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Characterization and Expression of *Pseudomonas Fluorescens* L-Arabinose Dehydrogenase: Genetic Elements in *Pseudomonas Putida*

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CHARACTERIZATION AND EXPRESSION OF *PSEUDOMONAS FLUORESCENS* L-ARABINOSE DEHYDROGENASE : GENETIC ELEMENTS IN *PSEUDOMONAS PUTIDA*

by

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Carol M. Wierenga, M.S.

Western Michigan University, 1995

An assay based on the spectrophotometeric properties of NAD/NADH was developed to detect the degradation of L-arabinose to L-arabinolactone. This assay was used to determine the expression of the L-arabinose utilization genes contained in *Pseudomonas fluorescens* and in the plasmid, designated pPZ221, contained in *Pseudomonas putida*. The results from this assay, in conjunction with various growth kinetics, indicate that pPZ221 is inducible and repressible for L-arabinose utilization. Thus, the *P. fluorescens* chromosomal insert in pPZ221 for L-arabinose utilization does appear to contain the regulatory genes necessary for controlling the expression of an L-arabinose operon.

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INTRODUCTION

Many studies have examined the growth and metabolic regulation in *Escherichia coli* grown on different carbohydrates. One system that has been well characterized in *E. coli* is that of L-arabinose utilization (Bustos & Schleif, 1993; Beverin *et al.*, 1971; Englesberg, 1971; Englesberg *et al.*, 1965; Hahn *et al.*, 1984; Heffernan & Wilcox, 1976; Hendrickson, Stoner, & Schleif, 1990; Horazdovsky & Hogg, 1989; Horazdovsky & Hogg, 1987; Hogg & Englesberg, 1969; Hou *et al.*, 1988; Kolodrubetz & Schleif, 1981; Lee *et al.*, 1981; Lichenstein *et al.*, 1987; Lobell & Schleif, 1990; MacInnes *et al.*, 1978; Novotny & Englesberg, 1966; Ogden *et al.*, 1980; Power & Irr, 1973; Redder & Schleif, 1991; Schleif, 1969; Sheppard, 1986; Sheppard & Eleuterio, 1976; Singer & Englesberg, 1971; Stoner & Schleif, 1983; Wilcox, 1974; Wilcox *et al.*, 1974). The known information about this pathway in this organism includes the genomic sequence, the catabolic pathway, and regulation of gene expression.

In *Pseudomonas*, however, utilization, transport, and regulation of various carbohydrates has not been well characterized. The L-arabinose utilization studies in *Pseudomonas* species have putative the catabolic pathway (Dagley & Trudgill, 1965; Doudoroff, 1962; Schmiz *et al.*, 1974; Weimberg & Dourdoroff, 1955). In order to examine the regulation and expression of enzymes in the L-arabinose utilization

prepared with the broad-host-range cosmid vector, pLA2917 (Allen & Hanson, 1985). The plasmid-containing strains were selected for L-arabinose utilization, and those strains that conferred L-arabinose utilization were furthered studied. In these experiments, we wished to determine if the regulatory elements for the expression of L-arabinose utilization were included in the chromosomal inserts that allowed *Pseudomonas putida* to utilize L-arabinose.

This objective was accomplished by developing reproducible assay conditions for L-arabinose dehydrogenase, and examining the induction of L-arabinose utilization, or lack thereof, in *P. fluorescens*, in *P. putida*, in *P. putida* containing pLA2917, and in *P. putida* containing pPZ221 by obtaining growth curves for these various organisms and testing for L-arabinose dehydrogenase activity.

REVIEW OF LITERATURE

Carbohydrate Utilization

Microorganisms are often able to utilize a number of different compounds as sole carbon and energy sources (Holt *et al.*, 1994). The range of compounds that each microorganism is able to use is characteristic for that organism (Holt *et al.*, 1994). *Pseudomonas fluorescens* is an aerobic fluorescent pseudomonad and is able to grow on a variety of carbohydrates as sole sources of carbon and energy (Lessie & Phibbs, 1984; Stanier *et al.*, 1966). Some of the carbohydrates that *P. fluorescens* grows on are unable to be utilized by *Pseudomonas putida*, a closely related aerobic fluorescent pseudomonad (Stanier *et al.*, 1966). L-arabinose is one such carbohydrate (Holt *et al.*, 1994; Lessie & Phibbs, 1984; Stanier *et al.*, 1966).

Carbohydrate Regulation

When an organism, such as *P. fluorescens* or *Escherichia coli*, is able to utilize a number of different sugars as carbon and energy sources, it must have control over the enzymes necessary for the degradation of each carbohydrate. There are several types of enzymatic regulation. The first such control is known as induction.

Induction is when an organism synthesizes an enzyme only in response to the presence of the substrate which the enzyme catabolizes (Brock & Madigan, 1988). If

the necessary substrate is not present in the medium, then the organism does not produce the enzyme. When a microorganism controls its enzyme production for a particular sugar in this fashion, the organism is said to be inducible for that substrate. Degradative enzymes are often inducible in microorganisms (Brock & Madigan, 1988).

Another mechanism microorganisms use to regulate enzyme production is known as repression. Repression is the process where the organism reduces, or totally inhibits, the synthesis of enzymes necessary for the degradation of one carbohydrate in the presence of another carbohydrate that is preferentially utilized (Brock & Madigan, 1988; Epstein & Beckwith, 1968; Magasanik, 1961; Paigen & Williams, 1970) In *E. coli*, glucose prevents the synthesis of the enzymes essential for the degradation of many other sugars (Epstein & Beckwith, 1968; Gottesman, 1984; McGinnis & Paigen, 1969; Paigen & Williams, 1970). It was later demonstrated that an intermediate in the glucose degradation pathway could repress the synthesis of enzymes for other carbohydrates in *E. coli.* (Magasanik, 1961).

Substrates, other than glucose, can inhibit enzyme production in other microorganisms. In *Pseudomonas* species, for example, succinate and other intermediates of the tricarboxylic acid (TCA) cycle have been found to repress the synthesis of enzymes essential for the degradation of other carbohydrates (Hamilton & Dawes, 1959; Hylemon & Phibbs, 1972; MacGregor *et al.*, 1991; Phillips &

Mulfinger, 1981; Temple *et al.*, 1994; Tiwari & Campbell, 1969; Wolff *et al.*, 1991; Zylstra *et al.*, 1989).

Methods for Studying Regulation

The fact that many microbes are able to utilize a large number of carbohydrates is intriguing because the regulation of gene expression may have multiple and varied mechanisms. How do these microorganisms control their various carbohydrate utilization pathways? In order to answer this question, several methods have been applied.

The first method of study is that of examining mutants of regulatory pathways. Such mutants have played a large part in the study of regulatory control. A mutant in a regulatory pathway can be used to identify the function of various components of the pathway. An early example of mutants serving in this capacity was a study by Perlman and Pastan (1969) using a strain of *E. coli* that was deficient in adenyl cyclase. This mutant was unable to grow on lactose, maltose, arabinose, mannitol, or gylcerol. It was only able to grow weakly on glucose, fructose, and galactose. When cyclic adenosine monophosphate (cAMP) was added to the medium, normal growth on these carbohydrates occurred. This observation allowed Perlman and Pastan to draw the conclusion that cAMP is necessary for *E. coli* to utilize a variety of carbohydrates (Perlman & Pastan, 1969).

Another mechanism which can be used to study regulation of carbohydrate utilization is isolating the genes of interest and the cloning them into a suitable expression vector. The vector may be inserted into appropriate surrogate hosts which normally do not express the trait that is contained in the vector (De Reuse & Danchin, 1991; Horazdovsky & Hogg, 1989; Horazdovsky & Hogg, 1987; Lichenstein *et al.*, 1987; Stoner & Schleif, 1983). This method can also be used to place the genes in mutant organisms to see if the inserted genes will complement the mutation of the host. This technique was used by Hendrickson *et al.* to characterize the *ara*FGH and *ara*J promoters of *E. coli* (1990) and Cuskey *et al.* to study genes for carbohydrate catabolism from *Pseudomonas aeruginosa* and *P. putida* (1985).

Of particular interest for this study is a cosmid cloning vector that was constructed by Allen and Hanson (1985). This vector, designated pLA2917, confers resistance to kanamycin and tetracycline. pLA2917 is a 21 Kb vector that was constructed from the broad-host-range pRK290. Allen and Hanson utilized pLA2917 to identify the genes that *Methylobacterium organoplium* need to grow on methanol (1985). This vector was utilized by Eisenberg and Phibbs (1988) to clone L-arabinose utilization genes from *P. fluorescens*. Eisenberg also used this vector to examine the *P. fluorescens* chromosome library for L-arabinose utilization genes. pPZ221 is the designation given to pLA2917 containing a 50 Kb chromosomal fragment from *P. fluorescens* that was examined in this project.

Other mechanisms scientists have used to investigate carbohydrate utilization pathways include the use of an analogue of the carbohydrate that is not metabolizable (Wilcox, 1974; Beverin *et al.*, 1971) or radioactively labeled carbohydrate in order to trace where it is shuttled in the cell (Hogg & Englesberg, 1969) or measurement of the mRNA transcription product of the genes (Power & Ітт, 1973).

Examples of Regulation Systems

lac Operon

There have been many regulatory systems that have been studied over the years. One of the most commonly studied systems is the *lac* operon in *E. coli*. The *lac* operon, consisting of the genes *lacI*, *lacZ*, *lacY*, *lacA*, is involved in the degradation of lactose to galactose and glucose. *LacI* is the regulator gene. *LacA*, *lacY*, and *lacZ* comprise the structural genes. *LacA* codes for thiogalactoside transacetylase. *LacY* and *lacZ* code for β-galactoside permease and β-galactosidase, respectively, which are essential for the transport and hydrolysis of lactose. These enzymes are coded for by a single mRNA molecule (Hartl, 1991; Epstein & Beckwith, 1968).

The *lac* operon has been shown to be subject to two types of control (Hartl, 1991; Schwartz & Beckwith, 1970). The first type of control is the negative control system of the *lac* operon. This system involves a repressor protein that is translated from the mRNA which was transcribed from the *lacI* gene (Hartl, 1991; Schwartz &

Beckwith, 1970). This protein interacts with the operator region of the *lac* operon and inhibits the transcription of *lacZ*, *lacY*, and *lacA* (Hartl, 1991; Jacob & Monod, 1961). The *lacI* gene product (repressor protein) is produced constitutively. When lactose is added to the medium, it complexes with the repressor. This binding alters the shape of the repressor so that it is unable to bind to the operator. This allows the transcription of the structural gene of the *lac* operon (Hartl, 1991).

The second regulatory mechanism is referred to as positive regulation, better known as either catabolite repression or the glucose effect. In the *lac* operon of *E. coli*, catabolite repression/glucose effect refers to the repression of structural genes transcription of the inducible *lac* operon when *E. coli* is grown in medium containing glucose. This repression occurs even if lactose is present in the medium (Cohn & Horibata, 1959; de Crombrugghe & Pastan, 1980; Hartl, 1991; Pastan & Adhya, 1976, Perlman *et al.*, 1970; Perlman *et al.*, 1969; Saier *et al.*, 1975; Schwartz & Beckwith, 1970; Zubay *et al.*, 1970).

How does glucose effect the transcription of the structural *lac* operon genes? Cohn & Horibata showed that glucose caused a decrease in the transcription of the *lac* mRNA (1959). Perlman *et al.* demonstrated that *lac* mRNA transcription resumed in glucose-repressed medium when cAMP was added (1970). Many experiments have shown that the concentration of cAMP in *E. coli* is low during growth on glucose. Conversely, in a medium which contains a carbon and energy source that is not utilized through the glucose degradative pathway, the concentration of cAMP is high (de

Crombrugghe, 1984; de Crombrugghe, 1990; Hartl, 1991; Schwartz & Beckwith, 1970). From these results, it was concluded that cAMP regulates the *lac* operon in some fashion (de Crombrugghe *et al.*, 1971; Eschenlauer & Reznikoff, 1991; Haggerty & Schleif, 1975; Hartl, 1991; Perlman *et al.*, 1969; Reznikoff, 1992; Schwartz & Beckwith, 1970).

