbon source (Brice and Kornberg, 1967). The repressor protein of the fructose operon, fruR, is a strong activator of PEP synthase (Chin *et al.*, 1987) and is an absolute requirement for the transcription of the *pps* gene (Geerse *et al.*, 1989). It may also exert a pleiotropic effect on the metabolism of pyruvate (Chin *et al.*, 1989).

The major role of the flux through PEP synthase is the reversal of the pyruvate kinase flux. One *mole* of hexose is formed from 2 *moles* of pyruvate at the expense of considerable amount of metabolic energy (Moat and Foster, 1988; Saier and Chin, 1990).

$$2\underline{CH_3\ CO\ COOH} + 4ATP + 2GTP + 2NADH + 2H^+ + 4H_2O$$

$$\downarrow$$

$$C_6H_{12}O_6 + 4ADP + 2GDP + 2NAD + 6Pi +$$

Energetics of Gluconeogenesis from Pyruvate

The acetyl-CoA–activated PEPc flux depended on quantitative throughput to acetyl-CoA and consumed 1 *mole* ATP. The K_m[acetyl-CoA] for activating PEPc was estimated at 140μM (Ashworth and Kornberg, 1966). Given that 40 to 50% of flux from acetyl-CoA was biosynthetic and excretory, there is a net balance of at least 24*mmol* g (dry weight)$^{-1}$ which can activate

PEPc and condense with the carboxylated output of its flux. The carbon dioxide (CO_2) given off during the conversion of pyruvate to acetyl-CoA, further enhances the carboxylation of PEP. This is more favourable to maximal activity of PEPc in the recombinant strain than during glycerol metabolism where stringent regulation may limit the output of acetyl-CoA. The vital role of PEPc in pyruvate metabolism was demonstrated in strains with lesion in the enzyme which could not utilize pyruvate (Kornberg, 1966).

PEP fed into fluxes catalyzed immediately by enolase (gluconeogenesis) PEPc (TCA cycle initiation) and phospho-2-keto-3-deoxyheptonate aldolase (biosynthesis of aromatic amino acids) (figure 5.3). The FBP synthesized during gluconeogenesis potentially favoured the activation of PEPc in synergism with acetyl-CoA to form more oxaloacetate. This will positively affect the quantitative oxidation of acetyl-CoA in the TCA cycle (Krebs, 1981). The flux which yields acetyl-CoA is sensitive to the energy balance of the cell and the status of the indicator-molecules (ATP/ADP; NAD^+/NADH; acetyl-CoA/CoA and phosphorylation/ dephosphorylation ratios); (Perham *et al.*, 1987; Patel and Roche, 1990). The activity of pyruvate dehydrogenase is therefore sensitive to the activity and outputs of the TCA cycle (Snœp *et al.*, 1990). The possibility of reduced CO_2 output due to inhibition of pyruvate dehydrogenase, could be circumvented by its increased output in the TCA cycle of the PEPc-overproducing strain (figure 4.22). In any case the output and availability of CO_2 was not likely to limit the carboxylation of PEP through PEPc.

It is proposed that the pattern of fluxes during aerobic pyruvate metabolism contain elements which can respond positively to the overexpression of PEPc and enhance the net efficiency of aerobic pyruvate utilization. The PEPc-overproducing strain excreted less acetate, grew faster and produced more biomass on pyruvate. The results indicate increased flux of PEP to oxaloacetate, or an increased evacuation of the output of the first reaction of pyruvate metabolism. This presumably affects the competing flux to acetyl-CoA and acetate excretion, and increases the ratio of PEP synthase: pyruvate dehydrogenase fluxes (Mansi and Holms, 1989). The role of repressor protein fruR is likely to enhance this ratio even further (Saier and Chin, 1990). The cumulative effect of the factors is likely to enhance the efficiency of aerobic metabolism of pyruvate, and is consistent with the findings of the present studies.

5.4.4 EVALUATION OF THE EFFECTS OF ENHANCED PEPc ACTIVITIES ON AEROBIC UTILIZATION OF GLYCEROL AND PYRUVATE

The present study showed that both growth rate and yield of *Escherichia coli* were higher on glycerol which is more reduced, than on pyruvate (Table 5.1). This is consistent with previous observations (Andresen and vonMeyenburg, 1980; Holms, 1986a). The higher growth rate was in spite of the stringent control of glycerol dissimilation (Postma *et al.,* 1984; Lin, 1987), even to the point of zero fluxes to acetate excretion. Glycerol and pyruvate are 3-carbon organic substrates. Only pyruvate sustained carbon fluxes to acetate excretion, but at a lower growth rate. In the *E. coli* ATCC15224-pJOE4 both growth rate and acetate excretion were improved on pyruvate. Even when allowance is given for some interconversion of acetate to acetyl-CoA when the flux through pyruvate dehydrogenase was inhibited in favour of gluconeogenesis (Brown *et al.*, 1977), the output of acetate remained high on pyruvate (figure 5.7). It is proposed that optimal activity of amplified phospho*enol* pyruvate carboxylase (PEPc) was limited by flux through PEP synthase during pyruvate metabolism. To test this hypothesis, an attempt was made to amplify the activities PEPc and PEP synthase concurrently. This required the cloning of the *pps* gene of *Escherichia coli.*

5.4.5. ATTEMPTS TO CLONE THE *pps* GENE

The gene bank (*see* section 3.1.2.) was screened for hybrid plasmids which were complementary to a PEP synthase lesion in *Escherichia coli* K2.It (*pps*); (table 5.3). Competent cells of *E. coli* K2.It (transformation efficiency =1.06- 2.60 x 10^6 *cft. µg.* pBR322^{-1}) were transformed with 1*µl.* of the gene bank preparation. Five different sets of transformations yielded 12,220cft *µl* (bank DNA prep.)$^{-1}$. Ampicillin-resistant transformants were screened for presumptive positive colonies on pyruvate plates and the amino acid requirements of the parent. Selection on lactate was not determinative, since both *E. coli* K2.1t and *E. coli* K2.1t-pBR322 grew on lactate plates within 48 hours (table 5.3). Four presumptive *pps*$^+$ isolates resulted from each batch of transformants. Second generation transformants of plasmid preparations from each isolate grew rather poorly after replication on pyruvate plates. Only four isolates merited further screening by restriction analysis of their plasmids (figure 5.10). Single digests with *Eco*RI and *Pvu*II were examined by agarose gel electrophoresis and compared with predicted patterns (Kohara *et al.,*

1987; Geerse *et al.,* 1989). A hybrid pBR322-*pps* plasmid was expected to possess two restriction sites for *Pvu*II and *Eco*RI, respectively. Each of the plasmids was at least 9.0kb in size (figure 5.10).

Although no plasmids produced transformants which grew significantly on replica-plates of pyruvate, electrophoresis of samples digested with *Pvu*II and *Eco*RI produced more promising results. Hybrid plasmids pJOE5, pJOE6 and pJOE 7 contained at least two restriction sites for *Eco*RI. Both plasmids pJOE7 and pJOE8 contained at least two restriction sites for *Pvu*II. On the basis of restriction analysis, each hybrid plasmid had some potentials for carrying the *pps* gene. However, none conferred significant ability to utilize pyruvate on *E. coli* K2.It. Explanations for the situation are only speculative at this stage. The leucine auxotrophy of *E. coli* K2.It may involve mutation at the 2 minute locus on the linkage map of *Escherichia coli* for the *leu A, B, C* and *D* genes (Bachmann, 1983; 1987). This is of interest to the present investigation, since Geerse *et al.* (1986) observed previously that the *fruR* gene was located between *leu* and *pbpB* (*penicillin-binding protein gene*) on the genome of *E. coli* and was cotransducible with the *leu* operon (Kornberg and Elvin, 1987). Thus a *leu* auxotrophy may also indicate *fruR* genotype.

5.4.5.1 Relationship between Leucine Auxotrophy, *FruR* and the Transcription of the *pps* Gene

The *pps* gene which mapped at 37 minutes on *E. coli* genome (Brice and Kornberg, 1967) required **fruR** (the repressor protein of the *fru* operon)for transcription (Geerse *et al.*, 1989a). A PEP synthase-negative mutant of *S. typhimurium* (strain PP1928; *pps*) expressed full PEP synthase activity after complementation with a hybrid plasmid containing the *pps* gene. However, PEP synthase activity in *S. typhimurium* (strain PP1927; *pps, **leu...***) which was similarly complemented, was barely detectable (Geerse *et al.*, 1989b). The pertinent mutational markers of the second strain were similar to *E. coli* K2.It (***pps, leu,*** *his, arg, thr*), and may provide *a priori* explanation for the failure to clone and/or express the *pps* gene using strain K2.1t, since complementation from the gene bank did not relieve leucine auxotrophy in *E. coli* K2.It-pJOE5, *E. coli* K2.It-pJOE6, *E. coli* K2.It-pJOE7 or *E. coli* K2.It-pJOE8.

In view of the reported role of fruR in the transcription of *pps*, a different PEP synthase-negative host whose marker-lesions do not interfere with the expression of the *pps* gene, would be more suitable for future cloning .

COMPONENT OF DEFINED MEDIA[♦]	STATUS[§] OF COMPONENT	*Escherichia coli* Strains		
		K2.1t	K10	ATCC15224
GLUCOSE	(+)	++	+++	+++
PYRUVATE	(+)	–	++	+++
LACTATE	(+)	±	++	+++
GLYCEROL	(+)	++	+++	+++
SUCCINATE	(+)	–	++	+++
THIAMINE	(–)	±	±	++
HISTIDINE	(–)	–	+	++
ARGININE	(–)	–	+	++
LEUCINE	(–)	–	+	++
THREONINE	(–)	±	+	++
AMPICILLIN	(+)	–	–	–

TABLE 5.3

COMPARISON OF NUTRITIONAL PHENOTYPES OF *Escherichia coli* STRAINS K2.1T, K10 AND ATCC15224

[♦] Plates were incubated for a total of 72 hours. Strains K10 and ATCC15224 were positive controls. Media contained glucose as carbon source except where indicated otherwise.

§ (+) Complete medium with sole carbon/Energy sources indicated (except ampicillin)

§ (–) Glucose medium lacking in the component indicated and ampicillin

\- No growth (or specific requirement for deficient nutrient)

± Weak growth, poor complementation

\+ } Different

++ } Degrees of

+++ } Positive Growth

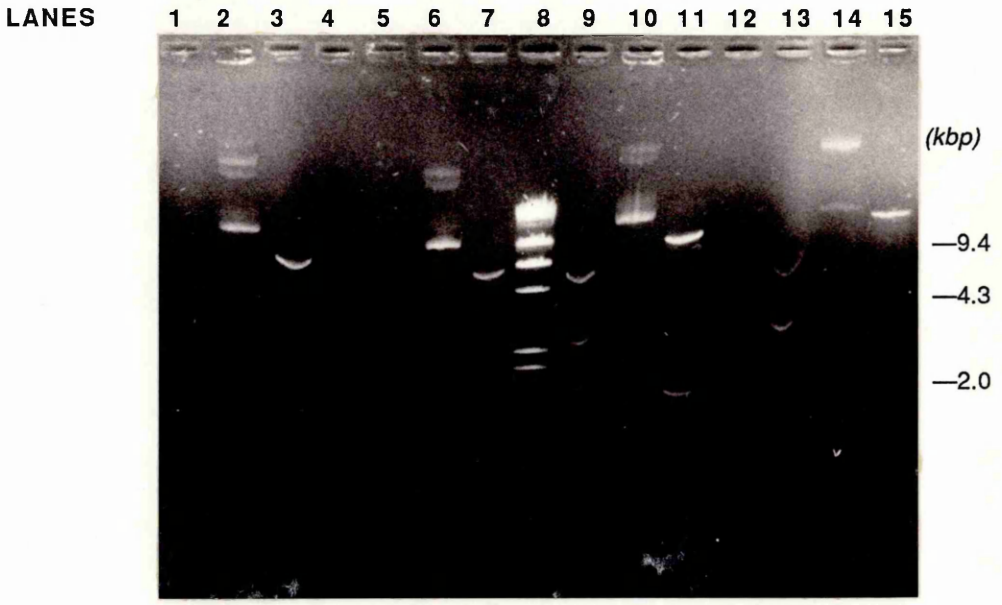


FIGURE 5.10

ELECTROPHORETIC PATTERNS OF *PvuII* AND *EcoRI* DIGESTS OF HYBRID PLASMIDS FROM TRANSFORMANTS OF *E. coli* K2.IT GROWING ON PYRUVATE PLATES

Samples were loaded on the lanes indicated in the following order :–

		*Pvu*II digests, untreated plasmids *Eco*RI digests	
pJOE5	(lanes 1-3)	pJOE6	(lanes 5-7)
pJOE7	(lanes 9-11)	pJOE8	(lanes 13-15)

The molecular weight marker (Bacteriophage λ digested with *Hin*dIII) ran on lane 8, between samples of Plasmids pJOE6 and pJOE7.

CHAPTER 6

EFFECT OF THE OVERPRODUCTION OF PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ON THE METABOLISM OF SELECTED NON-PTS, NON-GLYCOLYTIC SUGARS BY *Escherichia coli* ATCC15224

6.1. INTRODUCTION

Gluconate and glucuronate are C_1 and C_6 oxidation-derivatives of glucose respectively (Eisenberg and Gurin, 1952; Douglas and King, 1953). The latter is also an output of inositol metabolism and a precursor of L-ascorbic acid synthesis (Burns and Conney, 1966). Both are metabolized through the *Entner-Doudoroff* (ED) *Pathway* whose preliminary reaction steps are distinct from glycolysis (Entner and Doudoroff, 1952). Reactions of the ED pathway are partitioned at the 6-carbon intermediate, *2-keto-3-deoxy-6-phosphogluconate* (2-KDPG) (deZwaig *et al.*, 1973) which also forms the point of convergence of the metabolism of gluconate and glucuronate. Partition of flux at 2-KDPG results in two branches, both of which yield pyruvate eventually (figure 6.1). Thus, the metabolic fluxes during the utilization of C_6 compounds by the ED pathway generate 2 *moles* of pyruvate, one of which results from further metabolism of 1 *mole* of G-3-P which was produced along with the first pyruvate from the partition of flux at 2-KDPG (figure 6. 1).

Most members of the family *Enterobacteriaceae* possess inducible enzymes which enable them to use gluconate and glucuronate as sole carbon and energy sources by this pathway (Schlegel, 1988). Metabolism of substrates by the ED pathway yields less metabolic energy than by glycolysis (Dawes, 1986) but the various mechanisms for transporting D-gluconate and D-glucuronate by bacteria do not expend any of the energy output, or PEP (Booth and Morris, 1975; Gottschalk, 1986); hence, carbon flux is partitioned at PEP to aromatic amino acid synthesis, oxaloacetate and more pyruvate through pyruvate kinase. Pyruvate is oxidized to acetyl-CoA which may be incorporated into biosynthetic routes, oxidized in the TCA cycle or excreted as acetate.

This study was undertaken to determine the effect of amplification of PEPc activity on the metabolism of substrates which require fluxes through the Entner-Doudoroff pathway by growing *E. coli* ATCC15224 and *E. coli* ATCC15224-pJOE4 in aerobic batch cultures containing gluconate or glucuronate as sole carbon and energy sources. It was also envisaged that the findings would enhance the understanding of the effects of PEPc overproduction on glucose metabolism (chapter 4).

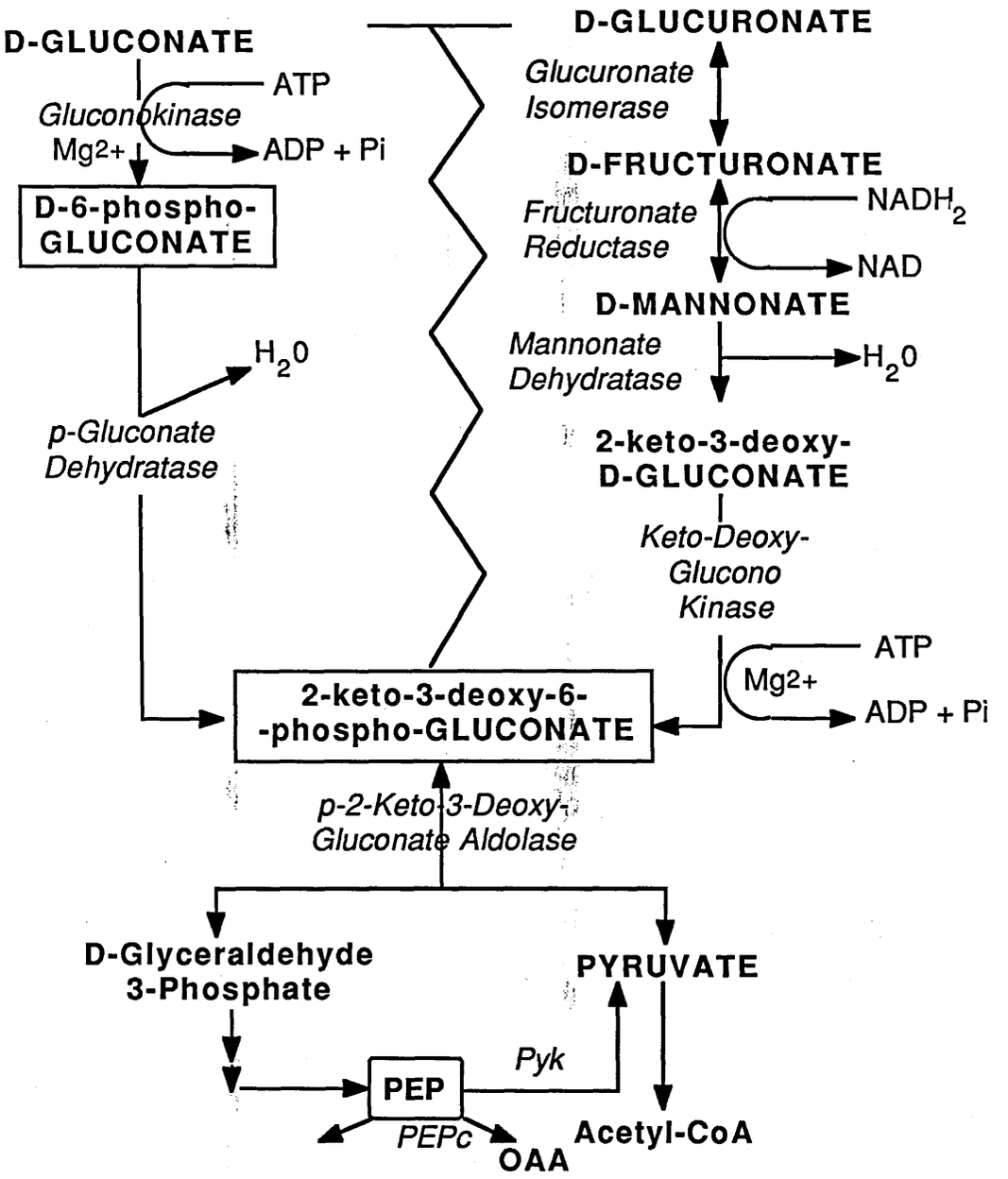


FIGURE 6.1

METABOLISM OF GLUCONATE AND GLUCURONATE BY THE *ENTNER-DOUDOROFF* PATHWAY OF *Escherichia coli*

Enzymes mediating individual reactions are represented in italics. Left- and right-hand reactions show routes which are unique to the metabolism of each substrate before convergence at 2-*oxo*-3-deoxy-6-phosphogluconate. The partitioned flux at 2-KDPG yields pyruvate directly and eventually by the metabolism of *D-glyceraldehyde-3-phosphate* .

6.2 Growth, Growth Yield and Acetate Excretion on D-Gluconate and D-Glucuronate

Parent and PEPc-overproducing strains of *E. coli* ATCC15224 were grown in batch cultures of 800ml, defined media containing 4mM D-gluconate or D-glucuronate as sole carbon and energy sources.

The growth of both strains on these substrates was characteristic of previously-adapted, substrate-limiting batch cultures. The concentration of substrate supplied, supported four generations of cell growth.

D-gluconate supported higher growth rates than D-glucuronate. Amplification of PEPc activity did not alter growth rate on D-gluconate (figure 6.2) but increased the growth rate on D-glucuronate marginally from 0.85 to 0.88 $hour^{-1}$ (figure 6.3; Table 6.1).