In order to understand how cAMP influences the transcription of the *lac* operon, *E. coli* mutants, which were unable to utilize lactose and other carbohydrates, were isolated (de Crombrugghe & Pastan, 1980). Two types of mutants were found. The first type of mutation had a defective adenyl cyclase enzyme and was unable to produce cAMP (Perlman *et al.*, 1969). When cAMP was added to these mutants, the *E. coli* cells were able to synthesize the inducible enzymes necessary to utilize various carbohydrates in the medium. The second type of mutation consisted of *E. coli* cells that were unable to synthesize the inducible enzymes even after cAMP was added to the medium (Schwartz & Beckwith, 1970).

This second type of mutant suggested that another regulatory factor was involved in the control of enzyme synthesis by cAMP (Zubay *et al.*, 1970). A protein, referred to as cyclic AMP receptor protein (CRP) or catabolite gene activator protein (CAP), was found that binds to cAMP (de Crombrugghe *et al.*, 1984; de Crombrugghe *et al.*, 1971; Hartl, 1991; Reznikoff, 1992). In the wild type organism, CRP directly regulates the synthesis of *lac* mRNA by binding to cAMP, forming a cAMP-CRP complex (de Crombrugghe *et al.*, 1984; Eschenlauer & Reznikoff, 1991;

Reznikoff, 1992). This complex binds at, or near, the *lac* promoter in order for transcription to occur (de Crombrugghe *et al.*, 1984; de Crombrugghe & Pastan, 1980; Eschenlauer & Reznikoff, 1991). Mutants have been identified where the cAMP-CRP complex is unable to form for various reasons (Brickman *et al.*, 1973; de Crombrugghe *et al.*, 1984; de Crombrugghe & Pastan, 1980; Eschenlauer & Reznikoff, 1991; Garges & Adhya, 1988; Hartl, 1991; Perlman *et al.*, 1969). These mutants have assisted in furthering the study of the cAMP-CRP positive control mechanism.

There are several models as to the exact mechanism of CAP activation. These include the idea that CAP is in direct contact with the RNA polymerase (de Crombrugghe *et al.*, 1984; Eschenlauer & Reznikoff, 1991; Reznikoff, 1992). The rationale behind this idea is that CAP stabilizes the DNA-RNA polymerase binding or changes the conformation of the polymerase so that the polymerase binds strongly. Another model is that CAP interacts directly with the DNA and causes a conformational change in the DNA. This could lead to contact of upstream DNA with RNA polymerase and would facilitate the initiation of transcription (de Crombrugghe *et al.*, 1984; Eschenlauer & Reznikoff, 1991; Reznikoff, 1992).

hexC Regulon

Another regulator system that has recently begun to characterized is the *hexC* regulon in *Pseudomonas aeruginosa* PAO. The enzymes involved in this regulon

include glucokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase (Temple 1994). These four enzymes play a role in the degradation of glucose to pyruvate and glyceraldehyde-3-phosphate (Temple 1994). The expression of these enzymes and others are subject to repression by succinate or other TCA cycle intermediates (Clarke & Ornston, 1975; Hylemon & Phibbs, 1972; Lessie & Phibbs, 1984; MacGregor *et al.*, 1991; Temple *et al.*, 1994; Wolff *et al.*, 1991).

Cuskey *et al.* devised a plasmid vector which contained the structural genes for 6-phosphogluconate dehydratase, glucokinase, glucose transport, and a regulatory gene for these three genes (1985). The regulatory gene was also shown to effect the genes for glucose-6-phosphate dehydrogenase and 2-keto-3-deoxy-6-phosphogluconate aldolase (Cuskey *et al.*, 1985). Results of studies with this plasmid supported the view that the genes for glucose active transport, glucose-6-phosphate dehydrogenase, 2-keto-3-deoxy-6-phosphogluconate aldolase, and 6-phosphogluconate dehydratase are clustered together on the chromosome of *P. aeruginosa* PAO (Cuskey *et al.*, 1985).

In another experiment dealing with the structural and regulatory genes for carbohydrate utilization in *P. aeruginosa* PAO, Temple *et al.* determined the order of the three structural genes, the glucose binding protein, glucokinase, and 6-phosphogluconate dehydratase (1990). By using plasmids containing cloned fragments of these genes, as well as glucose-6-phosphate dehydrogenase, and 2-keto-

3-deoxy-6-phosphogluconate aldolase, several regulatory genes, *hex*R and *hex*C were discovered (Temple *et al.*, 1990). *hex*R was found to be tightly linked to the 6-phosphogluconate dehydratase structural gene and *hex*C was found to cause a two- to nine-fold increase in the expression of glucokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydratase, and 2-keto-3-deoxy-6-phosphogluconate aldolase.

In a recent study, Temple *et al.* reported that they found that the gene for NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (*gap*) was physically close to the *hex*C locus and 6-phosphogluconate dehydratase. They also found that GAP activity showed glucose inducibility and was repressed by the presence of succinate (1994). The above observations suggested that *gap* might be a fifth gene in the *hex*C regulon. To investigate this possibility, Temple *et al.* showed that the concentration of GAP increased 6x in response to the presence of the *hex*C plasmid, an increase known as the *hex*C effect. This finding indicated that *gap* was affected by *hex*C in the same manner as the four known genes of the *hex*C regulon (1994).

Temple *et al.* then went onto determine the sequence of *gap* and of 6-phosphogluconate dehydratase (*edd*). The results of this revealed that the promoter region for *edd- gap* had features similar to other *Pseudomonas* species' promoter sequences which were functionally similar (Temple *et al.*, 1994). The exact mechanisms of how the *hex*C locus and catabolite repression control gene expression are under investigation at this time (Temple *et al.*, 1994).

Carbohydrate Transport

Carbohydrate regulation, as discussed previously, also deals with organisms preferentially producing enzymes in order to use one carbohydrate over another. Unstated in the previous statement are the ideas that an organism must be able to sense what carbohydrates are present in the surrounding environment and then be able to transport the carbohydrate into the cytoplasm in order to utilize that sugar as a carbon and energy source. There are three major categories of bacterial transport by which different substances can be shuttled into or out of the cytosol.

Carbohydrate Phosphotransferase System (PTS)

The first transport mechanism to be discussed here is the PTS. The PTS was first discovered in *E. coli* (Kundig *et al.*, 1964) and has been extensively studied in this organism since its discovery (Dills *et al.*, 1980; Meadow *et al.*, 1990; Postma *et al.*, 1993; Postma & Lengler, 1985; Saier, 1977). The PTS has been found to be widely distributed among Gram positive and other Gram negative organisms (Dills *et al.*, 1980; Meadow *et al.*, 1990; Postma *et al.*, 1993; Postma & Lengler, 1985; Saier, 1977).

The PTS is a group translocase process which involves moving sugars into the cell while simultaneously phosphorylating them during the transportation process. The general reaction equation of the PTS is: P-enolpyruvate (in) + carbohydrate (out)

—(PTS)→ pyruvate (in) + carbohydrate-P (in) (Dills *et al.*, 1980; Meadow *et al.*, 1990; Postma *et al.*, 1993; Postma & Lengler, 1985).

The transportation process of the PTS involves a carbohydrate specific protein, Enzyme II (EII), which is composed of three domains (IIA, IIB, IIC) and can only transport one, or at most, several different carbohydrates. The PTS also involves several non-carbohydrate-specific proteins, Enzyme I (EI) and Heat-resistant protein (HPr), which are necessary for the series of reactions that result in the phosphorylation of the carbohydrate as it is brought into the cytosol.

The PTS, in general, translocates a number of different sugars and must be able to selectively use a carbon and energy source if there is more than one present in the medium. To do this, the microorganisms have further specialized the EII proteins. Four classes of EIIs, comprised of genetically similar proteins, have been described. One class of EII includes the glucose proteins for *E. coli* (Postma *et al.*, 1993). The EII^{Glc} of *E. coli* consists of two proteins (Postma *et al.*, 1993). One is the soluble $EIIA^{Glc}$. The other protein is the membrane bound $EIICB^{Glc}$ protein (Postma *et al.*, 1993). The amino terminal end of the $EIICB^{Glc}$ protein is the $EIIC^{Glc}$ domain of this protein and carboxyl terminal end is the hydrophilic $EIIB^{Glc}$ domain (Postma *et al.*, 1993). The domains are joined by a linker (Postma *et al.*, 1993).

Another class of EII includes the mannitol proteins from *E. coli* (Postma *et al.*, 1993). EII^{Mtl} from *E. coli* is a large single protein (Postma *et al.*, 1993). The order of its protein domains is EIICBA (Postma *et al.*, 1993). The EIIC domain is membrane

bound and functions to bind and translocate the substrate (Postma *et al.*, 1993). The EIIA and EIIB domains contain highly conserved regions that are phosphorylated during translocase activities (Postma *et al.*, 1993).

The PTS is not strictly a transport system, but also serves a sensory function. The sensory aspects of the PTS allow the microbes to control its movement either towards or away from a substance. This movement allows the microbe to control which sugar is most abundant in the surrounding environment in nature. This enables the microbe to control which of its various metabolic pathways is being used (Postma *et al.*, 1993; Postma & Lengler, 1985).

Although many microbes appear to use the PTS to obtain intracellular concentrations of sugar-phosphates, not all organisms use this system. There are some cases where an organism will use the PTS to transport only a few of the many carbohydrates that the organisms is capable of using. A review article by Lessie and Phibbs states that fructose metabolism in *Pseudomonas* is distinct from other carbohydrates utilized by these bacteria (1984). In fact, fructose is the only carbohydrate that has been shown to be transported into *Pseudomonas* through PTS activity (Lessie & Phibbs, 1984). Once in the cell, the transported sugar is in the form of fructose-1-phosphate, and further metabolism converts this intermediate to fructose 1,6-diphosphate (Lessie & Phibbs, 1984). From this point, *Pseudomonas* can produce either pyruvate or fructose-6-phosphate (Lessie & Phibbs, 1984). According to MacGregor *et al.*, *Pseudomonas* differs from enteric organisms in their PTS in several

ways. The first is that *Pseudomonas* does not appear to have an HPr protein. The second difference is that the fructose PTS does not have a carbohydrate-specific EII (1991). *Pseudomonas* PTS is also unique in that fructose 1,6-diphosphate is used as a catabolic enzyme (Lessie & Phibbs, 1984). Uptake of other carbohydrates that *Pseudomonas* utilize is by active transport (Lessie & Phibbs, 1984). However, there may be other carbohydrates that *Pseudomonas* utilize by the PTS that have not yet been classified (Meadow *et al.*, 1990).

The other two categories that bacteria use to translocate carbohydrates included membrane-bound transport (MBT) and binding transport (BPT) active transport systems. These active transport systems differ from PTS in that the carbohydrate which is accumulated in the cytoplasm is chemically unchanged from the carbohydrate in the extracellular medium (Brock & Madigan, 1988; Saier, 1977).

The two active transport systems differ from each other in several ways. In the MBT system, transport of the carbohydrate via ion gradients is driven by chemical or electrical energy sources which are directly derived from the proton motive force (Dills *et al.*, 1980; Saier, 1977). In the BPT, the energy source for transporting the carbohydrate is derived through the direct hydrolysis of ATP (Dills *et al.*, 1980; Saier, 1977).

The mechanisms through which these transport systems operate are also different from each other. The MBT system differs from the BPT in that the BPT utilizes periplasmic binding proteins in the transport process. This is demonstrated

through osmotic shock experiments where MBT still functions in the osmotic shock vesicles while the BPT system does not function in the vesicles. Another difference between these two systems is the gene structure of the permease involved in the transport. The permease involved in the MBT is one gene product. In BPT, the permease is synthesized from more than one gene.

Many organisms are capable of utilizing both the PTS and active transport systems in transporting the barrage of carbohydrates that they are able to metabolize. Often the mechanism that is used to metabolize a carbohydrate varies between different microorganisms. *E. coli* and *Pseudomonas* species are two types of microbes that are included in both of these categories.

L-arabinose Utilization in *E. coli*

L-arabinose utilization has been extensively studied in *E. coli* (Bustos & Schleif, 1993; Beverin *et al.*, 1971; Englesberg, 1971; Englesberg *et al.*, 1965; Hahn *et al.*, 1984; Heffernan & Wilcox, 1976; Hendrickson, Stoner, & Schleif, 1990; Horazdovsky & Hogg, 1989; Horazdovsky & Hogg, 1987; Hogg & Englesberg, 1969; Hou *et al.*, 1988; Kolodrubetz & Schleif, 1981a & 1981b; Lee *et al.*, 1981; Lichenstein *et al.*, 1987; Lobell & Schleif, 1990; MacInnes *et al.*, 1978; Novotny & Englesberg, 1966; Ogden *et al.*, 1980; Power & Irr, 1973; Redder & Schleif, 1991; Schleif, 1969; Sheppard, 1986; Sheppard & Eleuterio, 1976; Singer & Englesberg, 1971; Stoner & Schleif, 1983; Wilcox, 1974; Wilcox *et al.*, 1974). Throughout these

studies, many facets of the metabolic pathway, transport, and regulation of the pathway for L-arabinose have been elucidated.

L-arabinose Pathway in *E. coli*

In *E. coli*, L-arabinose is transported into the cell through a permease transport system. Once inside, L-arabinose is converted into D-xylulose 5-phosphate through a series of reactions which involve three enzymes (Englesberg, 1971; Englesberg *et al.*, 1965; Lee, 1980; MacInnes *et al.*, 1978), as shown in Figure 1 (Lee, 1980). The end product that is shown in Figure 1, D-xylulose 5-phosphate, has been shown to be metabolized by transketolase, a constitutive enzyme in *E. coli* (Englesberg, 1971).

The three enzymes involved in L-arabinose metabolism are L-arabinose isomerase, L-ribulokinase, and L-ribulokinase 5-phosphate and are coded for by *araA*, *araB*, and *araD*, respectively (Englesberg, 1971; Englesberg *et al.*, 1965; Lee, 1980; MacInnes *et al.*, 1978). These structural genes, along with the initiator region *(araI)* and the operator region *(araO)* for these genes constitutes the *araBAD* operon. This operon and others that are involved in the transport of L-arabinose are controlled by the *araC* gene product (Englesberg *et al.*, 1965; Englesberg, 1971; Hahn *et al.*, 1984; Hou *et al.*, 1988; Koisba & Schleif, 1982; Kolodrubetz & Schleif, 1981; Lee, 1980; Lobell & Schleif, 1990; Ogden *et al.*, 1980; Power & Irr, 1973; Sheppard, 1986; Stoner & Schleif, 1983; Wilcox *et al.*, 1974).

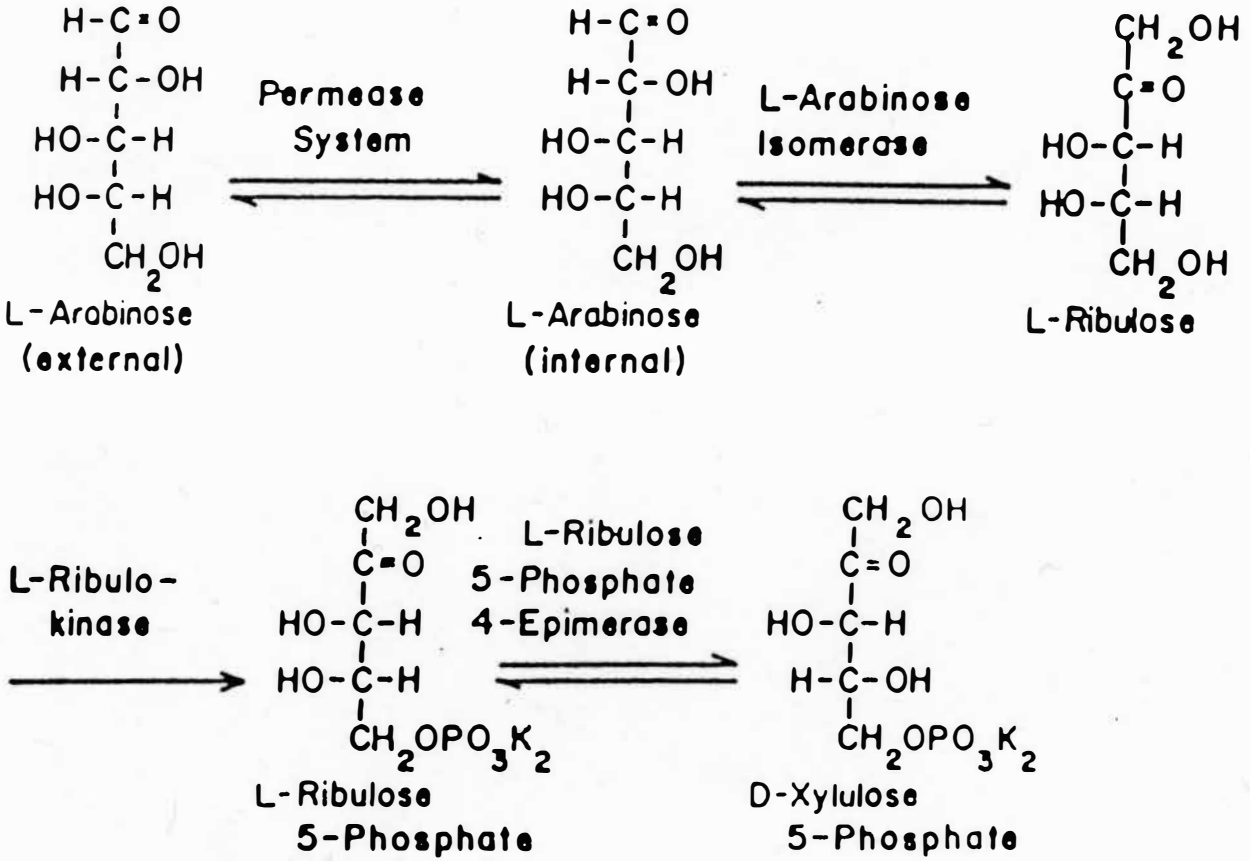

Figure 1. Proposed Pathway for L-arabinose Catabolism in *Escherichia coli* (Lee, 1980 and Reeder & Schleif, 1991).

L-arabinose Transport in *E. coli*

The transport of L-arabinose has been found to occur through two different permease systems (Englesberg, 1971; Horazdovsky & Hogg, 1989; Horazdovsky & Hogg, 1987; Kolodrubetz & Schleif, 1981a; Lee, 1980; Reeder & Schleif, 1991; Scripture *et al.*, 1987; Stoner & Schleif, 1983). The first system discovered was the low-affinity system. The structural genes for L-arabinose permease of this system mapped to the *araE* locus on the chromosome as shown in Figure 2 (Kolodrubetz & Schleif, 1981a; Lee, 1980; Novotny & Englesberg, 1966). This system is a MBT system. Other experiments on this system showed that it is induced by the presence of L-arabinose (Singer & Englesberg, 1971). It was also found that the *araC* gene product, in the presence of L-arabinose, served as a positive control for the L-arabinose permease (Englesberg, 1971; Englesberg, Irr, Power, & Lee, 1965). This permease may be a membrane-bound protein (Lee, 1980).

The second transport system of L-arabinose was characterized as a high-affinity system (Schleif, 1969; Singer & Englesberg, 1971). This system was also found to be inducible by L-arabinose (Hendrickson *et al.*, 1990; Horazdovsky & Hogg, 1989; Horazdovsky & Hogg, 1987; Reeder & Schleif, 1991; Schleif, 1969; Scripture *et al.*, 1987; Singer & Englesberg, 1987). Kolodrubetz and Schleif found that the high-affinity permease was the result of an operon which included the genes for *ara*F and *ara*G (1981a). A study by Horazdovsky & Hogg added another gene,

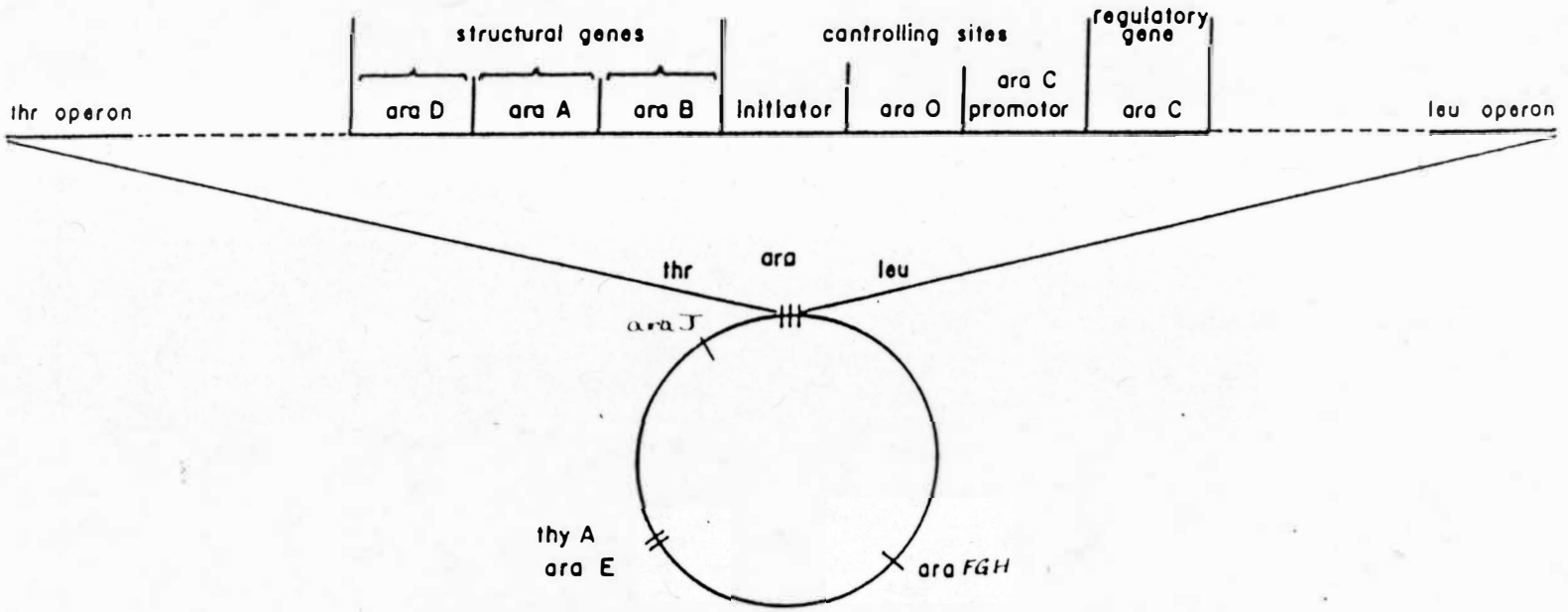

Figure 2. Proposed Arrangement of the ara Operon in *Escherichia coli* (Hou, Martin, & Schleif, 1988).

The structural genes, *araA, araB, araD* code for L-arabinose isomerase, L-ribulokinase, and L-ribulose 5-phosphate, respectively. *araE* codes for low-affinity transport , while *araFGH* codes for high-affinity transport.

araH, to this transport system (1987). The presence of multiple genes which code for the high-affinity permease suggests that this system is a BPT system.

Further characterization of this operon showed that *araF* coded for the L-arabinose binding protein (Horazdovsky & Hogg, 1989; Scripture *et al.*, 1987). As a binding protein, it was hydrophilic, soluble, located in the periplasm, and was released when the organism was osmotically shocked (Englesberg, 1971; Horazdovsky & Hogg, 1989; Schleif, 1969; Scripture *et al.*, 1987). Both *araG* and *ara*H were shown to be a membrane-associated complex for high-affinity transport of L-arabinose (Horazdovsky & Hogg, 1987). These proteins were shown to lack signal peptides (Horazdovsky & Hogg, 1989; Scripture *et al.*, 1987) which are normally necessary for proteins that are found on the membrane surface or in the periplasmic space. The lack of the signal peptide is interesting in that the hydrophilic *araG* protein has peripheral association at the inner surface of the cytoplasmic membrane (Horazdovsky & Hogg, 1987). The *araH* protein is hydrophobic and appears to exist as a β sheet (Scripture *et al.*, 1987). The *araG* and *araH* proteins have been found to be homologous to membrane-associated proteins in binding-protein dependent transport systems such as *hisM* for histidine transport and *rbsC* for ribose transport, respectively (Horazdovsky & Hogg, 1989).

As the studies continued, a promoter for another arabinose-inducible operon, *araJ*, was discovered by Hendrickson *et al.* (1990). Reeder and Schleif mapped and sequenced the *araJ* gene and found that the protein may have a signal peptide (1991).

They attempted to find a function for this gene, but found that no mutants had ever mapped to the *araJ* region of the chromosome (Reeder & Schleif, 1991). By using mutants, they showed that *araJ* is not vital for either the low-affinity or high-affinity transport systems, nor was it involved in the regulation of the organism's response to L-arabinose (Reeder & Schleif, 1991).