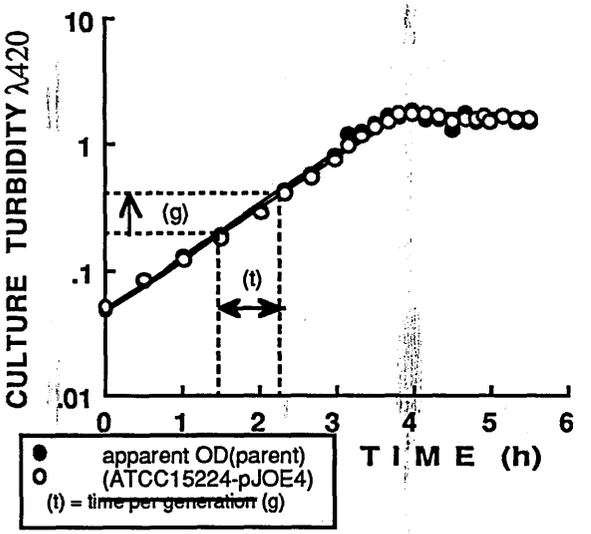


FIGURE 6.2 GROWTH OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4 ON D-GLUCONATE

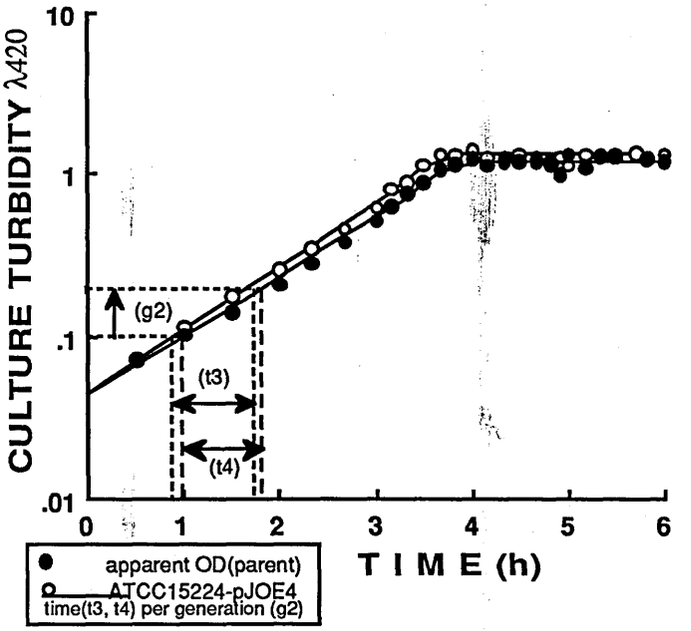


FIGURE 6.3 GROWTH OF *E. coli* ATCC15224 AND *E. coli* ATCC15224-pJOE4 ON D-GLUCURONATE

Gluconate supported higher biomass yield than glucuronate. Amplified PEPc activity resulted in marginal increases in yield on both substrates (Figure 6.3 & Table 6.1).

The parent strain excreted 0.69*mol.* acetate*/mol.* D-gluconate compared to 0.54 *mol. /mol.* in the PEPc-overproducing strain which was equivalent to 22% reduced throughput of carbon to acetate excretion (Table 6.1; figure 6.4). Throughput to acetate excretion by *E. coli* ATCC15224 during aerobic growth on D-glucuronate was estimated at 14.4 *mmol. g.* dry cell biomass^{-1}. Increase in PEPc activity reduced throughput to acetate by 24% to 11.8*mmol. g.*$^{-1}$ (Table 6.1).

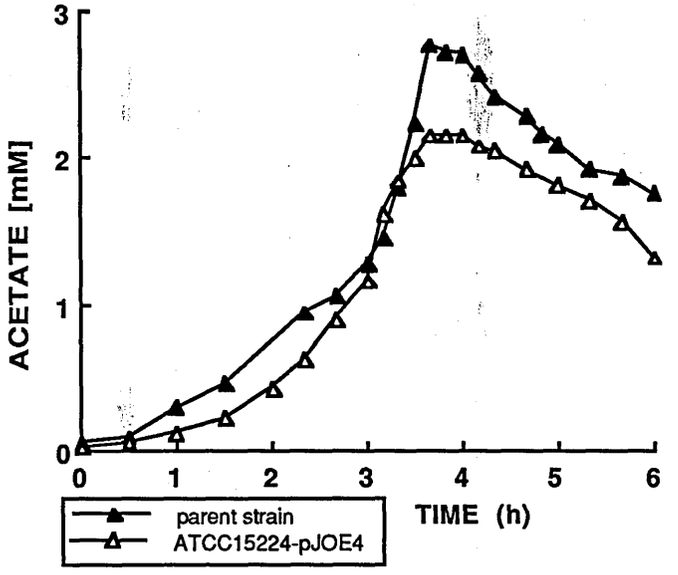


FIGURE 6.4 ACETATE EXCRETION BY *E. coli* ATCC15224 AND ATCC15224-pJOE4 GROWING ON D-GLUCONATE

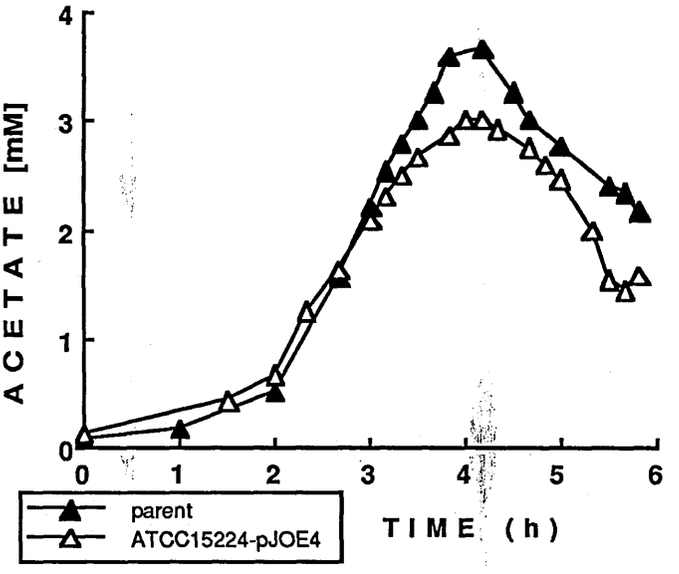


FIGURE 6.5 ACETATE EXCRETION BY TEST ORGANISMS GROWING AEROBICALLY ON D-GLUCURONATE

Carbon/ Energy Source	Growth Rate (μ) (h^{-1})	Carbon Input (*mmol.* C. *g.* dry weight.$^{-1}$)	Acetate Output (*mmol.* *g.* dry weight.$^{-1}$)	$Y_{(SUBSTRATE)}$ (*g.* dry weight *mol.* substrate.$^{-1}$)	PERCENTAGE DIFFERENCE IN GROWTH YIELDS‡
		Escherichia coli ATCC15224 (*i*$^-$, *z*$^+$, *y*$^+$, *a*$^+$)			
GLUCONATE	0.94	70.5	8.11	85.1	- \ (36.6)
GLUCURONATE	0.85	96.2	14.4	62.3	- \ (-)
		Escherichia coli ATCC15224-pJOE4 (*i*$^-$, *z*$^+$, *y*$^+$, *a*$^+$; pBR322-*ppc*$^+$)			
GLUCONATE	0.94	68.5	6.14	87.6	3.0 \ (34.4)
GLUCURONATE	0.88	92.0	11.8	65.2	4.7 \ (-)

TABLE 6.1

COMPARISON OF METABOLIC ACTIVITIES DURING THE AEROBIC UTILIZATION OF D-GLUCONATE AND D-GLUCURONATE BY *E. COLI* ATCC15224 BEFORE AND AFTER OVEREXPRESSION OF PEPC ACTIVITY

‡Differences in the $Y_{substrate}$ of parent and recombinant strains were expressed as percentage functions of $Y_{substrate}$ in the parent. Values in parentheses are differences between growth on gluconate and glucuronate as functions of $Y_{glucuronate}$ in each strain.

6.3 Discussion

During the metabolism of D-gluconate and D-glucuronate by *E. coli* ATCC15224, carbon fluxes were operated through the Entner-Doudoroff (ED) Pathway which is distinct from glycolysis in most reaction steps. Fluxes from both substrates converged at *2-keto-3-deoxy-6-phosphogluconate* which was split by 2-keto-3-deoxy-6-phosphogluconate aldolase into two C_3 intermediates — pyruvate and glyceraldehyde-3-phosphate, in equimolar amounts. Only the latter transmitted carbon flux through reactions from which the triose phosphates glyceraldehyde-3-phosphate and phospho*enol*pyruvate were derived (figure 6.1).

The central biosynthetic precursor, glucose-6-phosphate is not a direct intermediate of the ED pathway when gluconate and glucuronate are the sole carbon sources. Fluxes must exist towards this output from other intermediates. In *Pseudomonas sp.* which is most commonly associated with the ED pathway, the synthesis of hexose-phosphates is feasible thermodynamically (Hollmann, 1964; Burns and Conney, 1966). Extension of this model to *E. coli* would involve a cyclic process from triose phosphates, akin to gluconeogenesis (figure 6.7). The specific substrate for PEPc, phospho*enol*pyruvate, is also produced from glyceraldehyde-3-phosphate in reactions common to glycolysis. One *mole* of PEP was produced per *mole* of D-gluconate or D-glucuronate. Fluxes from PEP did not service the PTS system but were partitioned to biosynthesis, PEPc and pyruvate kinase; the last yielded pyruvate. Pyruvate was also produced directly from 2-KDPG by 2-KDPG aldolase (figure 6.1). The total flux to pyruvate by the parent strain was therefore 13.88*mmol. g.* dry biomass^{-1}. *h.*$^{-1}$ on D-gluconate (figure 6.8) or 19.83*mmol. g.*$^{-1}$. *h.*$^{-1}$. on D-glucuronate (figure 6.10). The fate of pyruvate included fluxes to acetyl-CoA (11.7 and 17.86*mmol g.*$^{-1}$. *h.*$^{-1}$ on gluconate and glucuronate respectively) and acetate (7.62 and 12.24*mmol. g.*$^{-1}$. *h.*$^{-1}$. respectively; figure 6.8).

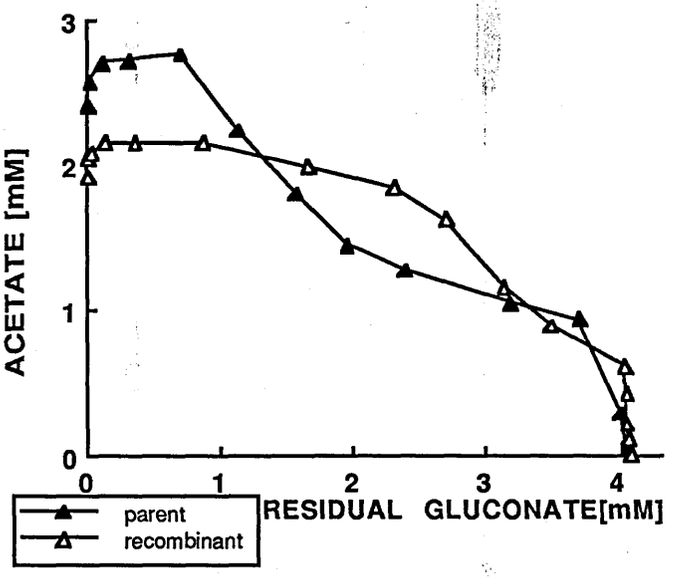


FIGURE 6.6 (a): RELATIONSHIP BETWEEN THE EXCRETION OF ACETATE AND UTILIZATION OF D-GLUCONATE

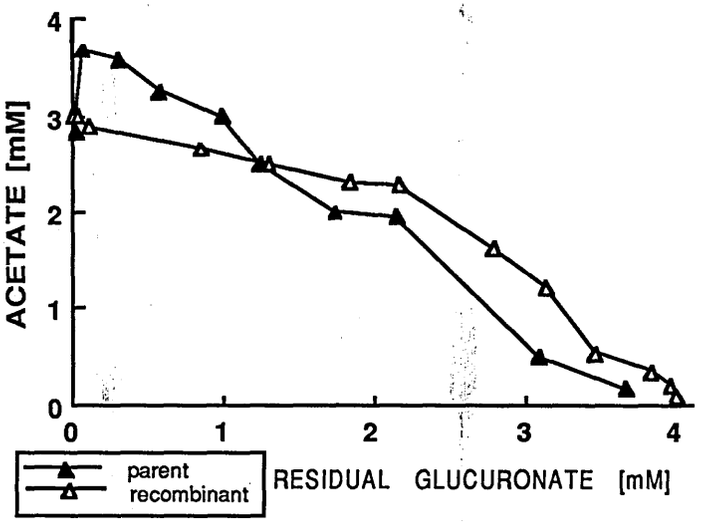


FIGURE 6.6 (b): RELATIONSHIP BETWEEN THE EXCRETION OF ACETATE AND UTILIZATION OF D-GLUCURONATE

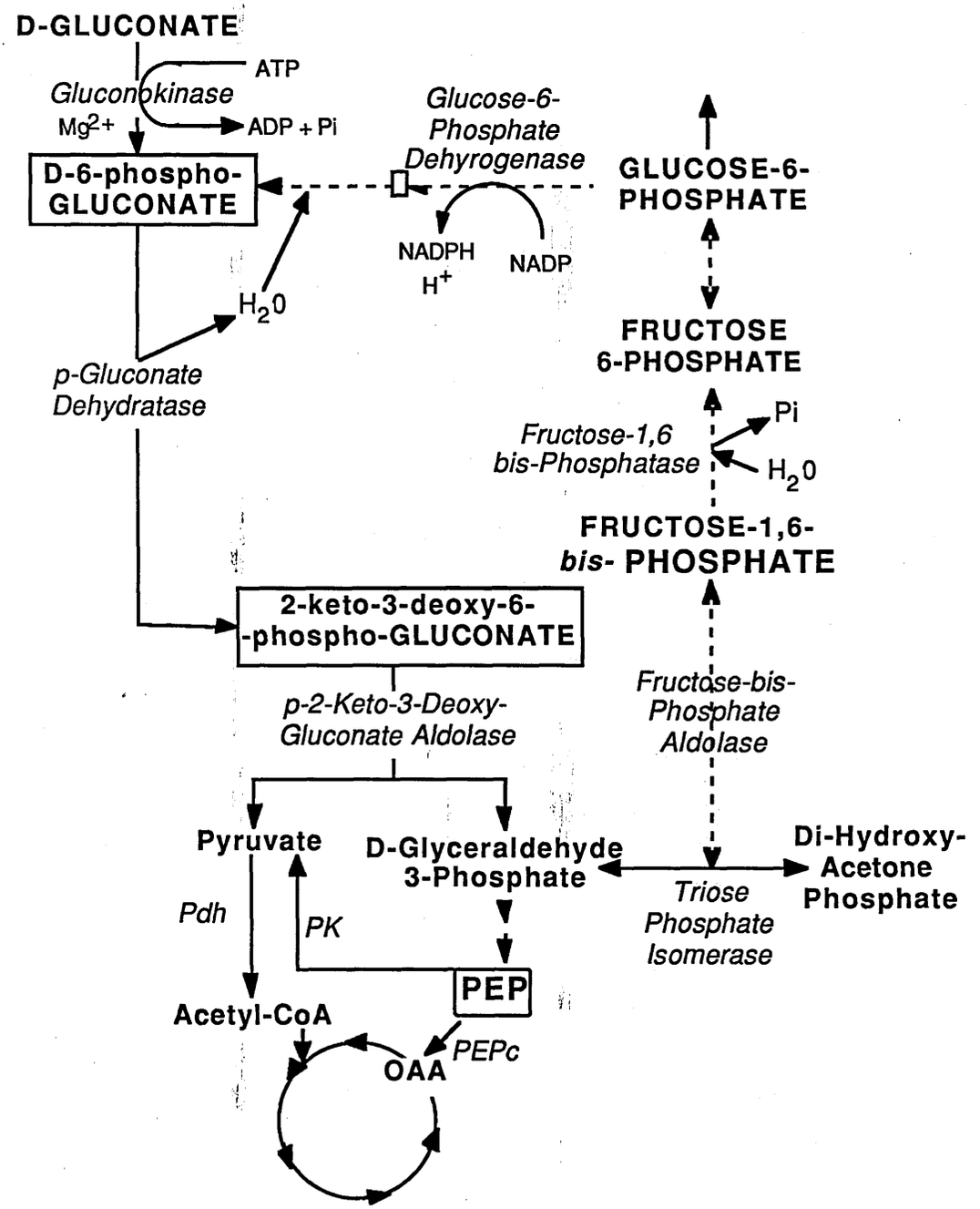


FIGURE 6.7 SYNTHESIS OF GLUCOSE-6-PHOSPHATE FROM C_3-INTERMEDIATES OF THE ENTNER-DOUDOROFF PATHWAY

The reactions shown are typical of D-gluconate metabolism.The routes to the left are reactions of the EDP while those to the right in dashed arrows, are mostly glycolytic in the forward direction and gluconeogenic in reverse. Enzymes mediating individual steps are shown in italics. Glucose-6-phosphate (above) is first converted to D-6-phospho-glucono-δ-lactone, then D-6-phosphogluconate by *aldonolactonase* to complete a cycle.

6.3.1 RELATIONSHIP BETWEEN OVEREXPRESSION OF PEPc AND THE METABOLISM OF D-GLUCONATE

Amplification of PEPc activity diminished flux to acetate excretion by 20% from acetyl-CoA. As observed during pyruvate metabolism (section 5.2.2), partition of fluxes at PEP favoured diminished fluxes to acetate excretion. This contributed 3% to growth yield on D-gluconate. This minimal increase must be viewed against the background of the partition of carbon flux at 2-KDPG which yields only 1*mol.* (PEP)|*mol.*; (figure 6.1). It is apparent that only this half of the partitioned flux is likely to be sensitive to amplification of PEPc activity. In addition, the metabolic burden on this route is enormous, since throughputs to over half of the biosynthetic precursors (triose phosphates, phosphoglycerates, PEP, G-6-P; some pyruvate, acetyl-CoA, oxaloacetate and 2-*oxo*glutarate; figure 1.2) depended on it (figure 6.7). The impact of amplification of PEPc activity cannot be optimal unde this condition. Theoretical carbon flux to pyruvate through 2-KDPG aldolase was more than sufficient for the entire cellular requirement for pyruvate. It is apparent that overproduction of PEPc competitively reduced carbon flux through pyruvate kinase, but could not abolish it altogether (figure 6.9). Further improvement of growth yield on gluconate must involve strategies that take flux through 2-KDPG aldolase into consideration.

FIGURE 6.8

CARBON FLUXES DURING THE AEROBIC METABOLISM OF D-GLUCONATE BY *E. coli* ATCC15224

Values are expressed in
mmol. (input, output or intermediate)
g. (dry biomass)$^{-1}$ hour^{-1}.

Growth Rate (μ) = 0.94*h.* $^{-1}$

Carbon Input = 70.5 *mmol.* C. *g.* (dry biomass)$^{-1}$

The pathways shown are *Entner-Doudoroff* and the TCA cycle. Fluxes to biosynthesis are enclosed in dashed boxes and were derived by expressing throughputs to biosynthesis as functions of the growth rate. The throughputs were based on the quantitative drainage of the pertinent intermediate metabolites to the synthesis of amino acids and nucleotides as illustrated in figures 1.2 & 1.3. Primary input was derived from $Y_{GLUCONATE}$ and flux to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced and specific growth rate (table 6.1).