Study of the structure of the *araJ* protein revealed that it is mostly hydrophobic (Reeder & Schleif, 1991). When the DNA and protein sequences were compared to others found in databases, there were no homologous sequences that were found. Upstream of the sequence for the *araJ* protein, there is an open reading frame that has been found to contain a myosin-like repeat (Reeder & Schleif, 1991).

The function of the *araJ* gene product is still unknown. The hypothesis that Reeder & Schleif propose is that *araJ* may be involved in the transport or processing of arabinose polymers (1991). Their support for this hypothesis is the probable signal peptide which would be necessary for the *araJ* gene product to cross the inner membrane to function in either transport of L-arabinose at the outer membrane or processing of L-arabinose in the periplasmic space (Reeder & Schleif, 1991). They discredit a theory suggesting that the possibility that *araJ* may be involved in chemotaxis since *E. coli* does not seem to respond to L-arabinose by chemotaxis. These investigators do not think that *araJ* is involved in arabinose catabolism because only one pathway for L-arabinose utilization is known to exist in *E. coli* (Reeder & Schleif, 1991).

Regulation of L-arabinose Pathway in *E. coli*

The most recent model that has been proposed for the mechanism of regulation of L-arabinose uptake by *E. coli* is described by Hou *et al.* (1988) and by Lobell and Schleif (1990). These investigators proposed that, without the presence of L-arabinose, most of the *ara* regulatory regions contain a loop that is located between the $araO_2$ and *araI* regions of the chromosome. They showed that this loop is generated by AraC protein molecules that bind both to the $araO_2$ and *araI* regions (Hou *et al.*, 1988; Lobell and Schleif, 1990). The loop appears to prevent the AraC protein from entering the induced state. This results in the level of the *araBAD* gene expression being held at a low level (Hou *et al.*, 1988). The loop may limit the AraC protein's access to the $araO_1$ and also limit the RNA polymerase's access to the promoter for the AraC protein. A diagram of this model is shown in Figure 3 (Hou *et al.*, 1988).

Hou *et al.* suggest that when L-arabinose is added to the system and is bound to the AraC protein, a series of reactions occur (1988). First, the $araO_2$ - *araI* loop opens (Hou *et al.*, 1988; Lobell and Schleif, 1990). This allows the majority of the AraC protein that is bound to the low-affinity $araO_2$ to be released(Hou *et al.*, 1988). The AraC protein that remains bound to *araI*, in conjunction with the bound L-arabinose, forces another AraC protein into a configuration that allows the expression of the *araBAD* promoter (Hou *et al.*, 1988). The opened loop and the new configuration of the AraC protein on the DNA strand allows the RNA polymerase to

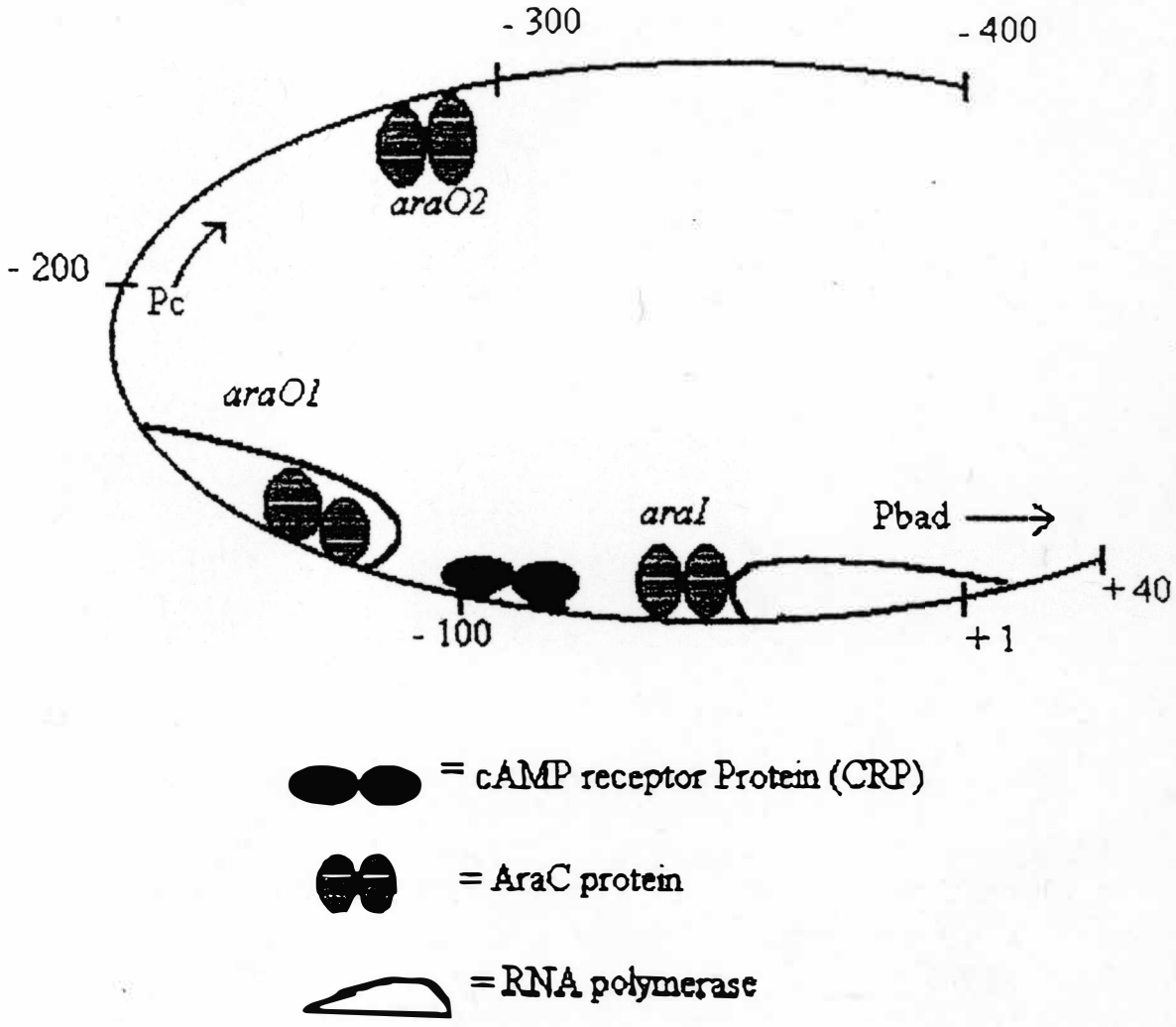

Figure 3. Proposed Binding Sites for CRP, AraC protein, and RNA Polymerase on the araCBAD Operon (Hou, Martin, & Schleif, 1988).

Numbering of the base pairs is relative to the P_{BAD} transcription start site which starts at +1.

have increased access to the promoter (Hou *et al.*, 1988). Within ten minutes after L-arabinose is bound to the AraC protein, most $araO_1$ sites are bound by AraC protein (Hou *et al.*, 1988). This binding leads to the formation of another DNA loop that involves the $araO_1$ and $araO_2$ sites on the DNA (Hou *et al.*, 1988). This loop or the presence of the AraC protein represses the synthesis of the AraC protein (Hou *et al.*, 1988).

The above model has suggested that the AraC protein has a varied role in the regulation of this pathway. Other factors that have been discovered about the AraC protein that concur with the versatility of this protein include the fact that the protein has been shown to regulate all of the operons for L-arabinose utilization at the transcriptional level (Englesberg, 1971; Hahn *et al.*, 1984; Koisba & Schleif, 1982; Sheppard, 1986), that binding sites for this protein have been found on all of the operons (Hahn *et al.*, 1984; Ogden *et al.*, 1980; Sheppard, 1986; Stoner & Schleif, 1983), and that the AraC protein binds to *araI*, $araO_1$, and $araO_2$ (Hahn *et al.*, 1984; Hou *et al.*, 1988; Lee, 1980; Lobell & Schleif, 1990; Sheppard, 1986).

These binding sites were found to be involved in repression of the L-arabinose operons (Hahn *et al.*, 1984; Hou *et al.*, 1988; Lee, 1980; Ogden *et al.*, 1980; Sheppard, 1986; Stoner & Schleif, 1983). Some interesting properties that have been discovered about the AraC protein are that protein is induced by L-arabinose and repressed by *araO* (Englesberg, 1971; Englesberg *et al.*, 1965; Hou *et al.*, 1988; Koisba & Schleif, 1982; Lee, 1980; Lobell & Schleif, 1990; MacInnes *et al.*, 1978;

Ogden *et al.*, 1980; Power & Irr, 1973; Wilcox *et al.*, 1974), and transcription of AraC occurs in the opposite direction than that of *araBAD* (Englesberg, 1971; Hahn *et al.*, 1984; Lee, 1980; Lichenstein *et al.*, 1987; Ogden *et al.*, 1980).

The *araBAD* genes are the structural genes in the L-arabinose operon. Expression of this operon is dependent on the concentration of L-arabinose, the metabolism of L-arabinose past ribulose 5-phosphate, and AraC protein (Englesberg, 1971; Hou *et al.*, 1988; Lobell & Schleif, 1990). The fact that metabolism of L-arabinose influences the expression of *araBAD* indicates the mechanism of this operon (Englesberg, 1971). AraC protein represses the *araBAD* operon when binding at two sites (*araI, and* $araO_2$) (Hahn *et al.*, 1984; Hou *et al.*, 1988; Lee, 1980; Lobell & Schleif, 1990; Sheppard, 1986).

The two transport systems, *ara*E and *ara*FGH, are similar in some aspects of regulation, but different in others. Both of these systems are catabolite repressible (Englesberg, 1971; Koisba & Schleif, 1982, Kolodrubetz & Schleif, 1981b). However, the extent of the repression is less in *ara*E than in *ara*FGH (Kolodrubetz & Schleif, 1981). The binding sites for AraC proteins vary slightly between these two transport systems. In *ara*E, the binding sites are homologous to those in *ara*BAD (Koisba & Schleif, 1982; Stoner & Schleif, 1983). However, the AraC protein binding sites in *ara*F have no homology to those in *ara*BAD. Both of the transport systems require CRP for full induction and both are regulated by the AraC protein (Englesberg,

1971; Koisba & Schleif, 1982, Kolodrubetz & Schleif, 1981b; Stoner & Schleif, 1983).

The L-arabinose operons involve an initiator (Englesberg, 1971). This is acted upon by the activator, L-arabinose, which causes stimulation of the operon's expression (Englesberg, 1971). *Ara*I initiates the transcription of *ara*BAD (Lee 1980). AraC protein tightly binds to *ara*I, which helps form the repression loop in the presence of L-arabinose, as discussed in the model above (Hou *et al.*, 1988). The operator is coded for by *araO* (Lee 1980). *AraO* forms part of the repression loop (Hou *et al.*, 1988). This loop allows the repression of the AraC protein since the AraC protein is unable to penetrate the looped DNA structure and initiate transcription (Figure 3) (Hou *et al.*, 1988).

CRP and cAMP have been shown to be involved in regulation of all of the L-arabinose operons (Englesberg, 1971; Lichenstein *et al.*, 1987; Koisba & Schleif, 1982; Ogden *et al.*, 1980; Stoner & Schleif, 1983). The complex that these molecules form is involved in activating the L-arabinose operon (Englesberg, 1971; Koisba & Schleif, 1982; Ogden *et al.*, 1980). However, this complex is catabolite repressible (Englesberg, 1971). The active site for the complex is between the *ara*B and *ara*O regions on the DNA (Englesberg, 1971).

L-arabinose Pathway in *Pseudomonas*

When the research on the L-arabinose pathway first began, it was thought the pathway was similar to that of glucose. However, in 1955, Weimberg and Doudoroff reported that, in *Pseudomonas saccrophila*, L-arabinose is converted to α-ketoglutarate through reactions that do not involve the TCA cycle nor intermediates that are phophorylated. They reported that L-arabinose is oxidized to L-arabinolactone with NAD-specific L-arabinose dehydrogenase (LAD). L-arabinolactone is metabolized into L-arabinonate which is catabolized into an unstable intermediate that is quickly processed into α-ketoglutarate (Weimberg & Dourdoroff, 1955). They proposed that either NAD or NADP acts as the electron acceptor for this metabolic system. Several years later, Weimberg showed that another *Pseudomonas* species, *Pseudomonas fragi*, also utilized pentose sugars through a similar process (1961). Doudoroff showed that *P. saccharophila* used NAD to metabolize L-arabinose to L-arabinolactone in cell-free extracts (1962).

Dagley and Trudgill showed that cell-free extracts from *P. saccharophila* converted L-arabinonate into 2-ketoglutarate semialdehyde when the assay was run without NAD. When NAD was added to the assay, L-arabonate was metabolized into 2-ketoglutarate (1965). In 1974, Schmiz *et al.* showed the entire pathway for L-arabinose in *P. fluorescens*. As with the previous two species, L-arabinose is broken down into L-arabino-1,5-lactone. This product is metabolized into L-arabonate. L-

arabinate is degraded into 2-keto-3-L-arabonate. This is degraded into α-ketoglutarate semialdehyde, which is then reduced into α-ketoglutarate (Figure 4).

The transport system for L-arabinose has yet to be explained. However, the transport of many other carbohydrates that are utilized by *Pseudomonas* has been studied. An early experiment dealing with transport was carried out by Hylemon & Phibbs, in which they examined the glucose transport in *Pseudomonas aeruginosa* (1972). They showed that the glucose transport system was not formed during diauxic growth conditions of succinate and glycerol. The glucose transport system was induced when the cells were grown on a mixture of pyruvate and either 2-deoxyglucose or a-methylglucoside (Hylemon & Phibbs, 1972).

Information about another transport system in *Pseudomonas aeruginosa* was expanded upon by Eisenberg and Phibbs. The metabolic pathway for mannitol was known and differs from that of *E. coli* which uses the PTS to transport mannitol (Eisenberg & Phibbs, 1982; Postma *et al.*, 1993). In this study, Eisenberg and Phibbs were able to characterize a binding protein that was inducible by mannitol, and confirmed that osmotic shock eliminated glucose transport in *Pseudomonas aeruginosa* cells (1982).

The regulation of various metabolic pathways in the *Pseudomonas* species has been under investigation almost since the differences between these bacteria and *E. coli* was known. The difference between these two organisms became apparent early when Hamilton and Dawes did diauxic studies on *P. aeruginosa* (1959 and 1960) and noticed that *P. aeruginosa* preferentially utilized organic acids over glucose. Soon other studies

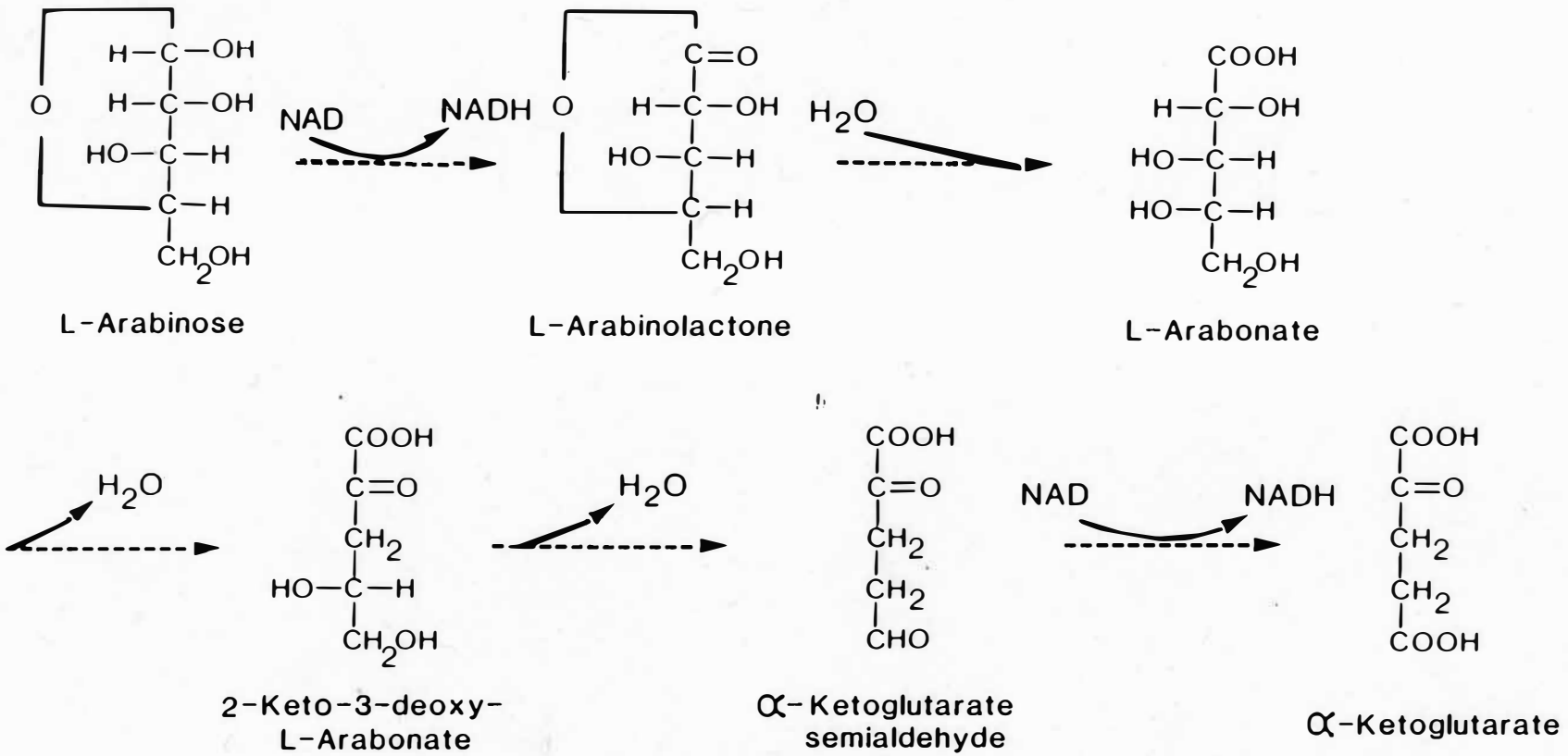

Figure 4. Proposed Pathway for L-Arabinose Catabolism in *Pseudomonas fluorescens* (Schmiz, 1974).

presented data in which *Pseudomonas* was shown to utilize succinate and other intermediates of the TCA cycle over glucose and other compounds such as tryptophan, histidine, glucose, gluconate, mannitol, glycerol, amidase, protocatechuate 3,4-dioxygenase, histidase (Clarke & Ornston, 1975; Temple *et al.*, 1990; Wolff *et al.*, 1991; Zylstra *et al.*, 1989; Phillips & Mulfinger, 1981).

In this study, we developed an assay for L-arabinose dehydrogenase and then examined pLA2917 containing genes for L-arabinose utilization from a library of the *P. fluorescens* chromosome for regulatory features.

MATERIALS AND METHODS

Source of Organisms

Bacteria and plasmids used are listed in Table 1.

Table 1

Strains and Plasmids Used in This Study

Strain or Plasmid	Description	Reference or Source
Escherichia. coli HB101	(See Maniatis, 1982)	P. V. Phibbs, Jr.
P. fluorescens ATCC 13525	Prototroph	ATCC 13525
P. putida ATCC 12633	Prototroph	ATCC 12633
pLA2917	Tc^r, Km^r, Broad host range cosmid cloning vector	B. W. Holloway via Allen & Hanson (1985)
pPZ221	Tc^r, Km^s, L-ara^+	Eisenberg & Phibbs (1988)

Preparation of Organisms

pLA2917 was extracted from *Escherichia coli* HB101 using an alkaline extraction and transferred into *Pseudomonas putida*.

The alkaline extraction procedure involved using 20 milliliters (ml) of cells with 50 µg/ ml tetracycline in sterile 50 ml plastic tubes, incubated at 37°C in a New Brunswick gyrator incubator shaker at 100 rpm for 14-16 hours. The cells were centrifuged in a Damon/IEC Division PR-6000 centrifuge at 2500 rpm for 5 minutes at 4°C (approximately 50 x *g*). The cells were then washed in ice-cold 15 mM Phosphate Buffer (pH 7.0). The cells were pelleted again and the supernatant was discarded. Next, the cells were resuspended in 125 µl of lysis buffer without lysozyme which contained 25 mM Tris (pH 8.0), 10 mM Ethylenediamine tetraacetic acid (EDTA), and 50 mM glucose (Sigma, St. Louis, MO).

The cells were transferred to a sterile Eppendorf tube and 125 µl of lysis buffer with lysozyme (10 mg/ml from Boehringer/Mannheim, Indianapolis, IN) was added and the solution was incubated for 5 minutes on ice. Following this incubation, 500 µl of alkali-sodium dodecylsulfate (SDS), composed of 0.2 N NaOH and 1% SDS, was added to precipitate DNA, RNA, and proteins. The Eppendorf tube was then inverted ten times. After incubation for 5 minutes on ice, 400 µl of chilled 3 M sodium acetate (pH 4.8) was added to neutralize the alkali in the reaction mixture and the tube was inverted ten times. A cloudy-white precipitate appeared which was composed of protein and large DNA.

The tubes were immediately centrifuged at room temperature for 5 minutes in a Brinkmann Eppendorf centrifuge, model 5414 at 1,400 rpm (approximately 50 x *g*) and then incubated on ice for 15 minutes. After this incubation, the tubes were centrifuged in a Brinkmann Eppendorf centrifuge, model 5414 at 1,400 rpm (approximately 50 x *g*) for 5 minutes. The resulting supernatant was placed in a fresh, sterile Eppendorf tube and mixed with 400 µl isopropanol to precipitate the plasmid DNA. The tubes were then placed at -70°C for 30 minutes. After this incubation, the precipitate was pelleted by centrifugation in a Fisher Microcentrifuge Model 235A 1,400rpm (approximately 50 x *g*) for ten minutes at 4°C. The resulting pellet was resuspended in 225 µl of distilled water and 125 µl of a 1:1 solution of phenol : chloroform saturated with 0.1 M Tris, pH 8.0 to remove the remaining proteins from the nucleic acids. The mixture was then centrifuged to separate the aqueous phase from the organic layer.

The supernatant (the aqueous layer) was transferred to a fresh, sterile Eppendorf tube and 200 µl of 7 M ammonium acetate was added to precipitate the RNA. This was followed by a ten minute incubation on ice. The mixture was then centrifuged in a Brinkmann Eppendorf centrifuge Model 5414 at 1,400 rpm (approximately 50 x *g*) for ten minutes at room temperature to pellet the RNA. The supernatant was then transferred to a new sterile Eppendorf tube and 800 µl of ice-cold 95% ethanol was added to precipitate the plasmid DNA. This mixture was incubated on dry ice for ten minutes and then centrifuged in a Brinkmann Eppendorf centrifuge Model 5414 at 1,400 rpm (approximately 50 x *g*) for ten minutes at room temperature.

The resulting supernatant was discarded and the pellet was resuspended in 135 µl of distilled water and 15 µl of 3 M sodium acetate. 450 µl of 95% ethanol was added. Next, the mixture was incubated for ten minutes on dry ice. After this, the solution was centrifuged in a Brinkmann Eppendorf centrifuge Model 5414 at 1,400 rpm (approximately 50 x *g*) for 15 minutes at room temperature and the supernatant was discarded. The pellet was resuspended in 25 µl of TE buffer, composed of 20 mM Tris, pH 7.4 and 1 mM EDTA, and stored at -20°C until needed.

Plasmid DNA Quantitation

Once pLA2917 was extracted from *E. coli* HB101, the amount of plasmid DNA in the TE buffer was estimated. The absorbance at 260 nm was determined using a Gilford 2000 recording spectrophotometer. This absorbance allowed quantitation of DNA in the sample, based on the assumption that 50 µg/ml of double-stranded DNA gives an optical density of 1 (Maniatis *et al.*, 1982, Stenesh, 1984).

Transformation of *P. putida*

Transformation of *P. putida* with pLA2917 was accomplished using the procedure of Durham *et al.*, 1984. Twenty-five ml of mid-log phase *P. putida* ATCC 12633 cells were chilled on ice for approximately 20 minutes. These chilled cells were centrifuged at 5,000×*g* in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge for 10 minutes at 4°C. Next, the cells were washed with 25 ml 100 mM

$MgCl_2$ and again centrifuged at 5,000×g for 10 minutes at 4°C. The supernatant was discarded and cells were resuspended in 12.5 ml of sterile ice cold 100 mM $CaCl_2$ / 50 mM $MgCl_2$ and incubated on ice for 1 hour.