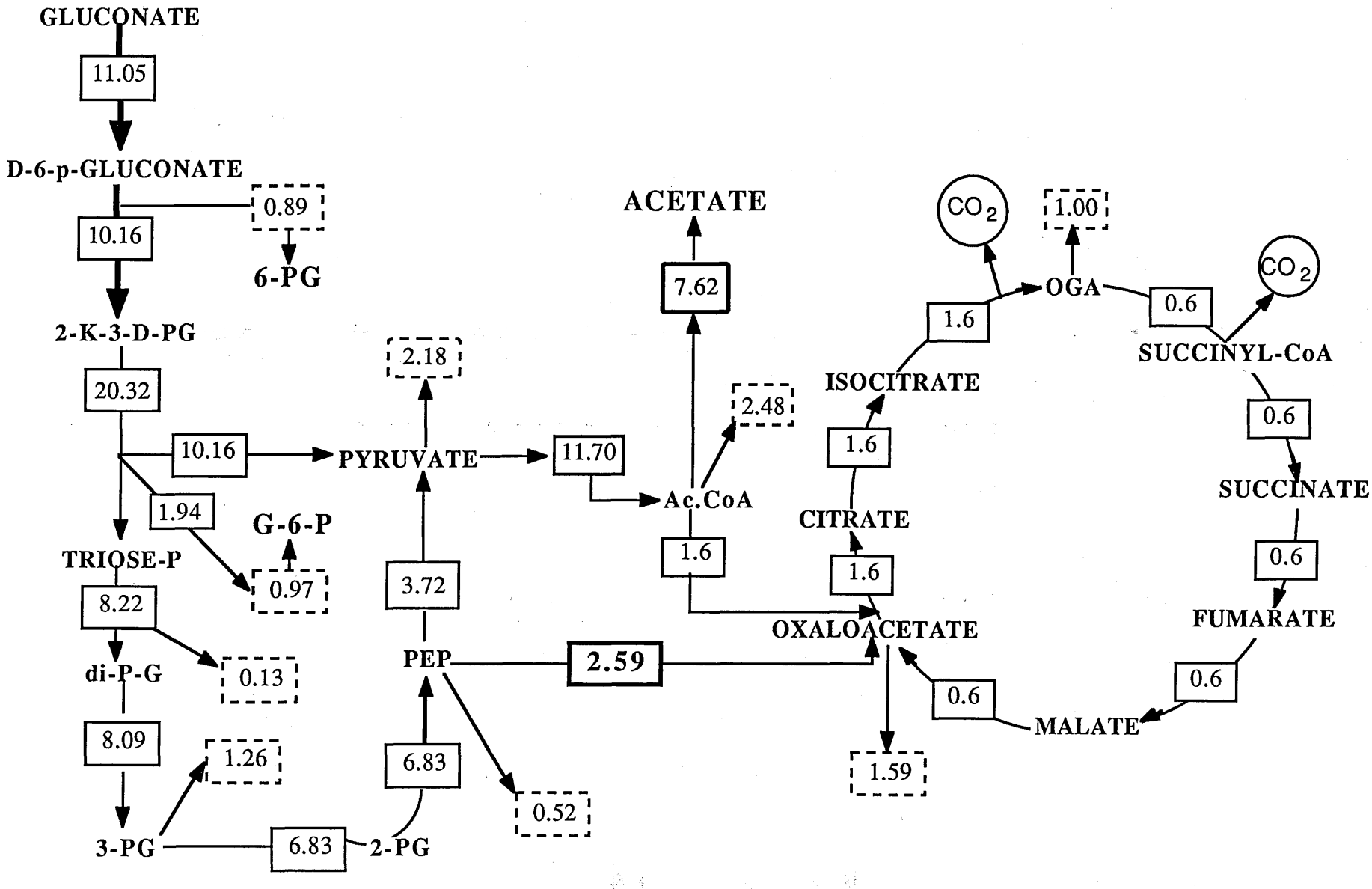

GLUCONATE
11.05
D-6-p-GLUCONATE
0.89
6-PG
10.16
2-K-3-D-PG
20.32
10.16
PYRUVATE
2.18
11.70
ACETATE
7.62
2.48
Ac.CoA
1.94
G-6-P
0.97
TRIOSE-P
8.22
0.13
di-P-G
8.09
1.26
3-PG
6.83
2-PG
3.72
PEP
0.52
6.83
1.6
1.6
CITRATE
1.6
OXALOACETATE
2.59
1.59
1.6
ISOCITRATE
CO2
1.00
OGA
0.6
CO2
SUCCINYL-CoA
0.6
SUCCINATE
0.6
FUMARATE
0.6
MALATE

FIGURE 6.9

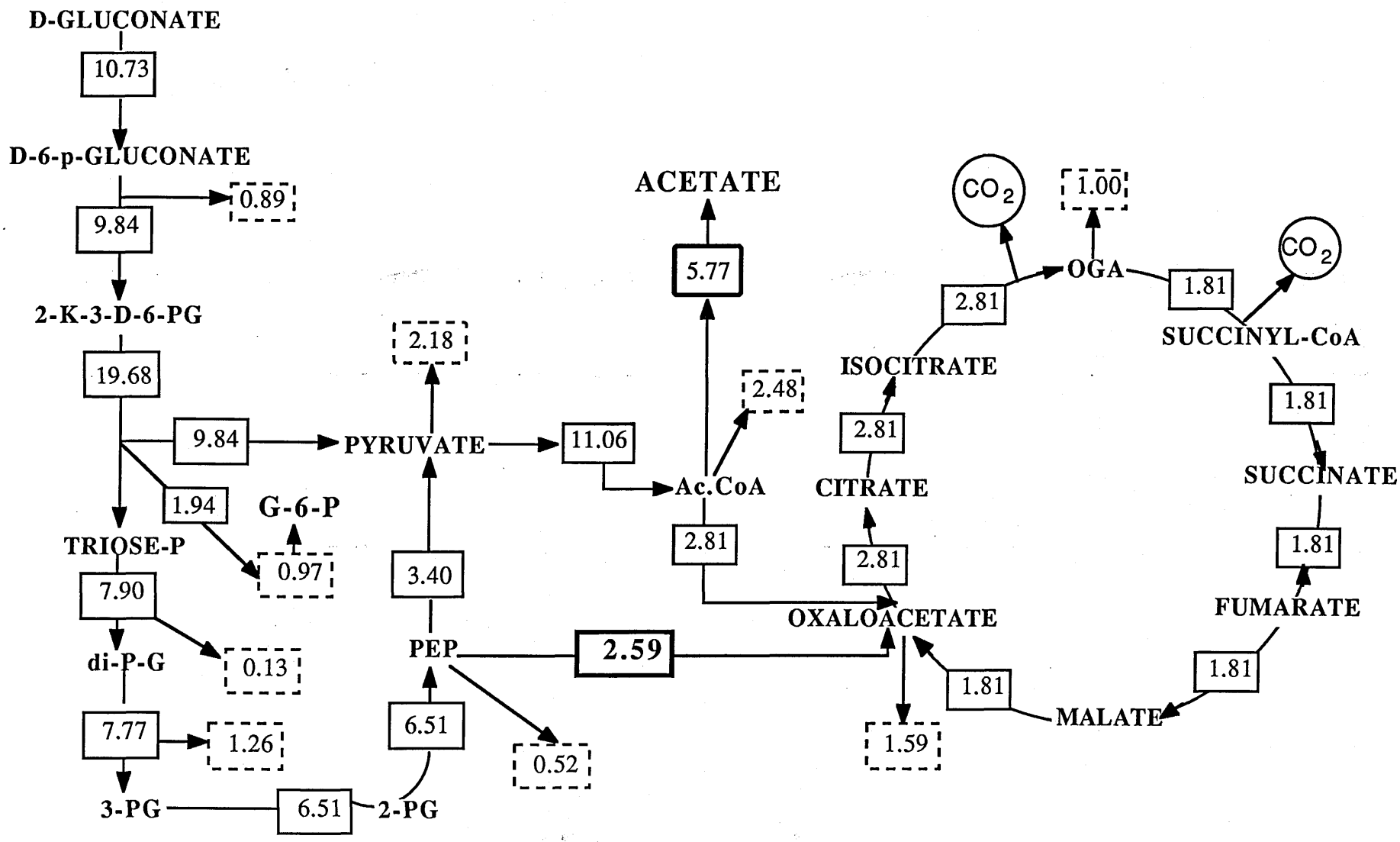
CARBON FLUXES DURING THE AEROBIC METABOLISM OF D-GLUCONATE BY *E. coli* ATCC15224-pJOE4

Values are expressed in
mmol. (input, output or intermediate)
g. (dry biomass)$^{-1}$ hour^{-1}.

Growth Rate (μ) = 0.94*h.*$^{-1}$

Carbon Input = 68.52 *mmol.* C. *g.* (dry biomass)$^{-1}$

The pathways shown are *Entner-Doudoroff* and the TCA cycle. Fluxes to biosynthesis are enclosed in dashed boxes and were derived by expressing throughputs to biosynthesis as functions of the growth rate. The throughputs were based on the quantitative drainage of the pertinent intermediate metabolites to the synthesis of amino acids and nucleotides as illustrated in figures 1.2 & 1.3. Primary input was derived from $Y_{GLUCONATE}$ and flux to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced and specific growth rate (table 6.1).


D-GLUCONATE
10.73
D-6-p-GLUCONATE
0.89
9.84
2-K-3-D-6-PG
19.68
9.84
PYRUVATE
11.06
1.94
G-6-P
0.97
TRIOSE-P
7.90
0.13
di-P-G
7.77
1.26
3-PG
6.51
2-PG
2.18
3.40
PEP
6.51
0.52
2.59
ACETATE
5.77
2.48
Ac.CoA
2.81
OXALOACETATE
2.81
CITRATE
2.81
ISOCITRATE
2.81
CO2
1.00
OGA
1.81
CO2
SUCCINYL-CoA
1.81
SUCCINATE
1.81
FUMARATE
1.81
MALATE
1.81
1.59

6.3.2 RELATIONSHIP BETWEEN OVEREXPRESSION OF PEPc AND THE METABOLISM OF D-GLUCURONATE

During the metabolism of D-glucuronate, the fluxes in operation were similar to D-gluconate, except the three steps required to produce 2-KDPG (figure 6.1). Since hexose-phosphates are intermediates of the glucuronic acid cycle (Burns and Conney, 1966), they could be produced through that route for biosynthesis.

Glucuronate sustained higher fluxes to acetate excretion but lower fluxes to biosynthesis compared to gluconate metabolism by *E. coli* ATCC15224 (Figures 6.8 & 6.10). Most of the biosynthetic precursors from glucuronate were derived from one of two fluxes which arose from 2-KDPG. Both however yielded pyruvate eventually, thereby sustaining substantial fluxes to acetate excretion. The PEPc flux could only compete directly for carbon from less than one-half.of fluxes from 2-KDPG. The most likely effect of an amplified PEPc activity would be a competitive reduction in further throughput to pyruvate *via* pyruvate kinase. In the recombinant strain, carbon flux through pyruvate kinase was diminished by 370μ*mol. g.* (dry biomass)$^{-1}$ *h.*$^{-1}$ in favour of the flux through PEPc. Reduced excretion of acetate could be a combination of cause-and-effect factors on carbon fluxes. From one perspective, diminished throughput to pyruvate could reduce fluxes to acetate excretion, as previously shown by Mansi and Holms (1989). In the present study, reduction of carbon flux through pyruvate kinase to pyruvate was achieved by competitive increase in flux of PEP through phospho*enol* pyruvate carboxylase to oxaloacetate (figure 6.11). Oxaloacetate is the carrier-molecule/co-reactant during the oxidation of acetyl-CoA in the TCA cycle. Its absence or deficiency affects further metabolism of acetyl-CoA through the TCA cycle (Krebs, 1981), but does not preclude its conversion to acetate. Flux analysis suggested an increased flux through the TCA cycle in the PEPc-overproducing strain during glucuronate metabolism (figures 6.10-11). This reflects an increase in the amount of acetyl-CoA oxidized through the cycle which can be monitored by measurement of CO_2 evolution. Some of this could have been converted to acetate and excreted. Increase in PEPc activity simultaneously reduced flux to pyruvate and fluxes from acetyl-CoA to acetate. A combination of both factors salvaged fluxes of carbon to acetate excretion and increased growth rate and biomass yield marginally.

FIGURE 6.10

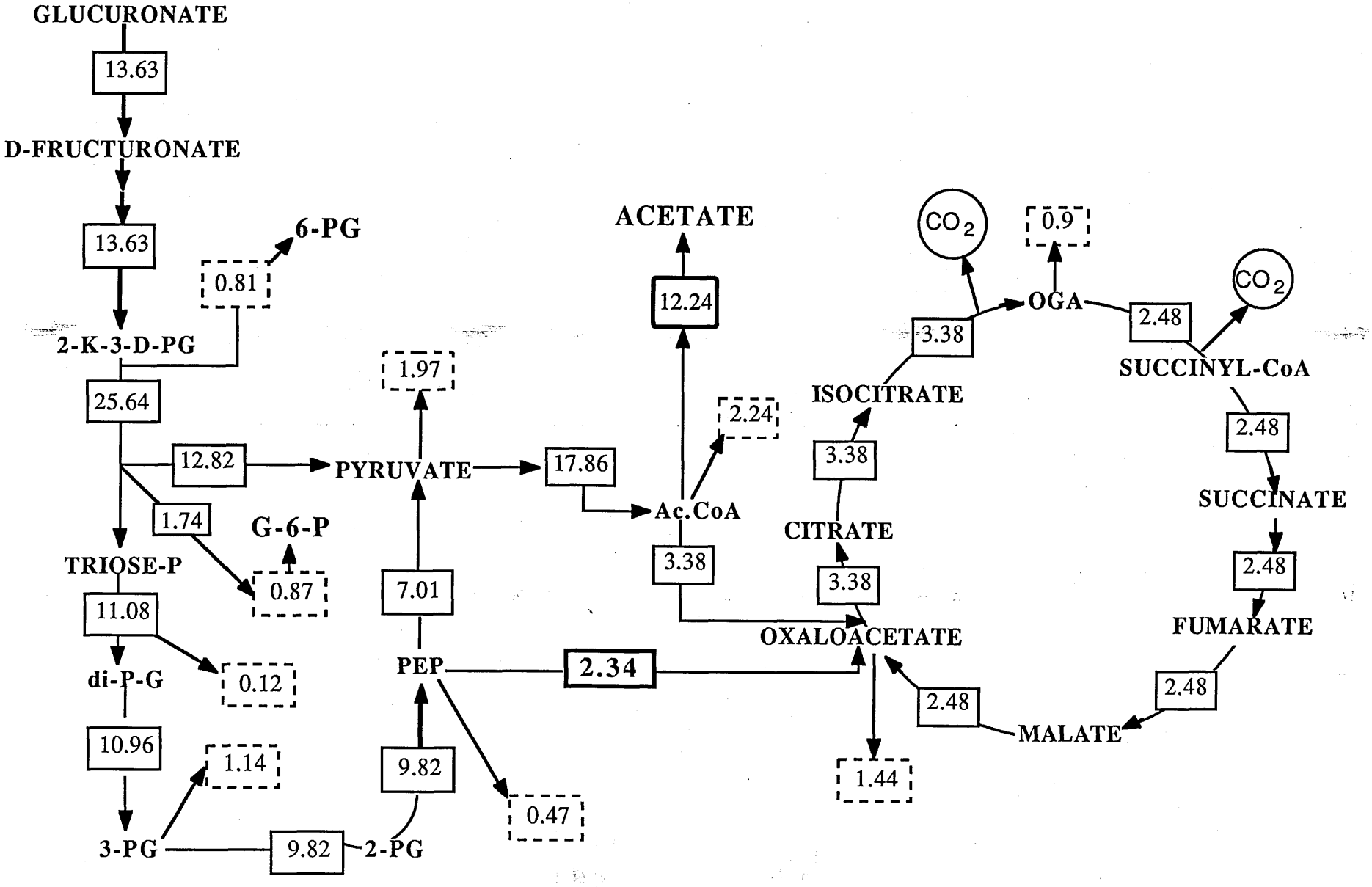
CARBON FLUXES DURING THE AEROBIC METABOLISM OF D-GLUCURONATE BY *E. coli* ATCC15224

Values are expressed in
mmol. (input, output or intermediate)
g. (dry biomass)$^{-1}$ hour^{-1}.

Growth Rate (μ) = 0.85*h.* $^{-1}$

Carbon Input = 96.24 *mmol.* C. *g.* (dry biomass)$^{-1}$

The pathways shown are *Entner-Doudoroff* and the TCA cycle. Fluxes to biosynthesis are enclosed in dashed boxes and were derived by expressing throughputs to biosynthesis as functions of the growth rate. The throughputs were based on the quantitative drainage of the pertinent intermediate metabolites to the synthesis of amino acids and nucleotides as illustrated in figures 1.2 & 1.3. Primary input was derived from $Y_{GLUCURONATE}$ and flux to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced and specific growth rate (table 6.1).


GLUCURONATE
13.63
D-FRUCTURONATE
13.63
0.81
6-PG
2-K-3-D-PG
25.64
12.82
PYRUVATE
1.74
G-6-P
TRIOSE-P
0.87
11.08
di-P-G
0.12
10.96
1.14
3-PG
9.82
2-PG
1.97
7.01
PEP
9.82
0.47
17.86
ACETATE
12.24
2.24
Ac.CoA
3.38
2.34
OXALOACETATE
1.44
CO2
0.9
3.38
OGA
2.48
ISOCITRATE
CO2
SUCCINYL-CoA
2.48
3.38
CITRATE
3.38
SUCCINATE
2.48
FUMARATE
2.48
2.48
MALATE

6.3.3 EVALUATION OF THE NET IMPACT OF AMPLIFICATION OF PEPc ACTIVITY ON ACETATE EXCRETION DURING GLUCONATE AND GLUCURONATE UTILIZATION BY *E. coli* ATCC15224

Although fluxes to acetate excretion were diminished by 24% on gluconate and 15% on glucuronate, throughputs to acetate excretion remained high in *E. coli* ATCC15224-pJOE4. In addition, improvements in biomass yields on both substrates could not be considered statistically significant. It is proposed that there must be other factors in the metabolism of gluconate and glucuronate, whose effects on acetate excretion and biomass yield are more prominent than the flux through PEPc. Not all however, will simultaneously reduce acetate excretion and increase growth yield. One of such reaction steps in the ED pathway, could be the *2-keto-3-deoxy-6-phospho-gluconate aldolase* flux. which is bidirectional reaction and likely to be highly regulated (Zwaig *et al.*, 1973). There is need to investigate its regulatory mechanism with a view to influencing the partition of fluxes between pyruvate and glyceraldehyde-3-phosphate (G-3-P), in favour of the latter. The metabolism of D-gluconate and D-glucuronate placed a heavy metabolic burden on G-3-P which was not commensurate with its supply relative to pyruvate (figures 6.7...6.11). The forward reaction of 2-KDPG aldolase produced both in equimolar proportions. This distribution favours acetate excretion and is unfavourable to outputs of new biomass or the amphibolic reactions. Although the reaction is reversible, the large flux to acetate excretion from gluconate and glucuronate suggests that the conversion of pyruvate to acetyl-CoA/acetate is more favoured thermodynamically than the formation of 2-KDPG from the condensation of pyruvate with G-3-P (figure 6.1). Selective redistribution of carbon flux from 2-KDPG to reduce its direct conversion to pyruvate would be a potential site for controlling carbon fluxes to increase the efficiency of metabolism of the substrates investigated. Flux control at PEP is clearly subordinate to this branch-point.

FIGURE 6.11

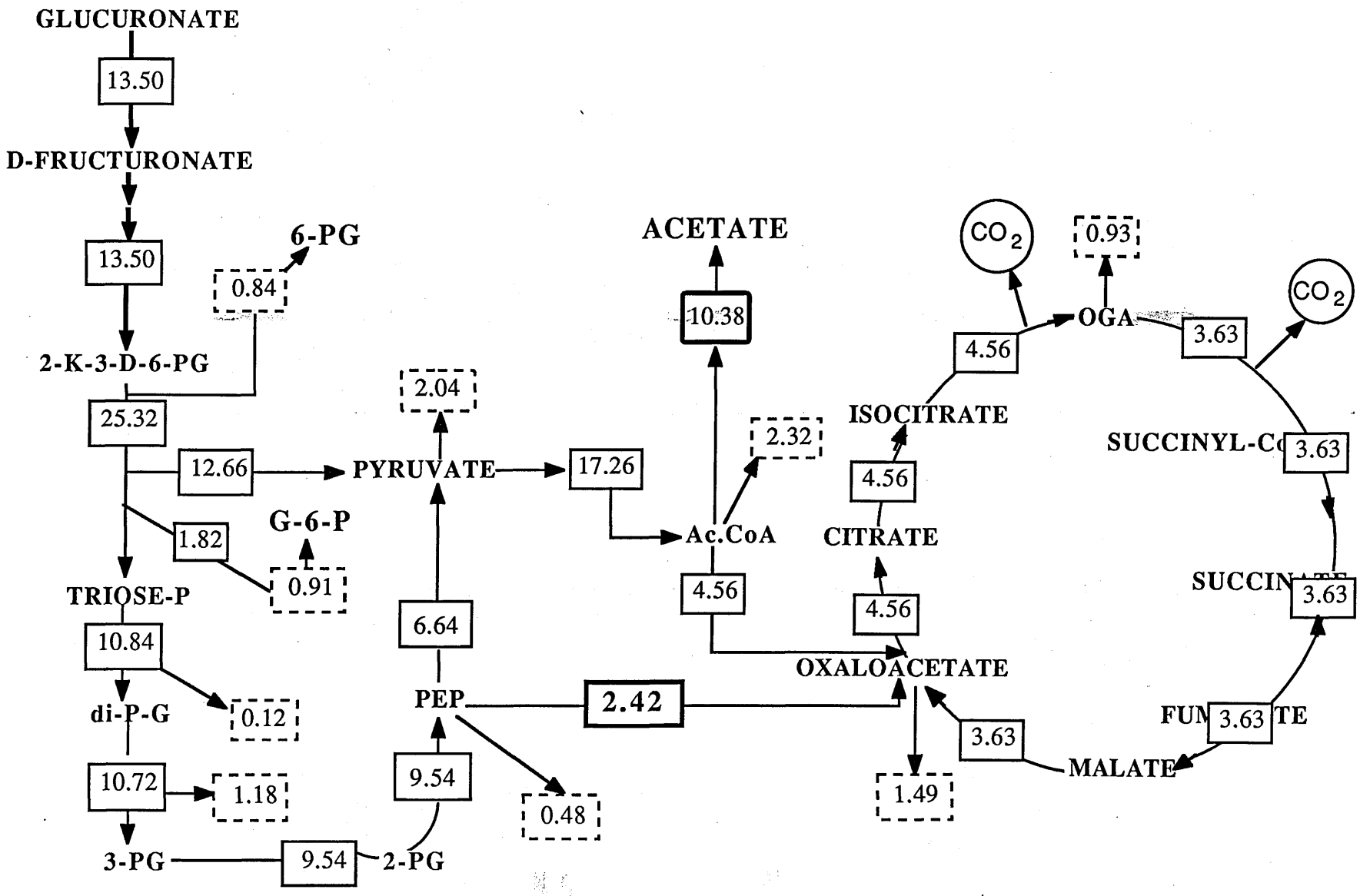
CARBON FLUXES DURING THE AEROBIC METABOLISM OF D-GLUCURONATE BY *E. coli* ATCC15224-pJOE4

Values are expressed in
mmol. (input, output or intermediate)
g. (dry biomass)$^{-1}$ hour^{-1}.