Cells were then centrifuged at 5,000×g for 10 minutes at 4°C and the pellet was resuspended in 0.5 ml of sterile, ice cold 100 mM $CaCl_2$ / 50 mM $MgCl_2$ and incubated on ice for 1 hour. Aliquots of 0.1 ml of this suspension were added to Eppendorf tubes. One aliquot was used to make dilutions for a cell count, another aliquot served as a control with no DNA added. The remaining aliquots received 3.7 μg of plasmid DNA. The suspension was then incubated for 20 minutes on ice. All the aliquots, except that used for the cell count, were heat-shocked at 42°C for 3 minutes in a Napco Model 210 water bath, put into an ice bath, and 1.0 ml Luria broth was added. The tubes were then put into a 37°C water bath and shaken for 90 minutes in a Lab-Line Orbit Microprocessor Shaker Bath.

After this incubation period, the cells were centrifuged for 10 minutes on high-speed in a Fotodyne microcentrifuge. The pellets were resuspended in 0.5 ml of sterile 50 mM Potassium Phosphate, pH 7.0 and aliquots of 1 μl, 10 μl, and 100 μl were put onto a selective media of Luria medium containing tetracycline (St. Louis, MO).

The aliquot of cells that was reserved for the cell count was diluted in the following fashion: 0.1 ml of the aliquot was added to 9.9 ml of sterile 50 mM Potassium Phosphate, pH 7.0. This resulted in a dilution of 1:100. This was finger vortexed for 1 minute and then 0.1 ml of this dilution was used to make a 1:100

dilution creating a final dilution of 1:10,000. This series of events was done until a final dilution of 1:10,000,000 was reached.

From this series of dilutions, an aliquot of 0.1 ml was put in the center of a hardened agar surface of Luria medium. The cells were then spread over the surface of the medium by moving a sterile bent glass rod over the surface of the medium, while rotating the petri dish. The bent glass rod was sterilized by dipping the rod into a beaker of 95% ethanol and then held in the flame of the bunsen burner to ignite the ethanol. After the flame had died out, the glass rod was cooled by touching it against the surface of the sterile medium (Maniatis *et al.*, 1982).

Transformation of *Escherichia coli*

One ml of an overnight culture of *E. coli* HB101 was used to inoculate 100 ml of Luria broth. The cells were then grown for 3 hours. After the culture was chilled on ice for 10 minutes, the cells were centrifuged in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge at 4000x *g* for 5 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 50 ml of an ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris (pH 8.0). After a incubation period of 15 minutes in an ice bath, the cells were centrifuged at 4000x *g* for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 6.6 ml of an ice-cold, sterile solution of 50 mM $CaCl_2$ and 10 mM Tris (pH 8.0).

From this cell suspension, 0.2 ml aliquots were put dispensed into chilled Eppendorf tubes and stored at 4°C for 12-24 hours. One tube was used to make dilutions for a cell count, one tube did not receive any plasmid DNA to function as a control to determine cell viability after transformation procedure. The tubes that contained the cells to be transformed received 3.7 μg of plasmid DNA in TE buffer.

After incubation in an ice bath for 30 minutes for all the tubes except the one used for the cell count, the Eppendorf tubes containing the cells were placed in a 42°C Napco Model 210 water bath for 2 minutes. One ml of Luria broth was added to each Eppendorf tube and incubated at 37°C for 30 minutes without shaking to allow the bacteria to recover and begin to express antibiotic resistance. Once this incubation period was over, 1, 10, and 100 μl amounts of the cells were spread onto Luria medium containing 100 μg of tetracycline and also onto Luria medium without the tetracycline, as a control, using the spreading procedure as described by Maniatis (1982). Once the liquid had been absorbed, the plates were inverted and incubated at 37°C.

Growth of Organisms

Stock cultures were maintained on basal salts medium (BSM) broth or Luria broth (LB). The BSM contained 10% 0.5 M potassium phosphate, 0.5% 3.0 M $(NH_4)_2$ SO_4, 0.1% 1.0 M $MgCl_2$, and 0.1% $FeSO_4$. The pH of the medium was 7.0. The LB contained (grams/liter): Bacto-Tryptone, 10 grams: Bacto-Yeast Extract, 5 grams: sodium chloride,

10 grams. The pH of the medium was 7.5. Carbon and energy sources and antibiotics were added as indicated. The bacteria were transferred weekly to new broth tubes containing the appropriate carbon and energy source and antibiotic, if necessary. Cultures were incubated at 27°C in a New Brunswick gyrator incubator shaker at 100 rpm.

Bacteria used for determination of enzyme activity were obtained by inoculating a loopful of stock culture into 10 ml of well-aerated BSM broth in a 50 ml Fernbauch flask. This culture was grown overnight and then used as a source of inoculum. The overnight culture was added to 1 liter (l) of BSM in a 2.8 l Fernbauch flask containing the indicated carbon source; cells used as inoculum were always grown in the same medium that they were being transferred into, unless otherwise indicated. The cultures were incubated overnight (about 14 hours) and then were harvested.

Bacteria used for determining growth curves were obtained by inoculating a loopful of stock culture into 5 ml of well-aerated BSM or LB. This culture was grown overnight and then used as a source of inoculum. A 1% inoculum was added to 50 ml of BSM in a 300 ml sidearm flask containing the designated carbon and energy source and, if necessary, antibiotic.

Preparation of Cell-Free Extract for Enzyme Assay

Bacteria were harvested by centrifugation in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge at 9150 × gravity (*g*) for 15 minutes. The cells were

rinsed three times in 0.5 M potassium phosphate buffer (pH 9.0). The pelleted cells were frozen at -4°C.

The frozen cells were resuspended in 0.5 M potassium phosphate buffer (pH 9.0) containing 0.1% 2-mercaptoethanol (BME). Cells were then disrupted with a Bronson model W185 sonifier at 50 Watts with three 10 second treatments. Ten seconds were allowed for the suspension to cool after each sonification period. Sonification vessels were held in an ice bath during cell disruption. Whole cells and cellular debris were removed from the cell extract by centrifugation in a Sorvall Superspeed RC-2B automatic refrigerated centrifuge at 20,000×*g* for 10 minutes. An aliquot of the cell-free extract was retained for protein analysis.

Protein Assay

Protein assays were done according to the Lowry and Kalb methods. Protein concentrations estimated by the Kalb *et al.* (1977) method used the following equation: $183 \times Ab_{230} - 75.8 \times Ab_{260}$ to determine the μg of protein per ml of sample. The absorbances were determined using a Gilford 2000 recording spectrophotometer.

The Lowry method that was used in this experiment is the modified Lowry Protein Determination as described by Alam (1992). A Bovine Serum Albumin (Sigma, St. Louis, MO) standard of 3 mg/ 10 ml was used to establish a standard curve. The assay involved 0.5 ml of unbuffered alkaline copper sulfate composed of 1.0 N NaOH, 0.16% Na-K Tartrate, and 0.05% copper sulfate, which was added to the sample and mixed via a

vortex. Next, 3.7 ml of 5 % Folin reagent [made from 100% (2N) Folin-Ciocalteu reagent] was added to the reaction mixture and the solution was vortexed. The absorbance of the solution was read after 10 minutes at 700 nm on a Gilford 2000 recording spectrophotometer.

Enzyme Assay for L-Arabinose Dehydrogenase

L-Arabinose dehydrogenase (LAD) activity was determined spectrophotometrically by observing the reduction of NAD on a Gilford 2000 recording spectrophotometer at 340 nm. The reaction mixture contained 0.5 M potassium phosphate, pH 9.0; 4 mM NAD; cell-free extract in 0.5 M potassium phosphate buffer, pH 9.0, plus 0.1% BME; 0.4 mM L-Arabinose (Sigma, St. Louis, MO); and the necessary amount of distilled water to bring the volume up to 1 ml. The reaction was measured at room temperature (22-25°C) for 1 minute. The reaction was initiated upon addition of the L-arabinose.

L-Arabinose Dehydrogenase Activity With Galactose as a Substrate

It was noted that there was some LAD activity in extracts of galactose grown *Pseudomonas fluorescens*.. In order to determine if LAD is the same enzyme that *P. fluorescens* needs to utilize galactose, cell-free extracts derived from galactose- or L-arabinose- grown cells were used to determine the optimal pH.

Growth Curves

Bacteria were grown on BSM with the indicated carbon and energy sources in order to determine growth curves and determine growth kinetics via the doubling times. The growth curves, based on the optical density of the media containing the bacteria, were determined using a Klett-Sommerson Photoelectric colorimeter with a #66 filter that has the spectral range of 640-700 millimicrons. Klett readings were taken at hourly intervals until the bacteria reached the stationary phase of growth. The data was used to determine the doubling time for the culture using the following equation: $t_d = \log 2 / r$, where t_d is doubling time and r is the slope from the graph of the log of the Klett readings versus time (hours).

The growth curves also indicated the preferential utilization of one carbohydrate when the bacteria were grown on a mixture of two carbohydrates and observed as a diauxic curve.

RESULTS

Assay Development

Saturation Kinetics for NAD

Catabolism of L-arabinose has been studied extensively in *Escherichia coli* (Bustos & Schleif, 1993; Reeder & Schleif, 1991; Hendrickson *et al.*, 1990; Lobell & Schleif, 1990; Huo *et al.*, 1988; Horazdovsky & Hogg, 1987; Lichenstein *et al.*, 1987; Sheppard, 1986; Hahn *et al.*, 1984; Stoner & Schleif, 1983; Kolodrubetz & Schleif, 1981; Lee *et al.*, 1981; Lee, 1980; Ogden *et al.*, 1980) and several species of *Pseudomonas* (Lessie & Phibbs, 1984; Schmiz *et al.*, 1974; Ornston, 1971; Stanier *et al.*, 1966; Weimberg, 1961; Weimberg & Doudoroff, 1955; Dourdoroff, 1948). In *Pseudomonas fluorescens*, L-arabinose is oxidized to α-ketoglutarate via a unique pathway illustrated in Figure 4.

The production of a Krebs cycle intermediate, α-ketoglutarate, by this pathway may involve some unusual regulatory properties since Krebs cycle intermediates normally function to repress synthesis of enzymes for carbohydrate catabolism (Lessie & Phibbs, 1984; Clarke and Richmond, 1975; Paigen & Williams, 1970). Thus we were interested in examining the regulatory and expression features of *P. fluorescens* genes coding for L-arabinose utilization that are cloned into a cosmid vector and expressed in a surrogate host (*Pseudomonas putida*) which is not capable of growing on L-arabinose.

The preliminary steps for this study involved developing a reliable assay for the first enzyme in the pathway, L-arabinose dehydrogenase (LAD). LAD activity was used to estimate induction of a putative L-arabinose operon (Figure 4). This reduction reaction of NAD to NADH is easily measured since NADH absorbs light at 340 nm, while NAD shows very little absorbency at this wavelength (Stenesh, 1984).

To determine the substrate saturation kinetics for NAD (Figure 5), the concentration of NAD (mM) was varied in the assay reaction mixture while L-arabinose was in excess and LAD activity was recorded. A unit of LAD activity is defined as one μmole of NAD reduced per minute. Results shown in Figure 5 demonstrate that 2×10^{-3} M NAD is saturating for NAD. In all subsequent LAD assays, 4×10^{-3} M NAD was used based on these results (Figure 5).

The insert in Figure 5 shows a reciprocal plot of mM NAD concentration versus units of LAD activity. From this graph, the apparent K_m (the substrate concentration required for observed half maximal activity) was calculated to be 6.65×10^{-7} M. The apparent K_m was calculated by solving for x in the equation of the line from the plot of the inverse of units vs. the inverse of the concentration of NAD (Cricket Software). The resulting number is the negative inverse of the apparent K_m. The correlation coefficient (r), which was 0.953, is included to show the experimental fit of these data to a straight line equation as determined by linear regression.