Growth Rate (μ) = 0.88*h.* $^{-1}$

Carbon Input = 92.04 *mmol.* C. *g.* (dry biomass)$^{-1}$

The pathways shown are *Entner-Doudoroff* and the TCA cycle. Fluxes to biosynthesis are enclosed in dashed boxes and were derived by expressing throughputs to biosynthesis as functions of the growth rate. The throughputs were based on the quantitative drainage of the pertinent intermediate metabolites to the synthesis of amino acids and nucleotides as illustrated in figures 1.2 & 1.3. Primary input was derived from $Y_{GLUCURONATE}$ and flux to acetate excretion was obtained by expressing the concentration of acetate excreted relative to the biomass produced and specific growth rate (table 6.1).


GLUCURONATE
13.50
D-FRUCTURONATE
13.50
6-PG
0.84
2-K-3-D-6-PG
25.32
12.66
PYRUVATE
2.04
17.26
ACETATE
10.38
2.32
Ac.CoA
4.56
1.82
G-6-P
0.91
TRIOSE-P
10.84
di-P-G
0.12
10.72
1.18
3-PG
9.54
2-PG
6.64
PEP
9.54
0.48
2.42
OXALOACETATE
4.56
4.56
CITRATE
4.56
ISOCITRATE
4.56
CO2
0.93
OGA
3.63
CO2
SUCCINYL-C
3.63
SUCCIN
3.63
FUM
3.63
TE
3.63
MALATE
1.49

CHAPTER 7

STUDY OF GLUCOSE METABOLISM BY A PYRUVATE KINASE-NEGATIVE STRAIN OF *Escherichia coli* OVERPRODUCING PEPc

7.1 INTRODUCTION:

TESTING THE THEORY OF PYRUVATE KINASE REDUNDANCY IN PEPC-OVERPRODUCING STRAINS

The preceding studies (chapters 4-6) demonstrated that amplification of the activity of phospho*enol*pyruvate carboxylase (PEPc) in *Escherichia coli* influenced its metabolism of carbohydrates by diminishing the excretion of acetate which was usually accompanied by increases in growth yields to various extents in aerobic cultures. The distribution of carbon in *E.coli* ATCC15224-pJOE4 with elevated PEPc activity has been computed together with the *a priori* fluxes which supported glucose metabolism in the strain (figures 4.16; 6.9; 6.11). Analysis of carbon and metabolic fluxes in the strain during glucose metabolism suggested a zero-flux of phospho*enol* pyruvate (PEP) through the unidirectional glycolytic reaction mediated by the enzyme *pyruvate kinase* (figure 7:1)

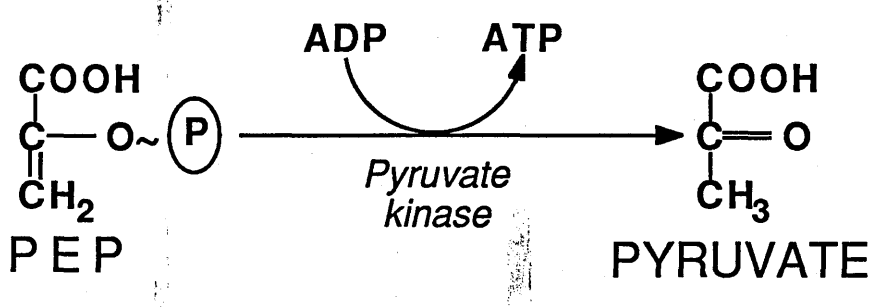


FIGURE 7.1
GLYCOLYTIC CONVERSION OF PEP TO PYRUVATE

In real terms, this presupposes that the synthesis of pyruvate in amounts commensurate with its various metabolic demands, occurred through alternative routes. From available evidence, 1*mol.* pyruvate was formed by the dephosphorylation of 1*mol.* PEP per *mol.* of substrate transported *via* the equilibrated phosphoenzyme reactions of the bacterial PTS system (Saier *et al.*, 1980; Postma and Lengeler, 1985; Postma *et al.*, 1989; Roseman, 1990). Incidentally, the pyruvate kinase flux, shared a common substrate (PEP) and product (pyruvate) with the non-specific *enzyme1* of the PTS system. Competition for PEP between these two fluxes would necessitate appropriate regulatory mechanisms to suit the organism and carbon source and would take other divergent fluxes to biosynthesis (figures 1.2; 1.3) and oxaloacetate into consideration (figure 7.2).

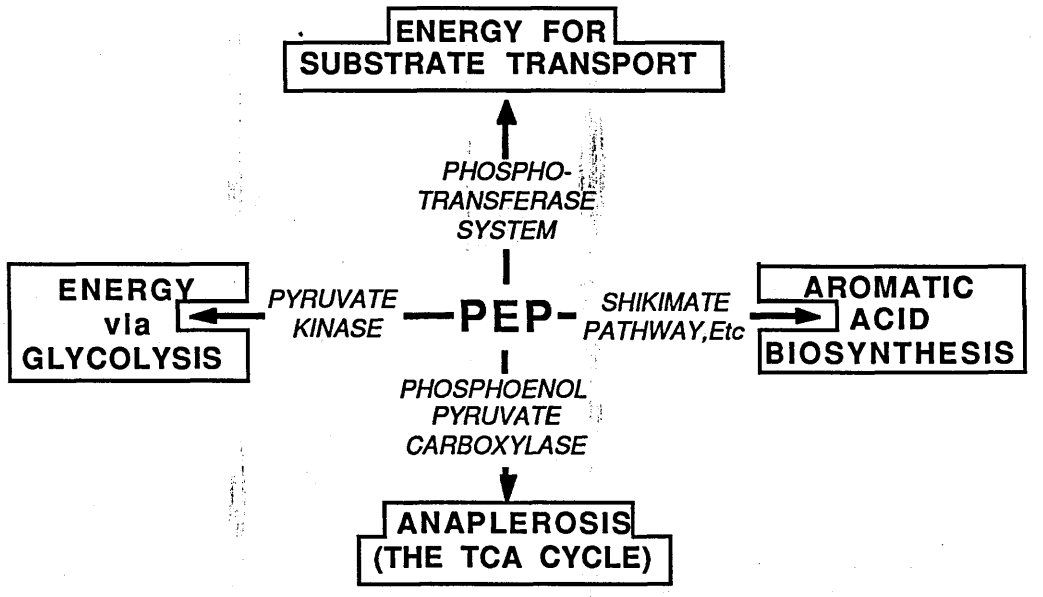


FIGURE 7.2 DISTRIBUTION OF PEP DURING THE METABOLISM OF PTS-SUGARS

The new steady-state established with overexpression of PEPc activity in *E.coli* ATCC15224-pJOE4, must reckon with the increased demand for PEP by amplified PEPc activity. Normally, a quantitative change of any of the enzymes of divergent fluxes from a branch-point, should first affect the control coefficient of the particular flux directly (Kacser and Burns, 1973; Acerenza and Kacser, 1990), while the magnitude of the effect is also distributed proportionally among the various divergent fluxes which *communicate* with the modified flux through a common central metabolite. This is consistent with the *relative sensitivity coefficient* of flux control (Crabtree and Newsholme, 1987). Appraisal of the fluxes from PEP (figure 7.2) suggested that the burden for balancing throughputs of PEP to its phosphorylated (PTS/glycolysis), biosynthetic and carboxylated routes, was borne overwhelmingly by pyruvate kinase (Thompson, 1987). Evidence from flux analysis (*see* figure 4.16) supported this view and showed that the magnitude of the burden at $Y_{GLUCOSE}$ of 114.4*mmol. g.*$^{-1}$ in *E. coli* ATCC15224-pJOE4, was capable of precluding flux through pyruvate kinase altogether.

7.1.1 APPRAISAL OF THE CHARACTERISTICS OF PYRUVATE KINASE AND THE IMPLICATION OF ZERO-FLUX THROUGH THE ENZYME

There are two forms of pyruvate kinase in *Escherichia coli* (Malcovati and Kornberg, 1969), each encoded by a distinct gene (Garrido-Pertierra and Cooper, 1983). Pyruvate kinase*A* which is the product of the *pykA* gene, is constitutively expressed (Maeba and Sanwal, 1968) and modulated by *AMP* (Sanwal, 1970) while the fructose-1,6-*bis* phosphate–activated *pyruvate kinaseF* is inducible and encoded by the *pykF* gene (Hess et *al.*, 1966). Unlike the mammalian variant, the two bacterial pyruvate kinases are not interconvertible forms of the same protein but since each form is capable of converting PEP to pyruvate independent of the other (Garrido-Pertierra and Cooper, 1977), only a double *pykA, pykF* mutation could guarantee a full lesion for pyruvate kinase in order to produce a mutant which was incapable of operating flux through pyruvate kinase (Garrido-Pertierra and Cooper, 1983).

The hypothesis of a redundant pyruvate kinase could be tested by amplifying PEPc activity in a strain which is impaired in the ability to transmit carbon fluxes through pyruvate kinase due to enzyme inhibition or mutations in the *pyk* genes. Alternatively, flux through pyruvate kinase could be blocked in wild-type strains with a view to enhancing flux through PEPc by default. A pyruvate kinase-negative derivative of *E.coli* ATCC15224 was not available neither was the strain amenable to necessary genetic manipulation. Both drawbacks undermined the otherwise excellent metabolic history of the test organism (Mandelstam, 1962; Holms, 1966; Koch and Wang, 1982; Holms, 1986a; b; 1987; Koch, 1988). As an alternative, PEPc activity was overexpressed in *E.coli* HW 1387, a pyruvate kinase-negative mutant derived from *E.coli* HW0760 strain which is a K-strain. In view of the pedigree of the mutant, it was necessary to determine the effects of overexpression of PEPc activity on glucose metabolism by a prototrophic K strain. *E.coli* K10, whose nutritional requirement for thiamine alone was typical of most K strains but otherwise analogous to *E.coli* ATCC15224, was used as prototrophic control for *E.coli* HW 1387. The present report includes a comparative assessment of important effects of amplified PEPc activity on glucose metabolism by *E.coli* K10 and *E.coli* ATCC15224. PEPc activity was also overexpressed in the parent strain of the pyruvate kinase-negative mutant as a second control for a *pykA; pykF* genotype during aerobic metabolism of glucose.

7.2 Preliminary Study of Glucose Utilization by *E. coli* K10

An inoculum was prepared as described for *E.coli* ATCC15224 (2.8.1). Growth was carried out in 1-litre flasks containing 800ml, 4mM-glucose minimal medium and monitored as described for *E.coli* ATCC15224 (2.8, 2.9).

Growth of cells, utilization of substrate and output of acetate were consistent with the pattern of substrate-limited, aerobic batch cultures (figure 7.3, a & b). Maximum biomass production and acetate excretion occurred at the same time and coincided with complete depletion of glucose.

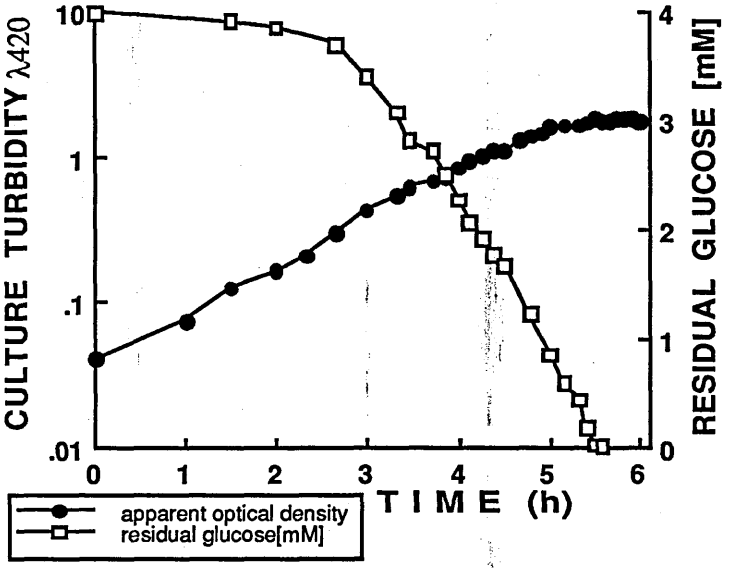


FIGURE 7.3 (a) GROWTH AND GLUCOSE UTILIZATION BY *E. coli* K10

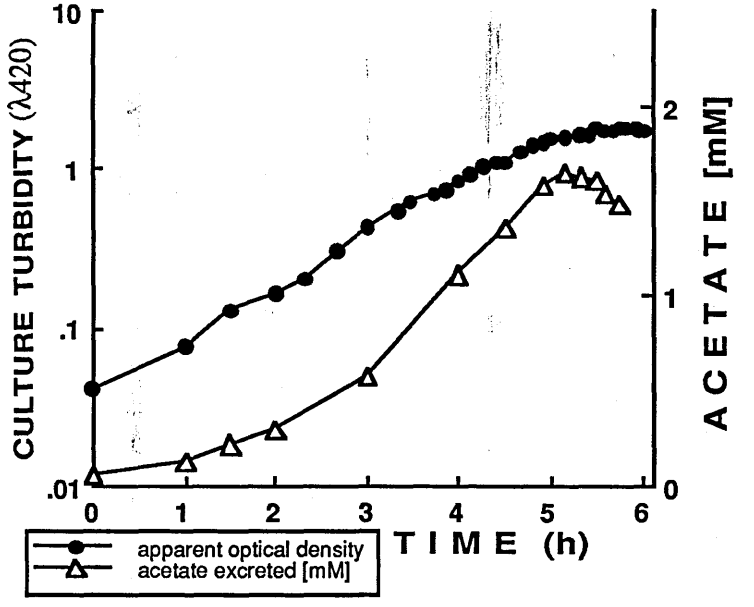


FIGURE 7.3 (b) GROWTH AND ACETATE EXCRETION BY *E. coli* K10

7.3 INDUCTION OF COMPETENCE AND TRANSFORMATION

An overnight L-broth culture of *E.coli* K10 was diluted ten-fold in 100ml prewarmed L-broth and grown with vigorous agitation at 37°C for just over an hour. Cells were recovered, made competent and stored as described for *E.coli* PA342 (see 2.5.4). The transformation efficiency of cells was determined using plasmid pBR322 (Table 7.1a). Competent cells were subsequently transformed with hybrid plasmid pJOE4, containing the *ppc* gene and incubated on selective plates with appropriate dilutions to obtain transformed colonies (Table 7.1 b).

Overnight stationary cultures from single colonies were sufficiently competent for transformation by reference and recombinant plasmids (Table 7.1). Competent cells remained viable after storage at -70°C with only 10% depreciation in transformation efficiency after thawing.

7.4 DETERMINATION OF PEPc ACTIVITY IN *E. coli* K10 AND DERIVATIVES

Ultrasonic cell extracts of 100ml, 10mM glucose cultures of *E.coli* K10-pBR322 and *E.coli* K10-pJOE4 were assayed for PEPc activities as described for *E.coli* PA342 (2.7.1). PEPc activity in the parent was 10.2*nmol. mg.* protein.$^{-1}$ min^{-1}. The activity was amplified 52 times in *E.coli* K10-pJOE4 (pBR322-*ppc*$^{+}$) (Table 7.2). The PEPc activity of *E.coli* K10-pBR322, the negative control for transformation, was not significantly different from the parent.

	TRANSFORMANTS (L-agar amp)		SURVIVORS (L-agar; *cfu*)		TRANSFORMATION* EFFICIENCY
OD_{600}	10^0	10^{-1}	10^{-5}	10^{-6}	(*cft. μg. DNA*$^{-1}$)
0.239	TNC	230	57	6	9.20×10^5
0.255	TNC	243	81	10	9.72×10^5

TABLE 7.1 (a) TRANSFORMATION EFFICIENCY OF COMPETENT CELLS OF *E. coli* K10

OD_{600} — OD of culture measured at λ_{600} before harvesting
L-agar — Non selective agar plate
L-agar amp — Selective plate for ampicillin resistance
TNC — Too numerous for visual counting
*Transformation efficiency was expressed as total number of transformants which produced colonies per *μg.* of reference plasmid; pBR322)

TRANSFORMATION EFFICIENCY OF COMPETENT CELLS (*cft. μg. pBR322*$^{-1}$)	TOTAL NUMBER. OF TRANSFORMANTS (*cft. ml.*$^{-1}$)
9.72×10^5	2268

TABLE 7.1 (b)

TRANSFORMATION OF *E. coli* K10 WITH PLASMID pJOE4

Cells which were made competent after growing in 100ml L-broth to OD_{600} 0.255 and whose transformation efficiency with pBR322 is shown in table 7.1a, were transformed with hybrid plasmid pJOE4, containing the *ppc gene*, and plated in 100μl aliquots after expression in 1ml L-broth. Transformants were selected for ampicillin resistance.

STRAIN of *E.coli* K10	PEPc ACTIVITY (*nmol. mg.*$^{-1}$. *min*$^{-1}$)
Parent	10.2 (±3.5)*
E. coli K10-pBR322	6.6 (±1.8)*
E. coli K10-pJOE4	533 (±16.0)*

TABLE 7.2

PHOSPHO*ENOL* PYRUVATE CARBOXYLASE ACTIVITIES OF ULTRASONIC CELL-EXTRACTS OF *E.coli* K10 STRAINS (PARENT, TRANSFORMANTS WITH PLASMIDS pBR322 AND pJOE4)

*Activities reported represent the mean of 3 independent assays monitored by the extinction of NADH at λ_{340nm}. The pBR322-transformant was used as vector control.