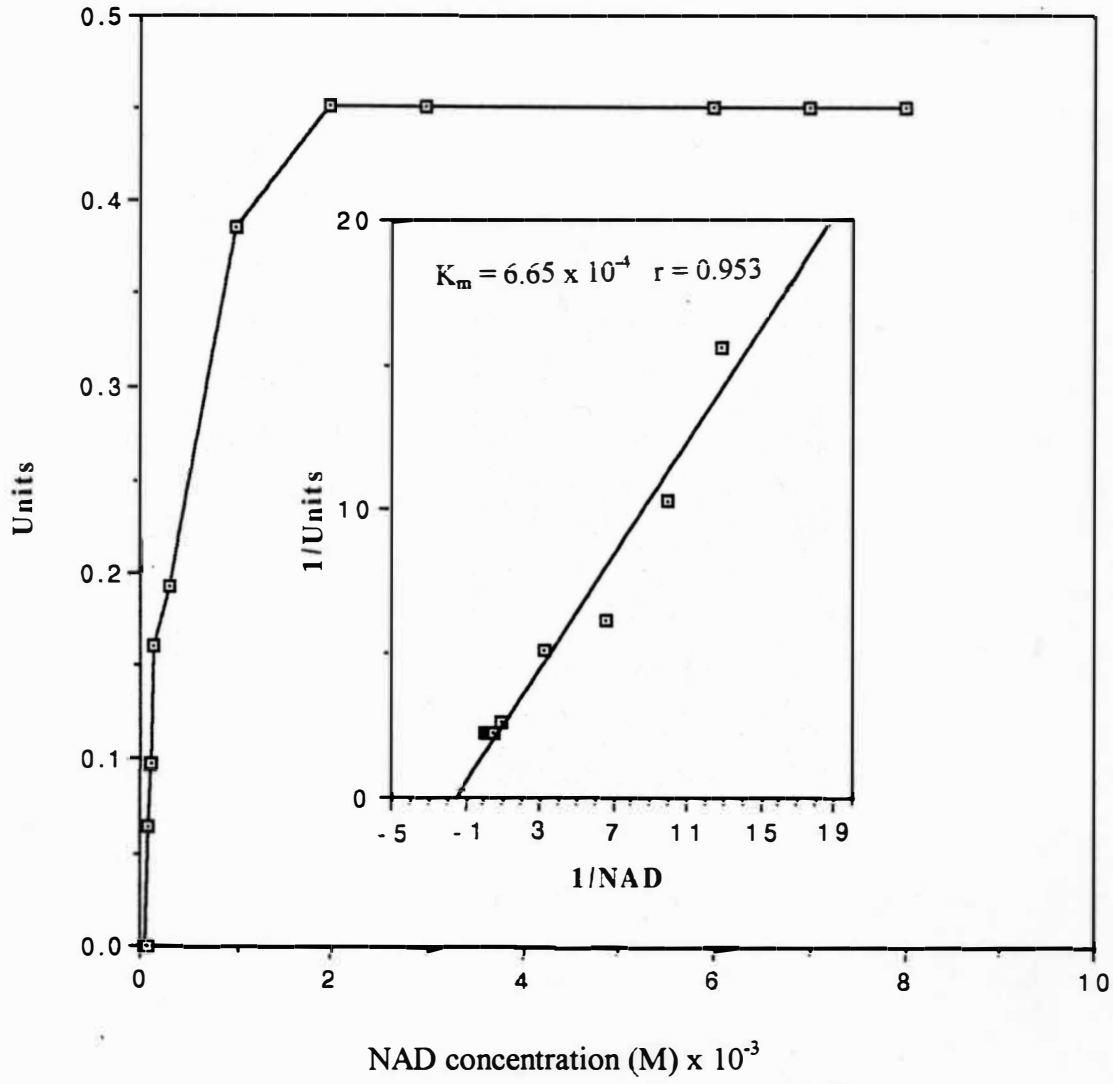

Figure 5. Effects of NAD Concentration on LAD Activity.

P. fluorescens was grown and cell-free extract prepared as described in Materials and Methods. A unit of LAD is defined as one μmole NAD reduced per minute.

The insert is a Lineweaver-Burke (1934) plot for the substrate saturation kinetics of NAD. The reaction mixtures contained 0.88 mg protein per ml and were assayed at 22°. The correlation coefficient (r = 0.953) is included to indicate good experimental fit of the data to a straight line equation as determined by linear regression.

Saturation Kinetics for L-Arabinose

Figure 6 shows the relationship between L-arabinose concentration (M) and units of LAD activity. These results show that the saturating concentration of L-arabinose for the assay used in this study was 2×10^{-4} M. In all subsequent assays, 4×10^{-4} M of L-arabinose was used based on these results.

The insert in Figure 6 is a reciprocal plot of L-arabinose concentration versus the units of activity of LAD activity. The apparent K_m was estimated as 1.63×10^{-5} M. The correlation coefficient (r) was 0.976, and is included to show the experimental fit of the data to a straight line equation as determined by linear regression.

pH Optima

The effect of varying the pH of phosphate buffer on the specific activity of LAD was determined as shown in Figure 7. At very acidic pH (pH 5.0 and pH 6.0) and very basic pH (pH 11.0 and pH 12.0), the specific activity was less than 0.001. The maximum specific activity for LAD occurred between pH 9.0 and pH 9.5. The optimum pH was determined with assays using the saturating substrate concentrations for NAD and L-arabinose, as described above. In all subsequent assays, a phosphate buffer with a pH of 9.0 was used.

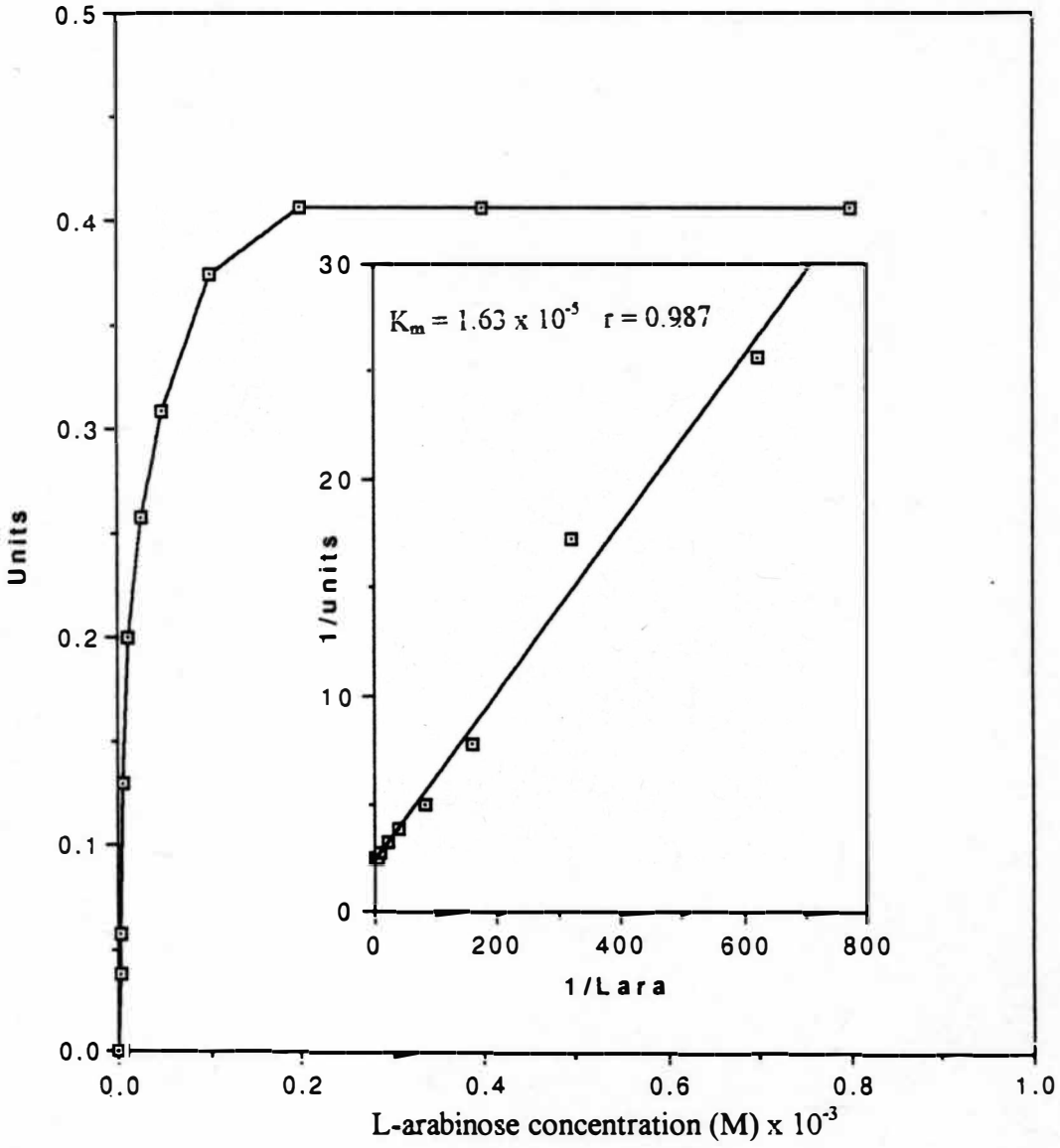

Figure 6. Effects of L-Arabinose Concentration on LAD Activity.

P. fluorescens was grown and cell-free extract prepared as described in Materials and Methods. A unit of LAD is defined as one µmole NAD reduced per minute.

The insert is a Lineweaver-Burke (1934) plot of the substrate saturation kinetics of L-Arabinose. The reaction mixtures contained 0.046 mg protein per ml and were assayed at 22°. The correlation coefficient (r = 0.987) is included to indicate good experimental fit of the data to a straight line equation as determined by linear regression.

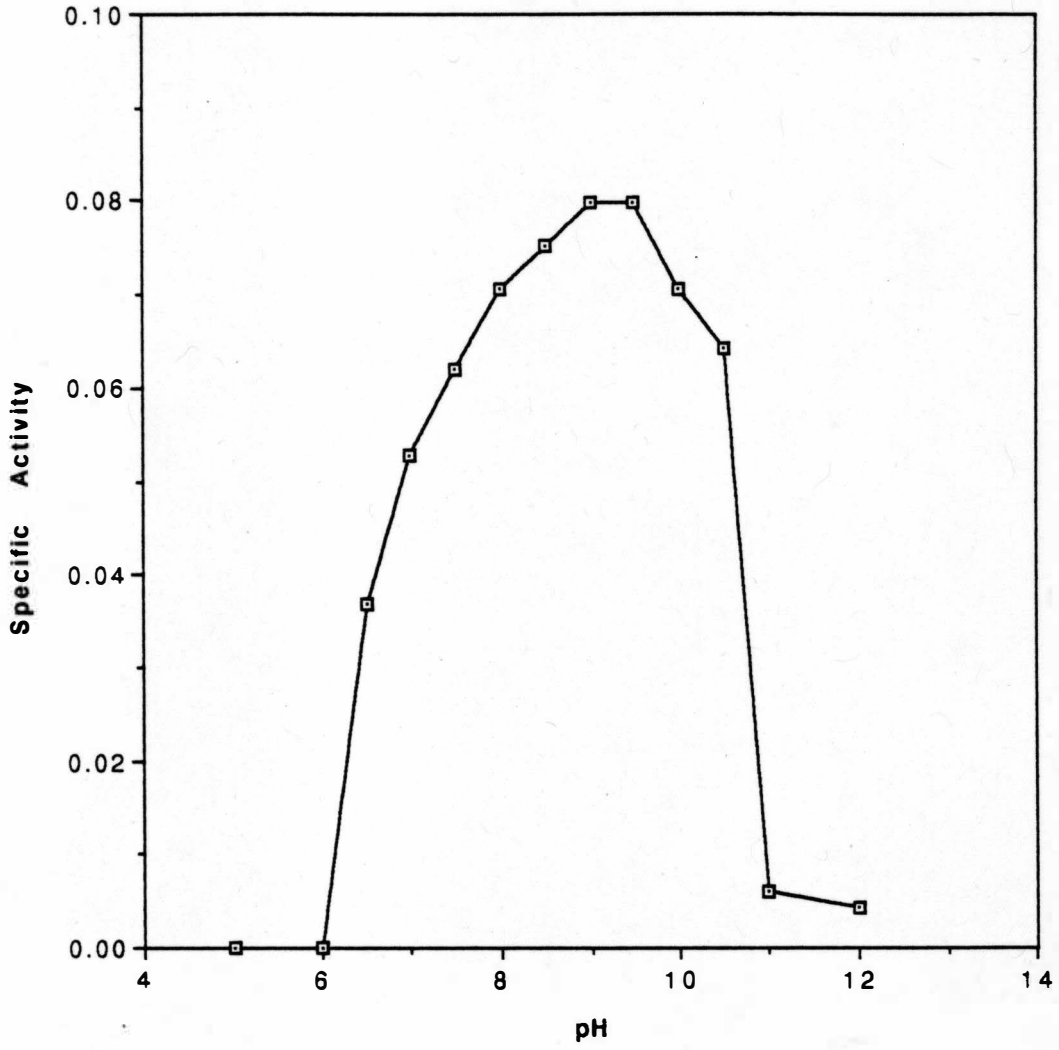

Figure 7. Effects of pH on LAD Activity.