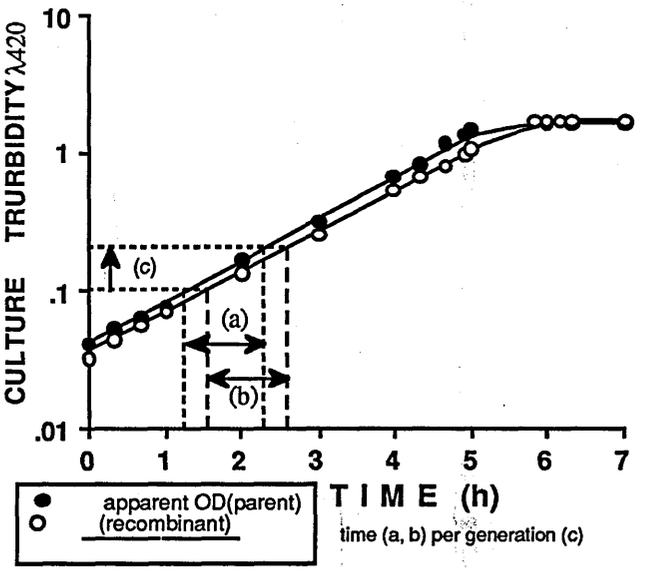
7.5 Comparison of Glucose Metabolism in Parent and Transformants

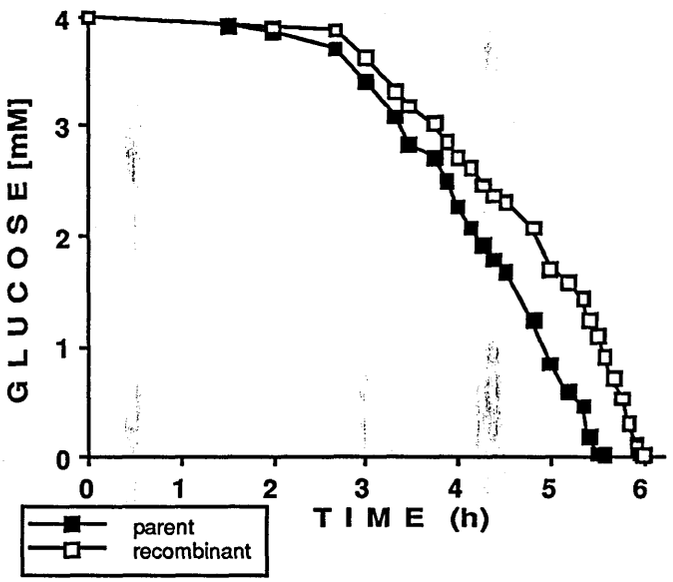
Biomass production and acetate excretion during aerobic glucose metabolism in 800ml batch (4mM) glucose cultures of parent and recombinant strains were measured and compared. The effect of the vector was determined by using the pBR322-transformant.

Growth rates were 0.69 and 0.67 *hour* $^{-1}$ in parent (K10) and recombinant (*E. coli* K10-pJOE4) strains respectively (figure 7.4). The growth rate of *E. coli* K10-pBR322 was 0.70 *hour* $^{-1}$.

E. coli K10-pJOE4 produced 7% more biomass than the parent (Table 7.3). Molar growth yields ($Y_{GLUCOSE}$) were 96.35 and 90.2 *g.* dry weight. *mol.* glucose respectively. These represented substrate inputs of 10.38 and 11.09 *mmol.* glucose-6-phosphate. *g.* dry weight.$^{-1}$ respectively.

In aerated batch culture, *E.coli* K10 excreted 0.389*mol.* acetate. *mol.* (glucose).$^{-1}$, compared with 0.25*mol.* acetate. *mol.*$^{-1}$ in *E.coli* K10-pJOE (figure 7.6). Reduction in acetate excretion due to overexpression of PEPc activity, was 35.7% (Table 7.3).





FIGURES 7.4 (a) & (b)

GLUCOSE METABOLISM BY *E. coli* K10 AND *E. coli* K10-pJOE4 IN AEROBIC BATCH CULTURES

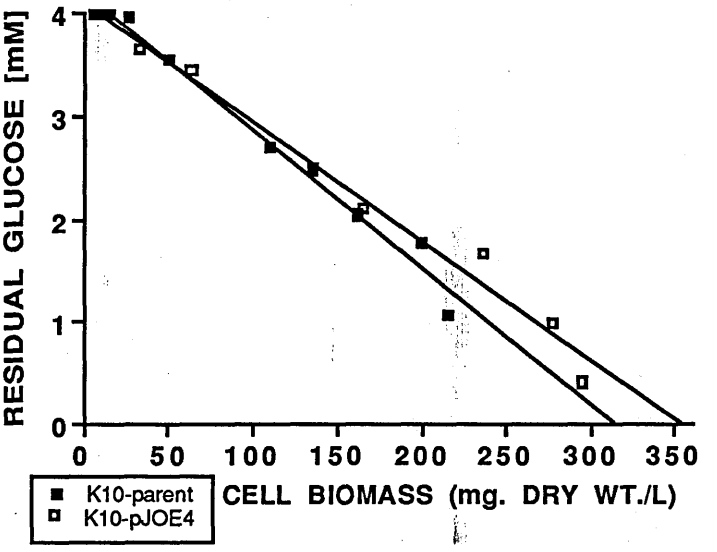


FIGURE 7.5 RELATIONSHIP BETWEEN GLUCOSE UTILIZATION AND BIOMASS PRODUCTION ($Y_{GLUCOSE}$)

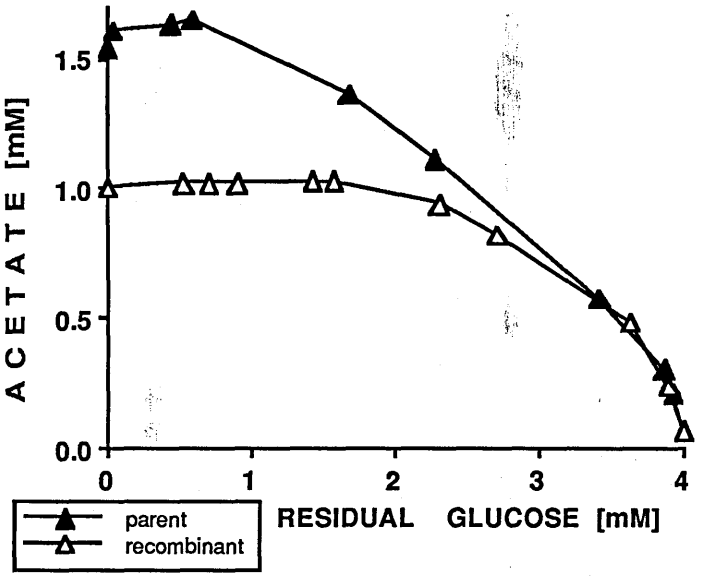


FIGURE 7.6 EXCRETION OF ACETATE BY *E. coli* K10 AND *E. coli* K10-pJOE4 GROWING AEROBICALLY ON GLUCOSE

7.6. Comparison of Enzyme Activities and Glucose Metabolism by *E. coli* K10 and *E. coli* ATCC15224

E.coli K10 required thiamine [3$\mu g.\ ml.^{-1}$] as an essential supplement for aerobic growth on glucose. This concentration did not contribute significantly to the derivation of throughputs. The growth rate on thiamine-supplemented glucose medium was 0.70 $hour^{-1}$, and was 28% slower than *E.coli* ATCC15224. Phospho*enol*pyruvate carboxylase activity in cells of *E.coli* K10 from glucose culture, was 42% less than the corresponding activity in *E.coli* ATCC15224. Both differences did not have significant effects on the difference in growth yields between both strains, which was 0.3%. However, there was a substantial difference in acetate excretion. Output of 4.31 *mmol. g.* dry weight.$^{-1}$ in strain K10 was 24.5% lower than strain ATCC15224, which was equivalent to the difference in growths between the two strains (table 7.3).

Amplification of PEPc activities in *E. coli* ATCC15224 and *E. coli* K10 by transformation with plasmid pJOE4 improved growth yield and reduced acetate excretion significantly at marginally lower growth rates. The observations suggested that the effects of amplified PEPc activity were not unique to *E.coli* ATCC15224. There were however, a few important quantitative differences in the effects. The level of overexpression of enzyme activities were 52 and 75-fold in strains K10-pJOE4 and ATCC15224-pJOE4 respectively. This had only minimal effects on growth rates and the net amount of acetate detected in culture, between the recombinant strains. When the outputs of acetate were expressed as functions of the growth yields of the respective parent strains, it was apparent that the effects of higher PEPc activities were more pronounced in *E.coli* ATCC15224. This was because of the 26% enhanced growth yield achieved in *E.coli* ATCC15224-pJOE4 in contrast to 7% in *E.coli* K10-pJOE4.

The most significant difference between *E.coli* ATCC15224 and *E. coli* K10 was the frequency of transformants with pBR322. The transformation efficiency of competent cells of *E.coli* ATCC15224 was only just above 1% of the corresponding experimental values obtained in competent cells of *E.coli* K10.

	Strains of *Escherichia coli*			
	PARENT		RECOMBINANT	
PARAMETER DETERMINED	‡K10	ATCC15224	§K10	¶ATCC15224
PEPc ACTIVITY *(nmol.mg.PROTEIN.$^{-1}$. min.$^{-1}$)*	**10.2**(0.42)	**24.4**	**533**(52)	**1819**(3.4)
GROWTH RATE *(hour^{-1})*	**0.70**(0.77)	**0.90**	**0.67**(0.95)	**0.87**(1.3)
$Y_{(GLUCOSE)}$ *(g. dry weight. mol.$^{-1}$)*	**90.2**(0.99)	**90.5**	**96.35**(1.07)	**114.4**(1.2)
ACETATE OUTPUT *(mmol. g. dry weight.$^{-1}$)*	**4.31**(0.76)	**5.69**	**2.59**(0.60)	**2.34**(0.90)
TRANSFORMATION EFFICIENCY *(cft. µg. pBR322.$^{-1}$)*	**9.72 x 10^5** (84.0)	**1.16 x 10^4**	—	—

TABLE 7.3

COMPARISON OF THE EFFECTS OF OVEREXPRESSION OF PEPc ON THE AEROBIC METABOLISM OF GLUCOSE BY *E. COLI* K10 AND *E. COLI* ATCC15224

Values in parentheses are relative to corresponding amounts in ‡(ATCC15224 PARENT) §(K10 PARENT) ¶(K10 RECOMBINANT)

7.7 Overexpression of PEPc Activity in a Pyruvate Kinase-negative Strain

7.7.1 Characterization of Strains Used

E.coli HW1387 (F^- λ^- *tyr A* del: :Km^R *pykF* : : Tn5 *pykA*) is a pyruvate-kinase (A & F)-negative mutant. Its parent strain is *E.coli* HW0760 (F^- λ^- *tyrA del*::Km^R). The parent was constructed from *E.coli* K-12 by forced recombination to delete the *tyrA* gene; kanamycin resistance was conferred by a *Bam*HI-*Bgl* II insert. The *pykF* mutation was produced by a Tn5 insertion (therefore *E.coli* HW 1387 carried a double kanamycin resistance determinant) while *pykA* was chemically induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG).

Colonies of *E.coli* HW 1387 and *E.coli* HW0760 were tested on solid minimal media to establish their characteristic nutritional requirements. Neither strain was impaired in glucose utilization, except on media deficient in L-tyrosine. In the absence of thiamine, growth was not significantly impaired. Both strains were resistant to kanamycin, but sensitive to ampicillin. Growth on sugars whose metabolism did not require fluxes through the PTS system, clearly differentiated the strains from each other (Table 7.4). *E.coli* HW 1387 was totally impaired in the ability to utilize D-glucuronate and L-arabinose, while *E.coli* HW0760 utilized both for normal growth (Table 7.4).

TEST MEDIA	STRAINS			
	HW0760		HW1387	
	24h	48h	24h	48h
L-AGAR KANAMYCIN	+	+ +	+	+ +
L-AGAR AMPICILLIN	–	– –	–	– –
GLUCOSE ((+) L-TYROSINE)	+	+ +	+	+ +
GLUCOSE ((-) L-TYROSINE)	–	– –	–	– –
GLUCOSE ((-)THIAMINE. HCl)	∓	±	–	±
L-ARABINOSE (+ KANAMYCIN)	±	+	–	– –
D-GLUCURONATE (+ KANAMYCIN)	+	+	–	– –

TABLE 7.4

SUBSTRATE UTILIZATION AND NUTRIENT REQUIREMENT BY *E.coli* HW0760 AND *E. coli* HW1387

Colonies of *E.coli* HW0760 and *E.coli* HW 1387 on L-agar were replica-plated on :

A) L-agar kanamycin (kan) [50μg. ml.$^{-1}$]
B) L-agar ampicillin [100μg. ml.$^{-1}$]
C) Complete glucose medium :
i) 10mM glucose in minimal salts
ii) 50μg. ml.$^{-1}$ L-tyrosine
iii) 3μg. ml.$^{-1}$ thiamine.HCl
iv) 50μg. ml.$^{-1}$ kanamycin
D) (C), less L-tyrosine
E) (C), less thiamine.HCl
F) (C), with 10mM glucuronate for glucose
G) (C), with 12mM L-arabinose for glucose

Plates were incubated for a maximum of 48 hours

(-)	Deficient Nutrient	(+)	supplement in medium
–	no growth (?)	+	growth (?)
--	confirmed negative growth	++	confirmed positive growth
±	weakly positive		

7.7.2 Transformation and Glucose Metabolism

Competent cells of E.*coli* HW0760 and *E.coli* HW 1387 (mean transformation efficiency. > 2.5 x 10^6 *cft.* $\mu g.$ pBR322.$^{-1}$), were transformed with hybrid plasmid pJOE4, containing the *ppc* gene. Transformants of each strain which overproduced PEPc were coded HW1387J (*pykA; pykF;* pBR322-*ppc*$^+$) and HW0760J (*pykA*$^+$ *pykF*$^+$*;* pBR322-*ppc*$^+$) respectively.

Cells of *E.coli* HW0760 and *E. coli* HW1387 (parent and PEPc-overproducing strains) were grown on glucose as described previously (section 2.8). Medium for the main batch culture consisted of 800ml, 4mM glucose, supplemented with thiamine-HCl, L-tyrosine, and kanamycin respectively. In addition, cultures of the recombinant strain were supplemented with 100$\mu g.$ *ml.*$^{-1}$ ampicillin to maintain the hybrid plasmid. Growth, substrate utilization and acetate excretion were monitored and analysed as described in previous chapters (4.2; 5.2; 6.2).

Growth and substrate utilization were typical of substrate- limited aerated batch cultures (Figure 7.7). The growth rate of the pyruvate-kinase negative mutant (HW1387) was 0.47$h.^{-1}$ (Table 7.5). This was marginally slower than the parent (HW0760). The difference was eliminated in the PEPc-overproducing strain, HW1387J. The amplification of PEPc activity also increased the growth rate of strain HW0760J by 5.6% (figure 7.9)

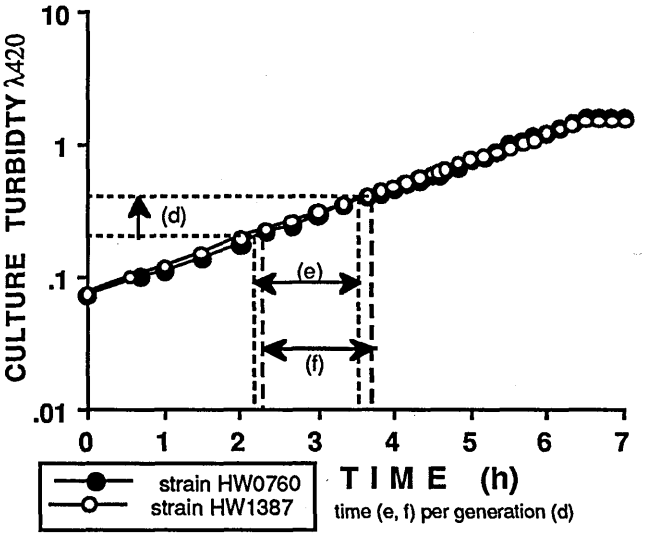


FIGURE 7.7 GROWTH OF *E. coli* HW1387 AND *E. coli* HW0760 IN AEROBIC GLUCOSE CULTURE

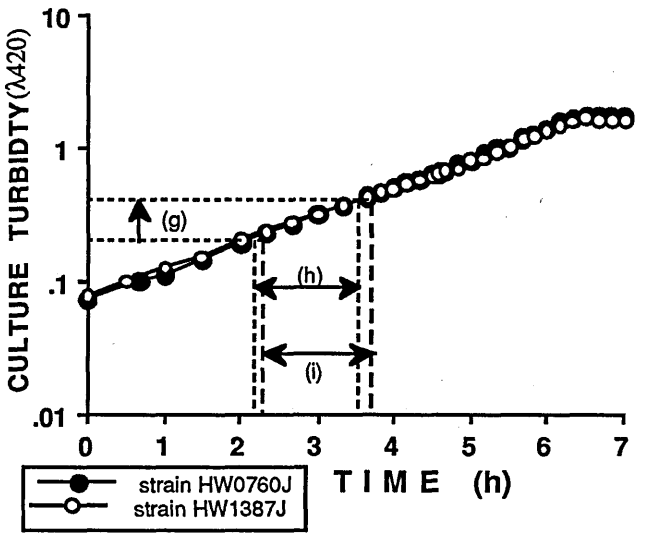


FIGURE 7.8 GROWTH OF PEPC-OVERPRODUCING STRAINS OF *E. coli* HW0760 AND HW1387 ON GLUCOSE

(g) generation time for (h) HW0760J; (i) HW1387J

7.7.3 SUBSTRATE UTILIZATION AND PRODUCTIVITY

Rate of substrate utilization increased marginally in PEPc overproducing strains (figures 7.9 and 7.10). Molar growth yields ($Y_{GLUCOSE}$) also increased marginally in the recombinant strains (Table 7.7). Growth yield was improved by about 3.0% in the pyruvate-kinase negative strain after transformation with hybrid plasmid pJOE4 (i.e.strain HW1387J). A functional pyruvate kinase activity increased $Y_{(GLUCOSE)}$ by only 1.% in strain HW0760. A combination of restored pyruvate kinase activity and amplified PEPc activity (i.e strain HW0760J) improved carbon utilization by 6.6% (Table 7.6). All improvements were however fractions of the $Y_{(GLUCOSE)}$ of *E.coli* K10 (Table 7.7).

A total of 172 samples taken over 7 hours were assayed by HPLC from the four cultures. All failed to show the presence of any acetate. No other significant excreted products were detectable.

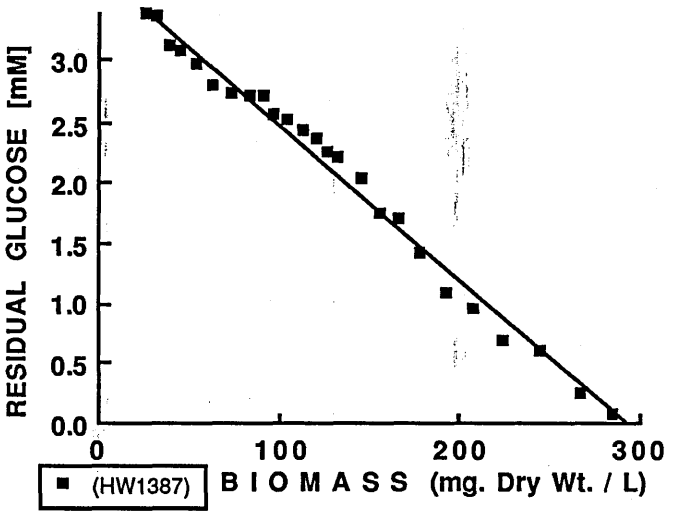


FIGURE 7.9 RELATIONSHIP BETWEEN GLUCOSE UTILIZATION AND BIOMASS PRODUCTION BY *E.coli* HW 1387

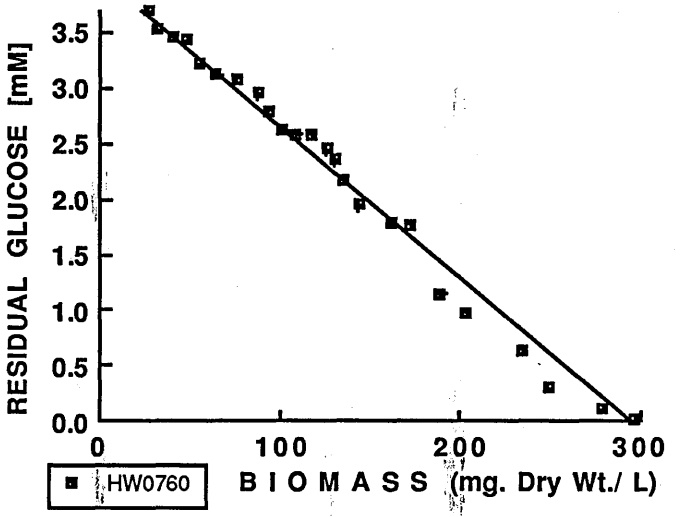


FIGURE 7.10 RELATIONSHIP BETWEEN SUBSTRATE UTILIZATION AND BIOMASS PRODUCTION BY *E.coli* HW 0760

Strain of *E. coli*	Relevant Genotype	Growth Rate (μ) (h^{-1})	Acetate Output (*mmol. g.* dry weight.$^{-1}$)	$Y_{(GLUCOSE)}$ (*g.* dry weight *mol.* substrate.$^{-1}$)	Percentage Difference in $Y_{(GLUCOSE)}$*
HW1387	(*pykA;pykF*)	**0.46(0.65)**	**0**	**71.3(0.79)**	—
HW0760	(*pykA*$^+$;*pykF*$^+$)	**0.47(O.66)**	**0**	**72.1(0.80)**	**1.1**
K10	(Prototype)	**0.70(1.0)**	**4.3**	**90.2(1.0)**	**22.6**
		TRANSFORMANTS			
HW1387J	(*pykA;pykF* ;pBR322-*ppc* $^+$)	**0.47(0.66)**	**0**	**73.1(0.81)**	**2.6**
HW0760J	(*pykA*$^+$;*pykF*$^+$; pBR322-*ppc* $^+$)	**0.49(0.70)**	**0**	**76.3(0.85)**	**7.0**
K10J	(*ppc* $^+$; pBR322-*ppc* $^+$)	**0.67(0.96)**	**2.6**	**96.4(1.07)**	**35.1**

TABLE 7.5

SUMMARY OF GROWTH RATES AND METABOLISM OF GLUCOSE BY *E. COLI* K10, *E. COLI* HW1387, *E. COLI* HW0760 BEFORE AND AFTER OVEREXPRESSION OF PEPC ACTIVITIES

(* Percentage differences in $Y_{GLUCOSE}$ are relative to the value in *E. coli* HW1387. Values in parentheses are relative to the equivalent amounts of parameter in *E. coli* K10. Strains with "J" carried the recombinant plasmid pJOE4 and overexpressed PEPc activities).

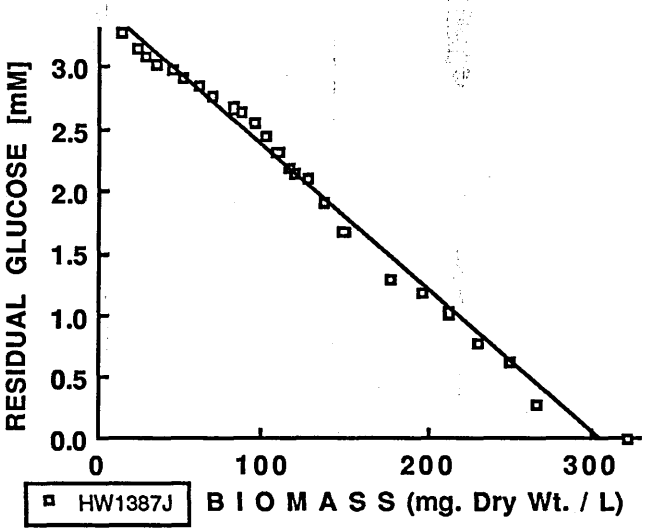


FIGURE 7.11 RELATIONSHIP BETWEEN SUBSTRATE UTILIZATION AND BIOMASS PRODUCTION BY *E.coli* HW 1387J

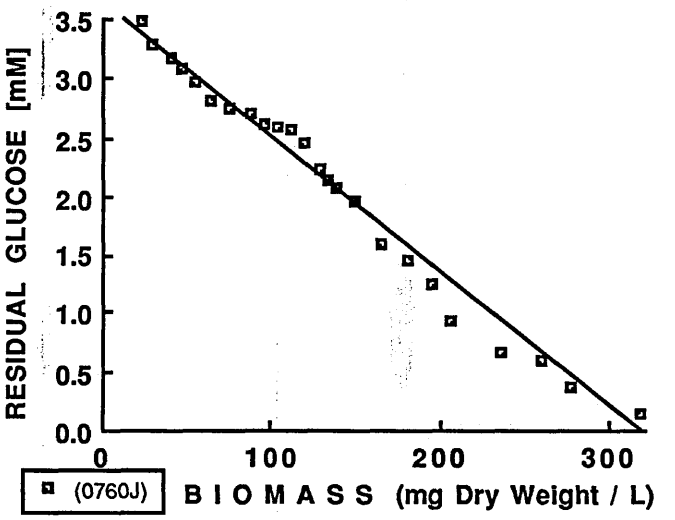


FIGURE 7.12 RELATIONSHIP BETWEEN SUBSTRATE UTILIZATION AND BIOMASS PRODUCTION BY *E.coli* HW 0760J

7.8 DISCUSSION

During this study, *E.coli* K10 was used as a prototrophic strain for comparative assessment of the effect of PEPc overexpression on the metabolism of *E.coli* HW 1387 which carried a lesion for pyruvate kinase. Its pedigree was closer to *E. coli* HW1387 than *E.coli* ATCC15224 which was selected initially because the excellent characterization of its physiology and metabolic fluxes provided a comprehensive framework for assessing the effect of any genetic alteration on enzymes of its metabolic pathways. However, this advantage was limited mainly because it was not easily manipulated genetically. The successful transformation of the cells during this study was the exception, not the rule and most competent cells frequently lost viability when preserved by standard methods (*see* 4.3.1; table 4.2). By contrast, *E.coli* K10 became artificially competent by non-fastidious methods. A stationary overnight culture from a single colony was sufficiently competent to yield a high frequency of transformed cells after the usual treatments even after prolonged storage at -70°C in 20% (v/v) glycerol. Although the intrinsic PEPc activity of *E.coli* K10 was 40% of that detected in crude cell extracts of *E.coli* ATCC15224, transformation with hybrid plasmid pJOE4 produced 50-fold enhanced activity and reduced acetate excretion by 36%. Some of the carbon redirected from acetate excretion was incorporated into new cell biomass, resulting in 7% increased growth yield.

Compared to strain ATCC15224, the percentage improvement of biomass yield was much less in *E.coli* K10 (figure 7.5). It was not possible to determine the fate of substrate carbon which was conserved by diminished flux to acetate excretion in the *recombinant* strain of *E.coli* K10 since throughput to biosynthesis was enhanced to a lesser extent than in *E. coli* ATCC15224. In addition, neither HPLC nor total organic carbon (TOC) analyses detected any other outputs which were quantitatively significant. It is however possible that additional outputs to amino acids may result from PEPc over-production. Sugita and Komatsubara (1989) constructed a threonine–*'hyperproducing'* strain of *Serratia marcescens* by amplifying its PEPc activity using a plasmid which contained a *ppc*gene-insert from *E.coli*. Increase in flux of oxaloacetate to ancillary metabolic pathways which did not result in new biomass was predictable in the strain. None of the amino acid-outputs originating from oxaloacetate was assayed in cultures of *E.coli* K10-pJOE4 growing on glucose.

Although some characteristics of *E. coli* K10 were quantitatively different from *E. coli* ATCC15224, it was a good reference strain for assessing the effect of overexpression of PEPc activity on glucose metabolism by *E. coli* HW1387 and *E. coli* HW0760. These effects included diminished carbon flux to acetate excretion and improved biomass yield in both strains. The throughputs underlying these events were derived for *E. coli* ATCC15224-pJOE4 (figures 4.15; 4.16) and flux analysis suggested that the observed events occurred with negligible flux of carbon through pyruvate kinase. The present study was designed to test this hypothesis.

Although pyruvate kinase was an important glycolytic enzyme, its absence was not critical to aerobic metabolism of carbohydrates which are transported by the phospho*enol*pyruvate:sugar phosphotransferase (PTS) system (Garrido-Pertierra and Cooper, 1977). Indeed, during starvation and gluconeogenesis the enzyme was practically inoperative (Cooper and Kornberg, 1967; Thompson, 1987). The throughput of PEP to pyruvate in the PTS system could meet all cellular requirements for pyruvate independent of the output of the pyruvate kinase flux (Garrido-Pertierra and Cooper, 1977). As such, the hypothesis of zero carbon flux through pyruvate kinase in *E.coli* ATCC15224-pJOE4 was not unrealistic, but required testing.

Glucose metabolism by *E.coli* HW 1387, a pyruvate kinase-negative mutant was investigated using *E.coli* HW0760 as a positive (*pyk*$^+$) control. Both strains grew slowly at μ of 0.46 and 0.47 $hour^{-1}$ respectively and failed to excrete acetate in aerated batch cultures. Enhancement of their PEPc activities by transformation with recombinant plasmid pJOE4 produced marginal increases in growth rates and eliminated the difference which previously existed between the growth rates and growth yields of the pyruvate-kinase negative mutant (HW1387) and its parent (figure 7.8). The effect of PEPc overproduction in HW0760J was equivalent to the effect on other prototrophic strains used in this study (section 7.5).

The generation time of all the HW-strains on glucose ranged from 85 to 91 minutes which represented very low growth rates. A major physical strategy for decreasing acetate excretion by bacteria was the reduction of their growth rates. Holms (1986a) had observed that reduction of the growth rate of *E.coli* ML308 on glucose from 0.94 to 0.72hour^{-1} in continuous culture prevented acetate excretion. Non-excretion of acetate by all the HW-strains was therefore predictable given their relatively low growth rates. It was difficult to explain why the growth rate of strain HW0760 which possessed a functional pyruvate kinase gene was substantially less than *E. coli* K10 and was barely different from the pyruvate kinase-negative mutant or why it did not excrete acetate (table 7.5).

The marginal increase in growth yield of *E.coli* HW 1387J was comparable to corresponding increases in *E.coli* ATCC15224-pJOE4 growing on gluconate and glucuronate where single fluxes operated from PEP to pyruvate as was the case in the pyruvate kinase-negative strain. The margin of increase in growth yield of strain HW0760J was comparable to K10-pJOE4 (Table 7.3).

Preliminary tests had demonstrated that absence of pyruvate kinase activity was critical only when non-PTS substrates were sole carbon and energy sources (Table 7.4). A higher PEPc activity in HW1387J did not alter this situation. During glucose metabolism however, there was no significant change in growth rate or growth yield as a result of absence of pyruvate kinase activity (table 7.5; cf. *E. coli* HW1387 and *E. coli* HW0760). This corroborated a previous observation that a functional PTS system was an adequate alternative route for generating the output of the reaction mediated by pyruvate kinase of *Escherichia coli* (Pertierra and Cooper, 1977). Consequently, strains which carried lesions for pyruvate kinase, such as HW1387 are not severely impaired in the ability to grow on glucose. Differences in growth rates and growth yields between (*pykA*$^{+}$,*F*$^{+}$) and pyruvate kinase-negative strains were increased after overexpression of PEPc activity (Table 7.7).

STRAIN	CARBON INPUT	‡ % DIFFERENCE
E.coli HW 1387	84.13	—
E.coli HW0760	83.25	1.1
E.coli HW 1387J	82.00	2.5
E.coli HW0760J	78.64	6.5
E.coli K10	66.48	21.0
E.coli K10J	62.22	26.0

TABLE 7.6

COMPARATIVE INPUT OF SUBSTRATE-CARBON INTO CENTRAL METABOLISM BY *E. coli* HW 1387 AND OTHER STRAINS GROWING AEROBICALLY ON GLUCOSE

Values were derived from $Y_{glucose}$ of each strain and represent the amounts of substrate-carbon used to produce biomass in each culture. The amounts were standardized for 1 gram of biomass and therefore expressed as *mmol.* substrate carbon *g.* dry biomass.$^{-1}$. Strains with "J" suffixes overproduced PEPc

‡ (% Difference) represents the expression of the differences in input-carbon into the central pathways between other test strains and strain HW1387-the pyruvate kinase negative strain as percentage functions of the input in HW1387.

A functional pyruvate kinase in *E. coli* HW0760 would compete with three other enzymes for PEP (figure 7.2) and increase throughput to acetyl-CoA by providing an additional flux-channel to pyruvate beside the PEP-PTS route. In the absence of pyruvate kinase PEP would be partitioned among the other three fluxes with the possibility of diminished fluxes to pyruvate, acetyl-CoA and possibly acetate excretion. Results obtained with *E. coli* HW1387 and *E. coli* HW1387J (*ppc*$^+$) suggested that the absence of pyruvate kinase precluded flux to acetate excretion possibly because of low throughput to acetyl-CoA. In the event that this level is too low for maximal activity of PEPc in strains transformed with plasmid pJOE4, synergistic activation by FBP would become more pronounced. However, comparison of the growth of the parent strains (*E. coli* HW1387 and *E. coli* HW0760) suggested that synergistic activation of PEPc by fructose-1,6-*bis* phosphate could not have influenced metabolism in the *pyk*$^+$ strains significantly. Moreover, analysis of samples from a glucose culture of *E. coli* HW0760 which had a functional pyruvate kinase showed absence of acetate, suggesting that the lesion of *E. coli* HW0760 also prevented the excretion of acetate. Thus, although the results presented have not disputed the underlying hypothesis of this study, the observations with the control strain—*E. coli* HW0760 make them equivocal.

The energetics of pyruvate production by the PTS and pyruvate kinase routes are different since the former is energy-consuming and the latter yields 1mol. of ATP (figure 7.1). The additional flux to pyruvate through pyruvate kinase could also enhance throughput to acetyl-CoA which would favour both amplified PEPc activity of *E. coli* HW0760J and a higher condensation ratio of acetyl-CoA and oxaloacetate which potentially favoured biosynthesis (Table 7.5). It is also proposed that these may account for the differences between the growth rates/yields of *E. coli* HW1387J and E. coli HW0760J (Table 7.7). The available acetyl-CoA would normally be limited by the absence of a pyruvate kinase flux in *E.coli* HW 1387 and *E.coli* HW 1387J, considering that there were no carbon fluxes to acetate excretion. This can potentially balance the ratio of acetyl-CoA to oxaloacetate and enhance carbon conversion coefficient.

	§PERCENTAGE DIFFERENCE (%)	
$\frac{(HW0760-HW1387)}{HW1387} \times 100$	**PARENTS** *(HW0760 & HW1387)*	**RECOMBINANTS** *(HW0760J & HW1387J*
GROWTH RATE	**2.2**	**5.6**
GROWTH YIELD	**1.05**	**4.1**

TABLE 7.7

COMPARISON OF GROWTH RATES AND GROWTH YIELDS IN (*pykA, F*) AND (*pykA*$^+$, *F*$^+$) HW-STRAINS

§ Values used were experimental differences between (*pyk*$^+$) and pyruvate kinase-negative mutants before and after transformation with pJOE4 and were expressed as percentage functions of the growth rates and growth yields of the pyruvate kinase-negative strains (HW1387 or HW1387J)

7.8.1 Evaluation of the Role of Pyruvate kinase in Glucose Metabolism by the HW-strains Used.

Apart from the characteristic differences in the abilities of strains HW1387 and HW0760 to utilize non-PTS sugars (Table 7.4), there were no remarkable differences in their metabolic activities during aerobic growth on glucose (figures 7.7, 7.9, 7.10). Consequently, evaluation of the role of pyruvate kinase in the differences between the growth rates and growth yields of *E. coli* HW1387J and *E. coli* HW0760J (Table 7.7) was ambiguous. It is obvious that the slow growth rate of *E. coli* HW1387 was inherited largely from its parent, *E. coli* HW0760 whose growth rate was only half of *E. coli* ATCC15224 and which also failed to excrete acetate (Tables 4.1; 7.5). Both observations in the parent have introduced a new dimension to the evaluation of the influence of amplified PEPc activity on carbon flux through pyruvate kinase in *Escherichia coli* particularly in relation to acetate excretion and biomass production. Although these results provided new information in themselves, it is not possible to use them to corroborate the deductions made from the analysis of carbon flux in *E. coli* K10/*E. coli* K10-pJOE4 or *E. coli* ATCC15224/*E. coli* ATCC15224-pJOE4. Nevertheless they suggest that a higher PEPc activity does not have adverse effects on aerobic metabolism of glucose by *Escherichia coli* even when the additional demand for PEP by PEPc precludes flux through pyruvate kinase in the process (figure 4.16). Most of the findings in *E. coli* HW1387 could not be attributed solely to the pyruvate kinase lesion since this study had the benefit of a closely-related control strain to eliminate secondary factors. The growth and metabolic patterns of the HW-strains with or without amplified PEPc require further investigation which is outside the scope of the present study.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

8.1 General Discussion

The initial objective of the work reported in this thesis was to evaluate the effect of increased phospho*enol*pyruvate carboxylase (PEPc) activity on the efficiency of fluxes through the central metabolic pathways during the aerobic utilization of selected carbon sources. The study was carried out primarily in *E. coli* ATCC15224. The enzyme catalyses a strategic metabolic flux which forms a unidirectional bridge between glycolysis and the tricarboxylic (TCA) cycle, mediating the direct throughput of a central metabolite of the phosphorylated amphibolic route to a carboxylated intermediate product. In this position, it competes for phospho*enol*pyruvate (PEP) whose partition is central to the output of about 70% of amino acids, 50% of nucleotides and other monomers for cellular biosynthesis (figure 8.1)

Carbon flux through the enzyme is therefore critical to general metabolism and its control is particularly crucial to the metabolism of facultatively anaerobic systems like *Escherichia coli*. The multiplicity of allosteric effectors associated with the enzyme (table 3.1; Teraoka *et al.*, 1974) may account in part for reports of its low level albeit, steady activity in *E. coli* (Cánovas and Kornberg, 1965; Izui *et al.*, 1981). This also means that the net throughput of PEP to oxaloacetate is maintained at a steady level (Sanwal, 1970) estimated at 2.75*mmol. g.* dry weight $^{-1}$ (Holms, 1986a). This step also influences the quantitative oxidation of acetyl-CoA, operation of the TCA cycle, net throughputs to 2-*oxo*glutarate, succinyl-CoA and therefore, the precise biosynthetic ratios of precursors drained from the amphibolic pathways (figures 1.2; 1.3). A combination of these diverse effects explains why the characteristics of PEPc-negative mutants (e.g. *E. coli* PA342) include inability to grow on glucose, glycerol or pyruvate (Ashworth and Kornberg, 1966).

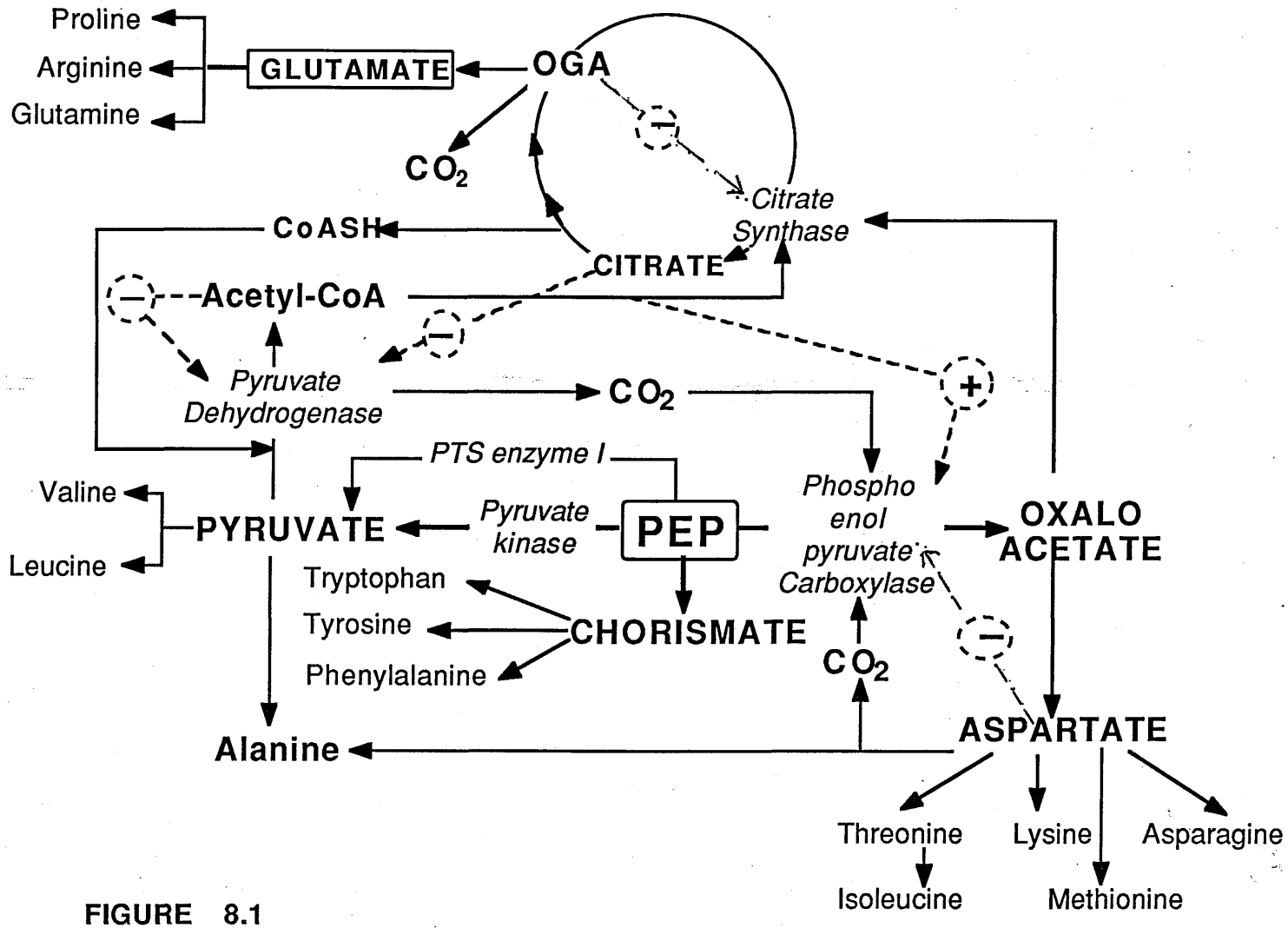


FIGURE 8.1

CENTRAL ROLE OF PEP IN THE DIRECT AND INDIRECT THROUGHPUTS TO SYNTHESIS OF MAJOR AMINO ACIDS

During aerobic metabolism of glucose, fructose, glycerol, pyruvate, gluconate and glucuronate, *E. coli* ATCC15224 operated fluxes through PEPc from PEP which varied according to the specific growth rates sustained by each substrate (table 8.1).

CARBON SOURCE	FLUX THROUGH PEPC (*mmol. g.$^{-1}$. h.$^{-1}$*)
GLUCOSE	**2.48** (0.90)
FRUCTOSE	**2.11** (0.77)
GLYCEROL	**1.98** (0.72)
PYRUVATE	**1.54** (0.56)
GLUCONATE	**2.59** (0.94)
GLUCURONATE	**2.34** (0.88)

TABLE 8.1

FLUXES OF PEP THROUGH PEPC DURING THE METABOLISM OF VARIOUS CARBON SOURCES BY *E. coli* ATCC15224

(Values in parentheses are specific growth rates in aerobic batch cultures during this study)

Given the considerable amphibolic demands on the TCA cycle, this flux required some augmentation not only by accelerated growth rate, but also by quantitative enhancement of net throughput. A simple approach to achieving this was to enhance enzyme activity by derepression or by gene amplification (Mortlock, 1982; Wöhrl *et al.*, 1990). The latter allowed greater freedom for technical manoeuvre. During this study, flux through PEPc was amplified by increasing the dosage of the *ppc* gene which encodes PEPc. The gene was cloned by complementing the *ppc* mutation of *E. coli* PA342, a PEPc-negative mutant, from a gene bank containing hybrid plasmids of pBR322 and *E. coli* genomic DNA. Plasmids from transformants which grew on unsupplemented glucose as sole carbon and energy source were screened by replica-plating,

restriction enzyme analysis and agarose gel electrophoresis resulting in the designation of recombinant plasmid pJOE4 as pBR322-*ppc*$^{+}$. The relevant segment of the restriction map of the plasmid was consistent with the published sequence of the *ppc* gene. The plasmid re-transformed *E. coli* PA342 and complemented its *ppc* lesion, confirming that the preliminary observation was not merely due to reversion of the *ppc* marker. The specific activity of PEPc in ultrasonic extracts of *E. coli* PA342-pJOE4 was 3.536μ*mol. mg.*$^{-1}$. *min.*$^{-1}$ compared with zero activity in the parent. The enzyme was stable at 4°C and required acetyl-CoA for quantifiable activity. Cells grown in the presence of L-aspartate showed 25% lower activity. These observations strongly suggested that the molecular characteristics of the enzyme-protein produced by the recombinant strains were similar to the properties of the enzyme in wild-type strains. Limited site-directed deletion of bases from the gene at a unique sequence recognized by the restriction enzyme *Sac*II, produced recombinant plasmid pJOE12 which carried a frameshift mutation which inactivated the gene. PEPc activity was barely detectable from cell-extracts of *E. coli* PA342-pJOE12 from glucose culture.

E. coli ATCC15224 had an intrinsic PEPc activity of 24.4*nmol.mg.*$^{-1}$*min.*$^{-1}$. Transformation with hybrid plasmid pJOE4 amplified the enzyme activity 75-fold to 1.820μ*mol. mg.*$^{-1}$. *min.*$^{-1}$. The metabolic activities of both strains were compared on selected carbon sources in aerated batch cultures. The metabolism of glucose, gluconate, glucuronate and pyruvate by *E. coli* ATCC15224 resulted in fluxes to acetate excretion in amounts which varied with both net throughputs and specific growth rates (table 8.2). These represented significant effluxes of input carbon from the central metabolic pathways and were symptomatic of a disproportionate partition of fluxes in the amphibolic pathways towards anabolic throughputs for the synthesis of precursors in precise biosynthetic ratios (figures 1.2; 1.3). The branch-point to acetate excretion diverged at acetyl-CoA, about 2.64 *mmol. g.*$^{-1}$ of which was required for biosynthesis of leucine and fatty acids at a ratio of 1:5.6 (figure 1.2; Morowitz, 1978; Holms, 1986a). Although the throughput of acetyl-CoA to the TCA cycle varied with carbon source and the conditions of culture, it depended invariably on the availability of oxaloacetate as a coreactant and carrier-molecule (Krebs, 1981; Fisher and Magasanik, 1984), and flux through PEPc.

Since acetyl-CoA and oxaloacetate are biosynthetic precursors and their relative throughputs to this demand are constant, the flux to acetate excretion would be an inverse function of the amount of acetyl-CoA oxidized in the cycle. The strategic location, flux and output of PEPc places it in an excellent position to influence acetate excretion quantitatively. Results obtained during this study showed that increased flux through PEPc with a predictable increase in throughput to oxaloacetate in *E. coli* ATCC15224-pJOE4, diminished carbon fluxes to acetate excretion by 25 to 60% on various carbon sources relative to the parent strain (table 8.2).

*Substrate	Fluxes to Acetate (*mmol. g.* dry weight.$^{-1}$. *h.*$^{-1}$)		§Output in Recombinant relative to Parent (%)
	Parent	Recombinant	
Glucose	5.12	2.04	41.1
Gluconate	7.62	5.77	75.7
Glucuronate	12.24	10.38	84.8
Pyruvate	18.2	12.73	66.5
Fructose	0	0	0
Glycerol	0	0	0
E. coli K10	3.02	1.74	60.1
E. coli HW 1387 (*pyk*)	0	0	0
E. coli HW0760 (*pyk*$^+$)	0	0	0

Table 8.2

Comparison of Fluxes to Acetate Excretion in Parent and PEPc-overproducing Strains of *E. coli* Growing on Selected Substrates

§ The output of acetate in each strain which overproduced PEPc has been expressed as percentage function of the corresponding value in its parent strain (column 4).

* The data presented for the 6 carbon sources relate to *E. coli* ATCC15224. *E. coli* K10, HW1387 & HW0760 were grown on glucose only.

The explanation for diminished flux to acetate excretion must reckon with *cause-and-effect* factors contributing to acetate excretion. Throughput to acetyl-CoA depended on the pivotal decarboxylation of pyruvate through the pyruvate dehydrogenase multienzyme complex (Patel and Roche, 1990), while pyruvate itself derived from dephosphorylation of PEP by unidirectional fluxes through pyruvate kinase and the equilibrated reactions of the phospho*enol* pyruvate:sugar phosphotransferase (PTS) system (figures 1.4; 5.1; 7.1). These reactions collectively effected the throughput of 80% of PEP to pyruvate (figure 4.15), leaving 20% for partition (1:5) between the biosynthesis of aromatic amino acids and throughput to oxaloacetate (figure 8.3).

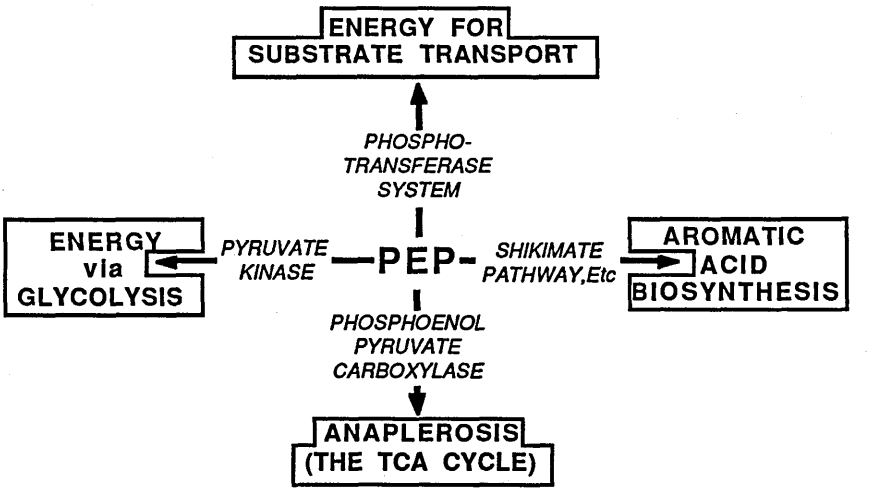


FIGURE 8.2 METABOLIC DEMANDS FOR PEP IN *Escherichia coli*

The large fluxes to pyruvate and acetyl-CoA were out of proportion to the biosynthetic ratios (Morowitz, 1978) which throughputs to other biosynthetic precursors could support and hence they could not be used optimally. The acetyl moiety of superfluous acetyl-CoA was converted to acetate. Since *E. coli* did not accumulate storage products (Linton, 1990; Wanner and Egli, 1990), acetate was excreted. However, when PEPc activity was overexpressed, throughput to oxaloacetate was significantly enhanced. This new steady state could only occur by increased flux of PEP through the carboxylating route which is thought to be *anaplerotic* , with a corresponding adjustment of fluxes through other divergent fluxes (figure 8.2) in proportion to the magnitude of increased flux through PEPc (Kacser and Porteous, 1987).

Analysis of fluxes in *E. coli* ATCC15224-pJOE4 suggested that the burden for balancing the partition of flux at PEP, while accommodating the increased demand due to overexpression of PEPc activity, was borne overwhelmingly by pyruvate kinase (Sanwal, 1970; Thompson, 1987). During growth on glucose, a zero-flux through this enzyme was possible without adverse impairment of growth or general metabolism (figure 4.16). Under this condition, throughput to pyruvate was effected *via* the PTS system (Pertierra and Cooper, 1977; 1983). The net effect of abolition of flux through a redundant pyruvate kinase was a net reduction in throughput of PEP to pyruvate which subsequently reduced throughput to acetyl-CoA and eventually diminished acetate excretion. Results in this study supported this postulate (figures 4.16; 6.9; 6.11).

Moreover, increased throughput to oxaloacetate had far-reaching effects on metabolism. Krebs (1981) observed that the availability of this intermediate metabolite was a key factor in the operation of the TCA cycle, where it fulfils multiple roles as a reactant, product, mandatory carrier molecule for acetyl-CoA and a precursor of citrate, 2–*oxo*glutarate, succinyl-CoA and biosynthesis (figures 1.2; 1.3; 4.21; 8.1; Krebs, 1937; 1940b; Krebs and Eggleston, 1940; Fisher and Magasanik, 1984). It is also a co-reactant in the flux through citrate synthase, which is rate-limiting for the TCA cycle (figure 4.22; Weitzman, 1987). Indeed, the entire *Krebs* cycle could be regarded as an insurance against the depletion of oxaloacetate (Krebs, 1942) which is primarily supplied through phospho*enol* pyruvate carboxylase or similar routes. This was clearly an improvement on the anaerobic system where two independent, non-cyclic fluxes diverged from oxaloacetate (Gest, 1987) to generate succinyl-CoA and α-ketoglutarate for biosynthesis (Spencer and Guest, 1987), without the capability to regenerate oxaloacetate (Luinenburg and Coleman, 1990). Frequently, the excretion of acetate in facultative anærobes was a sequel of the complete shutdown of aerobic metabolism, inhibition of enzymes of the TCA cycle (Namdari and Cabelli, 1990) and the possible derepression of anaerobic enzymes (Bell, Cole and Busby, 1990). This was capable of curtailing the entry and oxidation of acetyl-CoA in the TCA cycle and may signal a critical decline in the supply of oxaloacetate.

A key difference between aerobic and anaerobic metabolism is a functional TCA cycle. If as speculated, anaerobiosis predated aerobic metabolism (Gest, 1981), facultatively anaerobic systems like *Escherichia coli* could be viewed as advanced anærobes which have exploited molecular oxygen to develop a cyclic and more efficacious process for maintaining their internal pools of oxaloacetate (Krebs, 1981). However, flux analysis suggests that this advantage has not been exploited optimally as evidenced by excretion of acetate even under very generous aeration (Namdari and Cabelli, 1989; 1990). During the present study, cells were grown in batch cultures and aerated by vigorous magnetic stirring coupled with supplementary input of 200*cc.* compressed air/*min.* In spite of this, *E. coli* ATCC15224 excreted 0.52*mol.* acetate/*mol.* glucose and comparable amounts on pyruvate, gluconate and glucuronate (figures 4.5; 4.17; 5.7; 6.8; 6.10). Even when acetate was not excreted (e.g. during fructose & glycerol metabolism) this could not be attributed to aeration alone (tables 5.1; 8.2). Appraisal of *open* and *closed* flux control factors in central metabolism (Crabtree and Newsholme, 1987) and the multiple effectors of PEPc in particular, suggest that the allosteric regulation of the enzyme remains reminiscent of the anaerobic scenario in *Escherichia coli.* It is known that the absence of a cyclic pathway for generating biosynthetic precursors means that energy cannot be generated by that route under anaerobic conditions (Luinenburg and Coleman, 1990). However, the mandatory biosynthetic requirements for both 2-*oxo*glutarate and succinyl-CoA are met jointly by the unidirectional carboxylation of PEP to oxaloacetate via PEPc and the horizontal partition of flux at oxaloacetate between citrate synthase and malate dehydrogenase (Spencer and Guest, 1987). Fluxes to the C_4-dicarboxylic acids are initiated by direct NAD^+-mediated reduction of oxaloacetate to malate. The C_6-branch and parallel reduction to citrate required acetyl-CoA as a second reactant (figure 8.3). If (for the purpose of argument) the direct carboxylation of PEP via PEPc preceded its dephosphorylation via pyruvate kinase and oxaloacetate was produced before (and independent of) acetyl-CoA which required two major reaction-steps, partition of flux at oxaloacetate would severely undermine throughputs to the C_6-branch and favour the C_4-dicarboxylic acid branch overwhelmingly. To preempt this, flux through PEPc was virtually nil

until fluxes through the PTS, pyruvate kinase and pyruvate formate lyase (or pyruvate dehydrogenase in aerobes) produced acetyl-CoA in significant activating levels (Sanwal and Maeba, 1969; vanUrk *et al.*, 1989; 1990). Acetyl-CoA remains the single most powerful activator of PEPc activity in *E. coli* even during aerobic metabolism (Cánovas and Kornberg, 1969; Sanwal, 1970). During the present study, PEPc activity was difficult to demonstrate in ultrasonic extracts of cells from aerobic cultures when the assay cocktail (2.7.2; figure 2.1) did not include acetyl-CoA. The activity of the PEPc in *E. coli* K10 was only 10.2*nmol. mg.*$^{-1}$. *min.*$^{-1}$ and 24.4*nmol. mg.*$^{-1}$. *min.*$^{-1}$ in *E. coli* ATCC15224 (tables 4.1; 7.2). These levels were not substantially higher than those reported for some anaerobic organisms (Brune and Schink, 1990; Luinenburg and Coleman, 1990). Conversely, it is noteworthy that the most powerful group of inhibitors of aerobic PEPc activity (table 3.1) remained intermediates from the C_4-dicarboxylic branch of the primordial anaerobic pathways or their derivatives (figure 8.3).

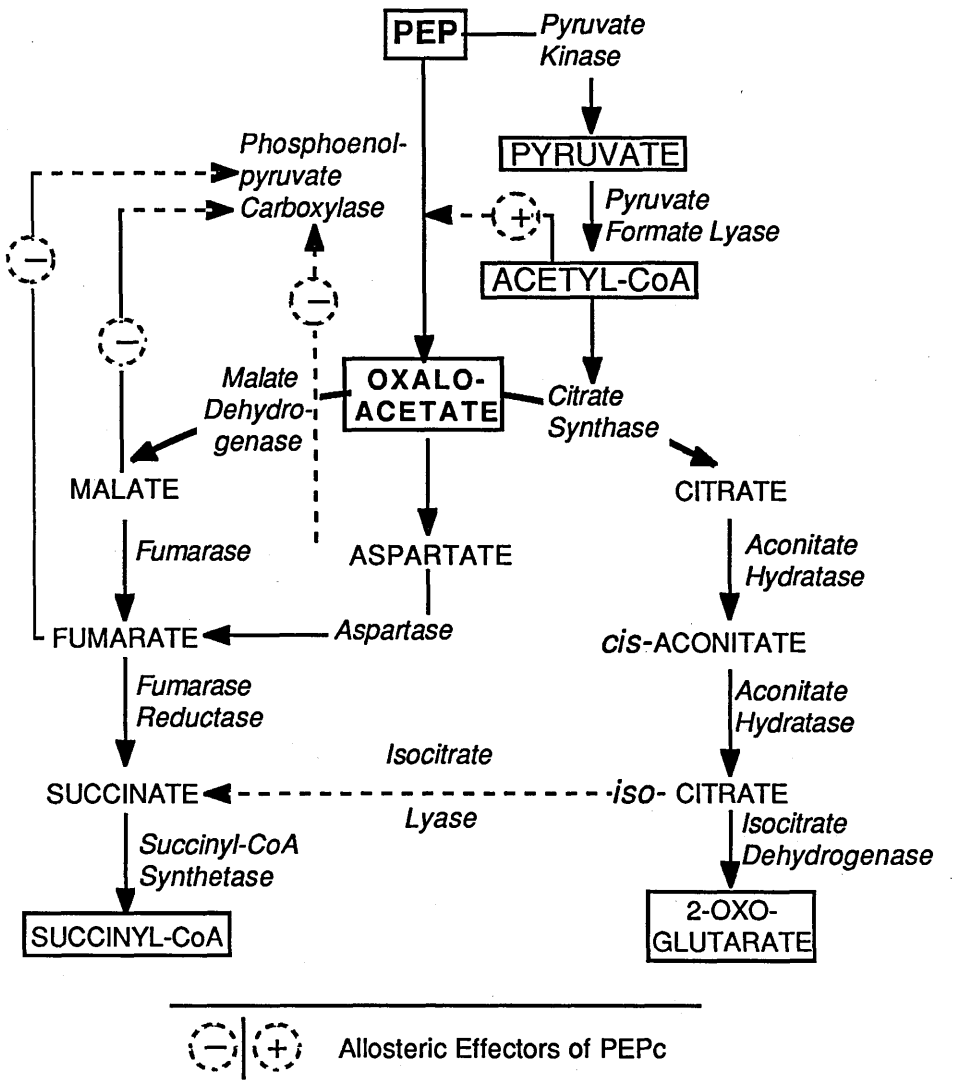


FIGURE 8.3

THE CENTRAL PATHWAYS OF ANAEROBIC METABOLISM IN *Escherichia coli* : EFFECTORS OF PEPc ACTIVITY

Biosynthetic precursors are highlighted by boxes. Dashed arrows with circles indicate the relationship between enzyme and effectors

In all probability, these inhibitors acted as subsidiary modulators to balance the partition of flux at oxaloacetate such that flux to the C_4-dicarboxylic acid half was not prodigal in the anaerobic state. The allosteric sensitivity of PEPc to inhibition by C_4-intermediates was balanced by its positive sensitivity to activation by acetyl-CoA which feeds into the alternative flux to C_6-intermediates and 2-*oxo*glutarate. The development of the TCA cycle clearly retained elements of this anaerobic regulation of partition of flux at oxaloacetate. Under physiological aerobic conditions and enhanced output of NADH, oxaloacetate has a threshold concentration below which its flux through malate dehydrogenase is highly unfavourable (Sanwal, 1970). Hence the TCA cycle resulted apparently from the physiological annexation of the C_4-half of the anaerobic flux from oxaloacetate by the C_6-section, while the rate-limiting role of citrate synthase is also reminiscent of the need to balance the formation of citrate with malate in anaerobic metabolism. It would be recalled that flux of oxaloacetate through malate dehydrogenase was initially repressed *per force*, to make flux through citrate synthase possible in anaerobes. In *E. coli* ATCC15224, *E. coli* K10, and *E. coli* HW0760 growing aerobically on glucose or other substrates, flux through PEPc was small but steady (Holms, 1986a) The magnitude of flux was positively correlated with increasing concentrations of acetyl-CoA (Ashworth and Kornberg, 1966) but negatively with increasing concentrations of aspartate, malate, fumarate or their derivatives, as in other strains of *E. coli* (Sanwal and Maeba, 1966; Yoshinaga *et al.,* 1970; 1974; Fujita *et al.,* 1984). In spite of the emergence of the TCA cycle for regenerating oxaloacetate and synchronizing fluxes to succinyl-CoA and 2-*oxo*glutarate, the allosteric modulation of PEPc on which primary throughput to oxaloacetate depended, did not improve on the anaerobic arrangement (*cf.* figures 4.21; 8.3). It is difficult to rationalize the operation of these control mechanisms during aerobic metabolism. Indeed, one possible diasdvantage of a preemptive flux of PEP to pyruvate & acetyl-CoA rather than oxaloacetate when cells grew aerobically on glucose, could be a gross flux to acetyl-CoA which took account of the need to activate PEPc, fulfil biosynthetic demands and react with oxaloacetate (Kornberg, 1966; Gest, 1981; Holms, 1986b). Although some of the acetyl-CoA which activated PEPc would be incorporated into general metabolism, the initial and feedback constraints on flux through PEPc was bound to limit its net utilization. On a

wider scale, constraints on throughput to oxaloacetate could limit the net synthesis of cell material by coupling available biosynthetic precursors at sub-optimal ratios (Holms, 1987). Predictably, the superfluous precursors would include pyruvate and acetyl-CoA. This provides some rationalization for *aerobic acidogenesis* (Andersen and vonMeyenburg, 1980; Andersen *et al.,* 1984; Holms and Bennett, 1971; Luli and Strohl, 1990; Namdari and Cabelli, 1990) or the bacterial *Crabtree effect* (Hollywood and Doelle, 1976) in *E. coli.* From these observations, it is proposed that the low levels reported for PEPc in prototrophic strains of *E. coli* (Cánovas and Kornberg, Yoshinaga *et al.*, 1970; Izui *et al.;* 1981) are necessary features of its facultatively anaerobic lifestyle (Spencer and Guest, 1987; Koch, 1988) and anaerobic heritage (Jayaraman *et al.*, 1989). Indeed, it is highly probable that *E. coli* switches prematurely to anaerobic mode of nutrition even during exponential aerobic growth (Sanwal, 1970; Dawes and Large, 1982; Koch, 1988). The existence and mechanisms of these switches are well documented (Griffiths and Cole, 1987; Iuchi and Lin, 1987; Spencer and Guest, 1987; Jayaraman *et al.,* 1988, 1989; Iuchi *et al.,* 1989; Bell *et al.,* 1990). If this is tenable, it is most unlikely that the highly elevated activities of PEPc detected in *E. coli* ATCC15224-pJOE and similar derivatives of the hybrid plasmid containing the *ppc* gene, were due to enhanced expressionof the *ppc* gene. Amplification of PEPc activity was most probably a function of increase in gene dosage as reported previously by Izui *et al.* (1981).

Diminished fluxes to acetate excretion in the recombinant strains grown aerobically on various carbon sources, coupled with improvement in growth yields (table 8.3) clearly demonstrated that higher throughput to oxaloacetate was beneficial to cellular biosynthesis and could not be detrimental to general metabolism. It would be of considerable interest to use techniques of protein engineering to relieve PEPc of some allosteric constraints and evaluate the effect of this on microbial productivity and acetate excretion during aerobic metabolism.

SUBSTRATE/ ORGANISM/	CARBON CONVERSION COEFFICIENT (g.dry wt./g substrate carbon)		% IMPROVEMENT
	PARENT	RECOMBINANT	
GLUCOSE			
E. coli ATCC15224	1.26	1.60	27
E. coli HW0760	1.00	1.06	6
E. coli HW 1387	0.99	1.01	2.02
E. coli K10	1.25	1.34	7.2
E. coli ATCC15224			
FRUCTOSE	1.48	1.62	9.5
PYRUVATE	0.49	0.53	6.9
GLYCEROL	1.42	1.44	1.4
GLUCONATE	1.18	1.22	3.4
GLUCURONATE	0.87	0.91	4.6

TABLE 8.3

COMPARISON OF THE EFFICIENCY OF CARBON CONVERSION IN *ESCHERICHIA COLI* STRAINS GROWING AEROBICALLY ON SELECTED CARBON SOURCES

The efficiency of the TCA cycle in energy generation has often been emphasized above its amphibolic status in general and anabolic function (which depended on flux through PEPc) in particular. This is in spite of several reports that energy generation is subordinate to the greater biosynthetic demand for the generation of 2-*oxo*glutarate and oxaloacetate as in anaerobes (Sanwal, 1970; Gest, 1981; Dawes and Large, 1982; Fisher and Magasanik, 1984; Holms, 1986b). The energy output was only required to supplement any reduction in output of energy from glycolysis (Guest, 1981b).

The results obtained during this study have demonstrated that requirements for biosynthetic precursors produced by the cycle may be more prominent in influencing net growth yield than is normally assumed. In support of this view, reports of the consequences of relevant lesions of some mutants of *Escherichia coli* on glucose, glycerol or pyruvate metabolism have been inspected critically.

PEPc-negative mutants actively catabolized pyruvate without appreciable growth (Ashworth and Kornberg, 1966). Inability to synthesize oxaloacetate meant that they were neither able to oxidize pyruvate beyond acetyl-Co nor initiate a functional TCA cycle. As a result, acetyl-CoA entered into ancillary fluxes yielding and excreting acetate. Mutants which were deficient in citrate synthase shared similar nutritional lesions with PEPc-negative mutants (Wilde and Guest, 1986). Although they possessed a functional PEPc enzyme and were competent in the production of oxaloacetate, they could neither metabolize it to generate 2-*oxo*glutarate nor regenerate oxaloacetate since they lacked a full and functional TCA cycle. Growth after nutritional supplementation with derivatives of 2-oxoglutarate was only possible by the energy generated largely from glycolysis, since the supplement could neither complement the *gltA*. mutation for citrate synthase nor restore a full/functional TCA cycle.

Fumarase-negative mutants dramatized the key role of the synthesis and regeneration of oxaloacetate through PEPc and the TCA cycle even more vividly. It was shown that they utilized malate as sole source of carbon and energy, but not acetate. On glucose, their growth rate and yield were nearly as good as the wild-type (Guest and Roberts, 1983). Lesion in fumarate hydratase did not impair the ability of fumarase-negative mutants to synthesize oxaloacetate, 2-*oxo*glutarate or succinyl-CoA but only diminished energy output by interrupting oxidative phosphorylation and the regeneration of oxaloacetate by the TCA. In that regard, the fumarase-negative mutant utilized the TCA cycle almost like anærobes (Luinenburg and Coleman, 1990).

The partition of carbon flux at PEP through pyruvate kinase & pyruvate dehydrogenase on the one hand, and PEPc on the other is a master plan for the parallel decarboxylation and carboxylation of PEP to yield the two principal reactants of the TCA cycle sequentially from glucose, fructose, glycerol, pyruvate. Although unique junctions and flux control mechanisms may exist to achieve this objective on each input (figure 8.4), it is evident that this is a reason why cells retain flux through citrate synthase as a rate-limiting step for the *Krebs* cycle (Weitzman, 1981) even though it is apparently a dispensable vestige of anaerobic metabolism. Comparison of fluxes to oxaloacetate and acetyl-CoA on various substrates revealed that the partition of fluxes in carbon sources which sustained fluxes to acetate excretion was disproportionately in favour of throughput to the latter. Hence acetate excretion was a safety valve for releasing the acetyl-CoA for which no oxaloacetate was available.

One substrate which generated more oxaloacetate than was required to oxidize acetyl-CoA and yield 2-*oxo*glutarate, was acetate itself (Holms, 1986b). Here, throughput to oxaloacetate actually depended on acetyl-CoA. It is possible to visualize the glyoxylate bypass as shunting the TCA cycle to produce oxaloacetate more quickly. The system was balanced between a net 45% flux to biosynthesis and 55% to energy generation with low growth rate (Holms, 1987). It is significant that this is one substrate where the primary throughput to oxaloacetate was not subject to the stringency of flux through PEPc. No flux to excreted products was therefore necessary. This contrasted with the metabolism of pyruvate where the initial output of oxaloacetate depended on two highly-regulated reactions. Flux through PEP synthase which initiated pyruvate metabolism, was also the rate limiting step (Saier and Chin, 1990). The PEP produced must then be carboxylated to oxaloacetate. In the meantime, partition of fluxes at pyruvate yielded acetyl-CoA which is a mandatory activator of PEPc. The intracellular concentrations of PEP synthase and PEPc are low (Cánovas and Kornberg, 1965; Cooper and Kornberg, 1965) and rely heavily on activators to function (Sanwal, 1970; Geerse *et al.*, 1989). As a result, the output of acetyl-CoA far exceeded the rate of supply of oxaloacetate. The same was applicable to glucose, gluconate and glucuronate. In the last two, the partition of flux at 2-KDPG was particularly unfavourable to PEP (hence oxaloacetate) production (figures 6.8; 6.10; 8.4; table 8.4). This was reflected in the large fluxes to acetate on both substrates.

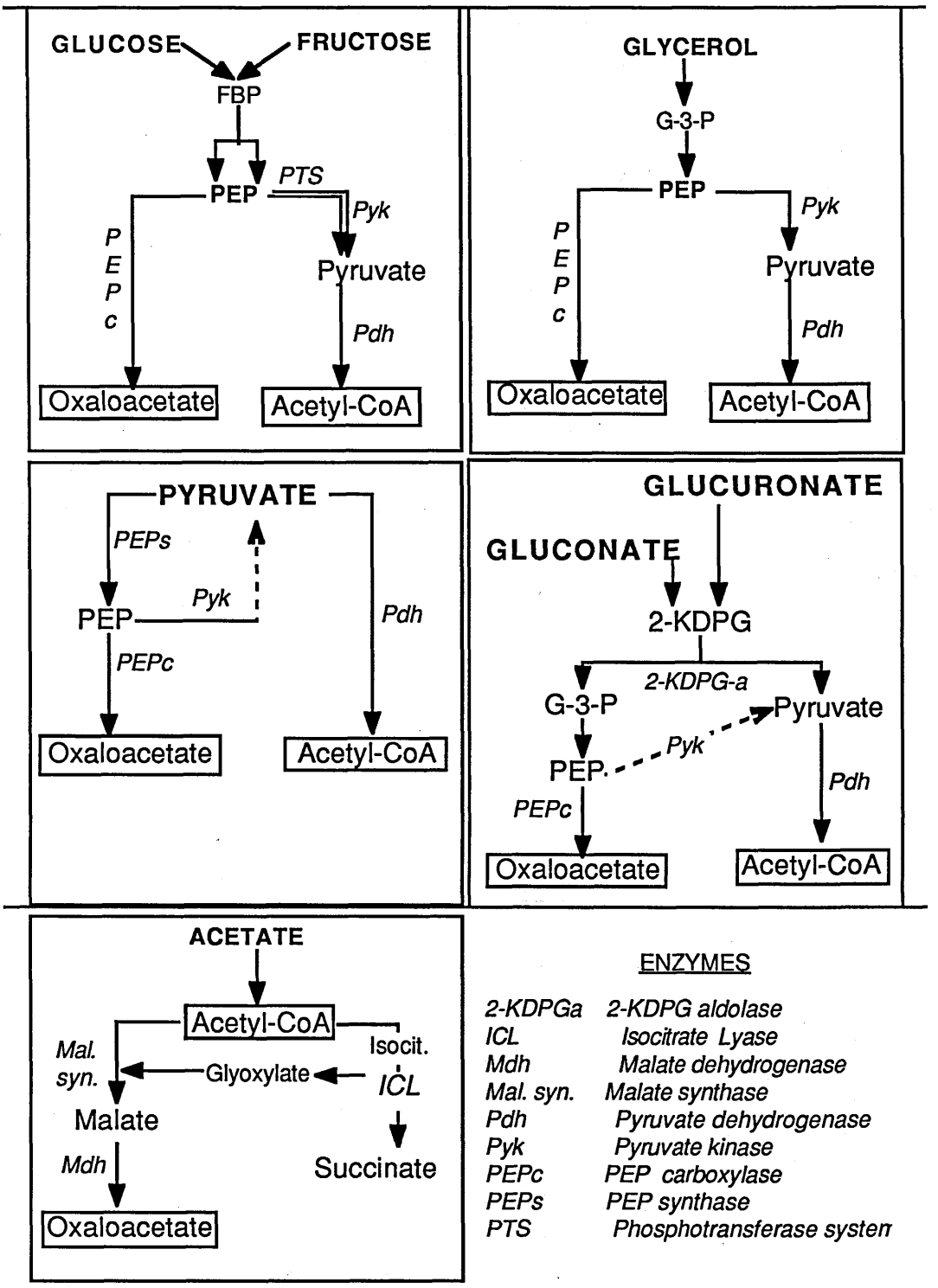


FIGURE 8.4: CARBON FLUXES TO ACETYL-COA AND OXALOACETATE DURING AEROBIC GROWTH ON DIFFERENT CARBON SOURCES BY *Escherichia coli*

8.1.1 THE ROLE OF ACETYL-CoA : OXALOACETATE RATIO IN THE REGULATION OF ACETATE EXCRETION

All the substrates used in this study produced both acetyl-CoA and oxaloacetate as precursors for biosynthesis (table 8.3). After making allowance for this requirement, the ratio of throughput to non-biosynthetic acetyl-CoA *vis-a-vis* oxaloacetate was critical to acetate excretion. For substrates where the ratio attained unity, acetate was not excreted. This was the case on fructose and glycerol as well as glucose metabolism by the pyruvate kinase-negative strain. On the other hand, throughput to acetate increased in proportion to increases in the *acetyl-CoA: oxaloacetate* ratios on glucose, pyruvate, gluconate and glucuronate (table 8.4). A simple approach to the problem of acetate excretion appears to be the reduction of the ratio of non-biosynthetic acetyl-CoA to oxaloacetate. Since the biosynthetic requirements are constant for each compound (figure 1.2), the ratio can be expressed as a simple function of the net flux of carbon to each intermediate. The strategy may involve the reduction of net throughput to acetyl-CoA and/or increasing fluxes to oxaloacetate. Previously, it had been observed that acetate excretion on pyruvate was a function of the ratio between fluxes to PEP synthase and pyruvate dehydrogenase (Mansi and Holms, 1989). It is proposed here that this ratio was effective only because it was instrumental in diminishing the more general acetyl-CoA: oxaloacetate ratio.

During this study, both goals were attained by controlling flux at PEP. Amplification of PEPc activity increased throughput to oxaloacetate. By computing the carbon fluxes of input through the known routes to the known outputs, by the known growth rate, it appeared that the level of overexpression of PEPc increased the demand for PEP to the extent that flux through pyruvate kinase was virtually abolished (figure 4.16). Overexpression of PEPc activity in *E. coli* HW 1387 (*pykA, pykF*) supported this computation. The net effect of this would include diminished throughput to pyruvate and acetyl-CoA. In all the recombinant strains which carried the plasmid pJOE4 (pBR322-*ppc*$^{+}$), the acetyl-CoA:oxaloacetate ratio was reduced significantly. As a result, PEPc-overproducing strains excreted between 25 and 60% less acetate than their respective parent strains. The reduction of the ratio was greater in substrates

which sustained larger throughputs to PEP, like glucose where acetate excretion was diminished by 58.9%. As for gluconate and glucuronate, the ED pathway yielded 1mol PEP *via* glyceraldehyde-3-phosphate. Overexpression of PEPc diminished acetate excretion by about 25%.

A decrease in the acetyl-CoA:oxaloacetate ratio also favoured increased flux to biosynthesis. As demonstrated in this study, diminished throughput to acetate was frequently accompanied by increased growth yield in strains where PEPc activity was enhanced genetically (Table 8.3). This was only possible through a generalized increase in the output of precursors for biosynthesis. Already, the bacterial *Crabtree* effect reflected high fluxes through glycolysis (vanUrk *et al.*, 1990) in contrast to diminished fluxes through the TCA cycle (Namdari and Cabelli, 1989). The observed inhibition of growth due to the *Crabtree* effect (Pan *et al.*, 1987) may be explained in part by a critical reduction in the output of some biosynthetic precursors, especially from the TCA cycle. Although oxaloacetate was not primarily *generated* from the cycle, its net yield depended on *regeneration* through the operation of the cycle. Moreover, the yield of 2-*oxo*glutarate relied largely on oxaloacetate (Krebs and Eggleston, 1940). Both intermediates were sole or joint-precursors for the synthesis of a number of amino acids and nucleotides (figures 1.2; 1.3; 8.1).

The excretion of acetate represented high flux through glycolysis (or parallel reactions) and a high yield of the biosynthetic precursors derived therefrom. Under the *Crabtree* effect, flux of acetyl-CoAinto the TCA cycle was minimal. Consequently, the acetyl-CoA:oxaloacetate ratio would be extremely high, thus making acetate excretion a necessary remedial efflux (Andersen and vonMeyenburg, 1980; Namdari and Cabelli, 1990). The resulting metabolic pattern simulated anaerobic growth where oxaloacetate was produced through PEPc but not regenerated by the TCA cycle. The observed increases in biomass yields when PEPc activity was overexpressed during this work (table 8.3) suggested that throughputs to both biosynthetic precursors were enhanced. This was reflected in net increase in growth yield which was not always complemented by increase in specific growth rate, except marginally on pyruvate and glucuronate (tables 5.1; 6.1).

CARBON SOURCE FOR AEROBIC GROWTH	NET THROUGHPUT§ (*mmol . g. dry wt.*$^{-1}$) Acetyl-CoA (Ac.CoA)	Oxaloacetate (OAA)	(Ac.CoA) / (OAA)‡
GLUCOSE	8.72	3.52	2.48
FRUCTOSE	7.46	7.46	1.00
PYRUVATE	42.41	16.33	2.59
GLYCEROL	4.32	4.32	1.00
GLUCONATE	9.81	1.70	5.77
GLUCURONATE	18.37	4.00	4.59
ACETATE	33.18	33.19	0.99

TABLE 8.4
COMPARISON OF THE NET THROUGHPUTS OF CARBON TO ACETYL-COA AND OXALOACETATE IN *E. coli* ATCC15224

§ "Net" refers to net balance of carbon throughput to metabolite in the parent strain, after making allowances for their relative biosynthetic demands

‡ net balance of throughputs to acetyl-CoA as function of oxaloacetate (acetate data based on Holms, 1986a)

Since oxaloacetate fed directly into the primary rate-limiting step of the TCA cycle (Weitzman, 1981; Wilde and Guest, 1986), citrate synthase could assume an even more significant regulatory function as throughput to oxaloacetate increased. In addition, the increased demand for substrate by the amplified PEPc could make PEP a limiting metabolite. It is known that unidirectional flux of PEP through pyruvate kinase regulates the net rate of glycolysis (Dawes, 1986; vanUrk *et al.*, 1989). As noted earlier (figure 4.16), the degree of overexpression of PEPc in this study could potentially make the pyruvate kinase reaction redundant. The growth rate of the pyruvate kinase-negative strain used during this study was as low as half the rate in prototrophic strains (table 7.5). The observed growth in the recombinant strain could therefore reflect a compromise between diminished flux of PEP through pyruvate kinase and its enhanced flux through PEPc to oxaloacetate.

Concluding Remarks

Evidence from the present study supports the view that aerobic cultures of *Escherichia coli* excrete acetate because flux control mechanisms were not designed to partition input carbon to intermediate metabolites required as biosynthetic precursors in proportion to the relative amounts at which they are required for biosynthesis.

It has been demonstrated during this study that carbon fluxes can be manipulated at PEP to decrease the inefficiency associated with the constraints highlighted above. Amplification of PEPc activity diminished throughput of carbon to acetate and enhanced throughput to biosynthetic precursors from the TCA cycle concurrently. The latter contributed to improved growth yield on a wide range of carbon sources.

Increased throughput of PEP to oxaloacetate competitively diminished the flux of carbon through pyruvate kinase and eventually reduced throughput to acetyl-CoA. Both effects favoured reduced output of acetate and increased output of biomass at the same time.

Amplification of PEPc activity enhanced the conservation of input carbon with regard to acetate excretion while also improving the net conversion of substrate-carbon to biomass-carbon. Both effects represent the benefits of applying the techniques of genetic engineering to the manipulation of metabolism to construct a more efficient bioflux system.

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