P. fluorescens was grown and cell-free extract prepared as described in Materials and Methods. The reaction mixture contained 0.029 mg protein per ml. A unit of LAD is defined as one μmole NAD reduced per minute. Specific activity is units per mg protein.

Induction Studies

Induction of LAD in *Pseudomonas fluorescens*

Once the LAD assay had been developed, the induction of LAD in *Pseudomonas fluorescens* ATCC 13525 was studied. Table 2 presents the data for the induction studies for LAD.

Table 2

Effect of Sole Carbon and Energy Sources on the L-arabinose Dehydrogenase Activities in *Pseudomonas fluorescens* [a]

Carbon and Energy Sources	Specific Activity [b]
D+ Xylose	<0.001
L-Malate	<0.001
D-Ribose	<0.001
Succinate	<0.001
Glucose	<0.001
Citrate	<0.001
D-Sorbitol	<0.001
D-Galactose	0.052
L-Arabinose	0.495

[a] *Pseudomonas fluorescens* was grown and cell-free extracts prepared as described in Materials and Methods.

[b] Specific activity is indicated as one µmole NAD reduced per minute per mg protein.

From this data, it can be seen that LAD is inducible since the highest specific activity for this enzyme was from L-arabinose grown cells. This data also shows LAD activity in cells that were grown on basal salts medium with D-galactose as a sole carbon and energy source. However, the activity is about ten-fold less on D-galactose than on L-arabinose.

This raised the question as to whether or not LAD was same enzyme that *P. fluorescens* needed to utilize D-galactose. In order to answer this question, the pH optimum was determined for a cell-free extract from *P. fluorescens* grown on L-arabinose and a cell-free extract from *P. fluorescens* that was grown on D-galactose (Figures 8 & 9, respectively). For the pH optimum determination, each extract was tested with a pH range of 5.0 to 12.0. Both the L-arabinose and the D-galactose extract were assayed using the developed LAD assay with L-arabinose as the substrate. The extracts were also assayed for D-galactose activity using the developed LAD assay which was modified by using D-galactose rather than L-arabinose as the substrate.

Figure 8 shows the pH optimum for LAD in the cell-free extract from *Pseudomonas fluorescens* grown on L-arabinose. By examining this figure, it can be seen that there is a shift in the optimal pH for the D-galactose oxidation (pH 10.0) in comparison to that of the enzyme for L-arabinose (pH 9.0) when the L-arabinose cell-free extract was assayed.

Figure 9 represents the data gathered from the pH optimum using the cell-free extract from cells grown on D-galactose. Because of the different pH optimum for the maximal enzyme activity and the large difference in maximal specific activity when cells are

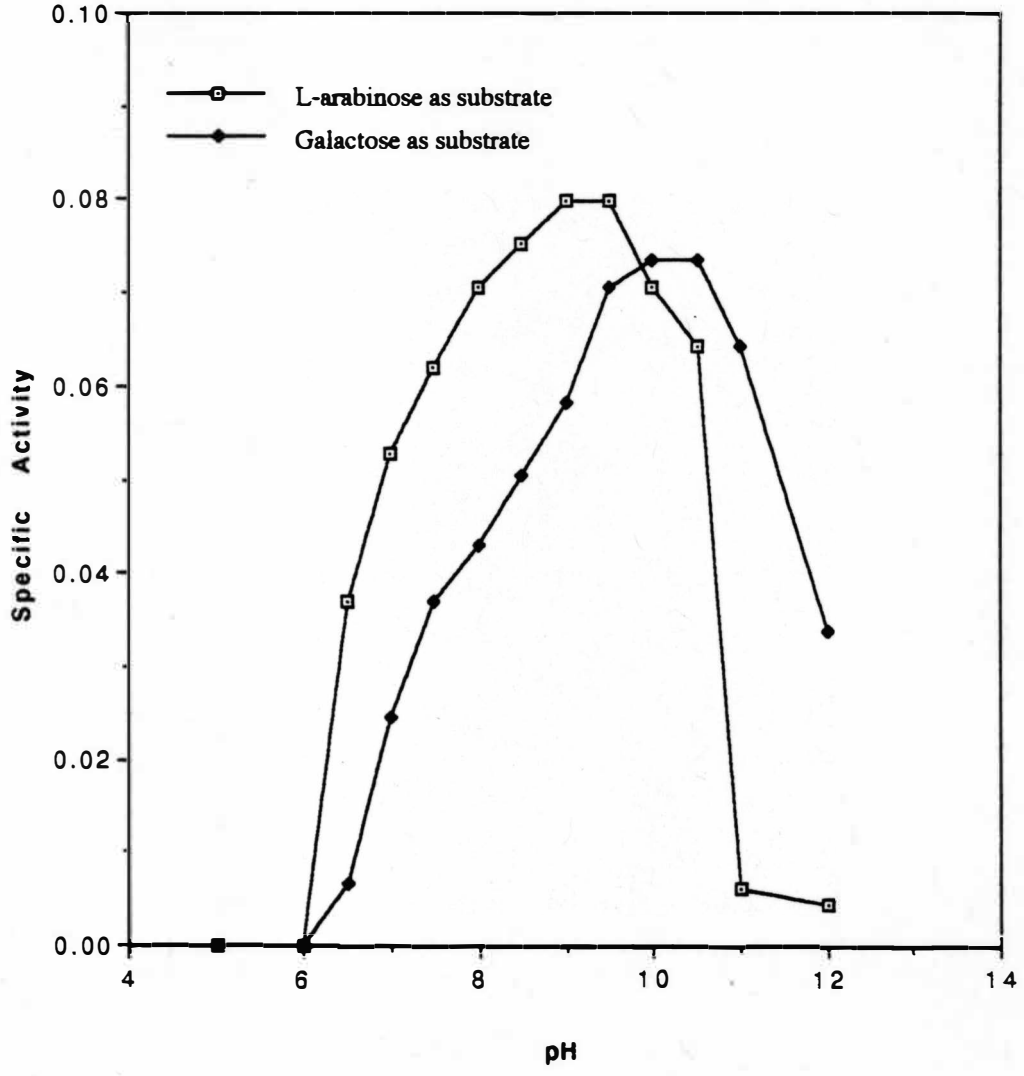

Figure 8. Comparison of the Effects of pH and Galactose and L-arabinose on Cell-Free Extracts Derived From *Pseudomonas fluorescens* Grown on L-arabinose.

Pseudomonas fluorescens were grown and cell-free extracts were prepared as described in Materials and Methods. The reaction mixtures contained 0.026 mg protein per ml and were assayed at 22 °. A unit of LAD is defined as one µmole NAD reduced per minute. Specific activity is units per mg protein.

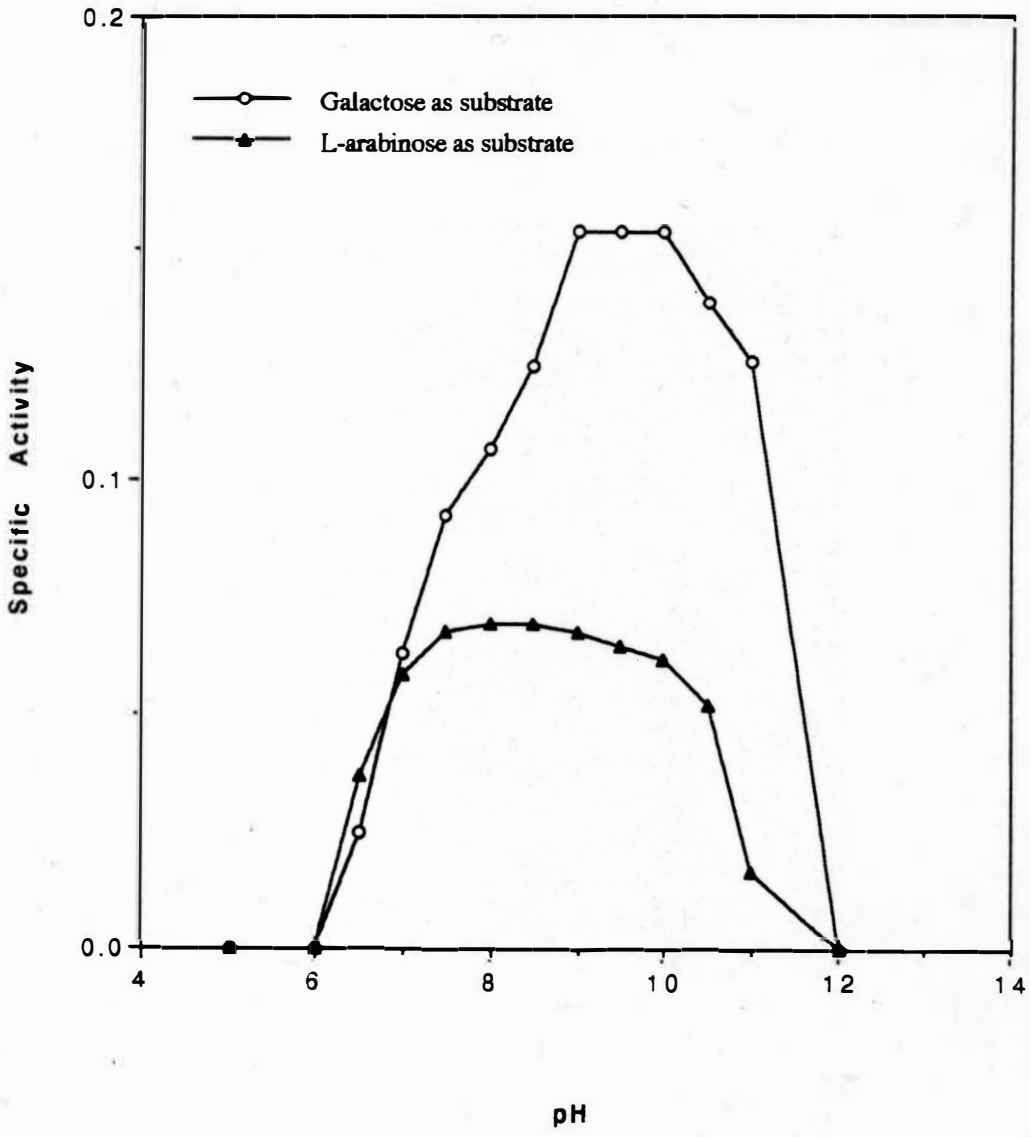

Figure 9. Comparison of the Effects of pH and Galactose and L-arabinose on Cell-Free Extracts Derived From *Pseudomonas fluorescens* Grown on Galactose.

Pseudomonas fluorescens was grown and cell-free extracts were prepared as described in the Materials and Methods. The reaction mixtures contained 0.026 mg protein per ml and were assayed at 22 °. One unit of LAD are defined as µmole NAD reduced per minute. Specific activity is units per mg protein.

grown on galactose, these data suggest that L-arabinose and D-galactose are oxidized by two different dehydratase enzymes. In order to conclude if there are two enzymes involved for the utilization of L-arabinose and galactose, we compared the specific activities of cell-free extracts from *P. putida* grown on L-arabinose and glucose when assayed with both L-arabinose and galactose. The results of these assays are shown in Table 3.

Table 3

Comparison of Specific Activity for *P. putida* Containing pPZ221 for L-arabinose and Galactose Utilization

Organism	Grown on	Assayed with	Specific Activity
P. putida containing pPZ221	glucose	L-arabinose	0.009
		galactose	<0.001
		L-ara / gal	0.008
P. putida containing pPZ221	L-arabinose	L-arabinose	0.154
		galactose	0.064
		L-ara / gal	0.129

Pseudomonas fluorescens Growth Curves

Figure 10 shows the growth curve for *P. fluorescens* on 5 mM succinate, 20 mM glucose, and on a mixture of these two sugars. These data suggest the previous results of (Clarke & Richmond, 1975; Hamilton & Dawes, 1960; Phillips & Mulfinger, 1981; Temple *et al.*, 1990; Wolff *et al.*, 1991; Zylstra *et al.*, 1989) that have shown that this organism preferentially utilizes succinate over glucose.

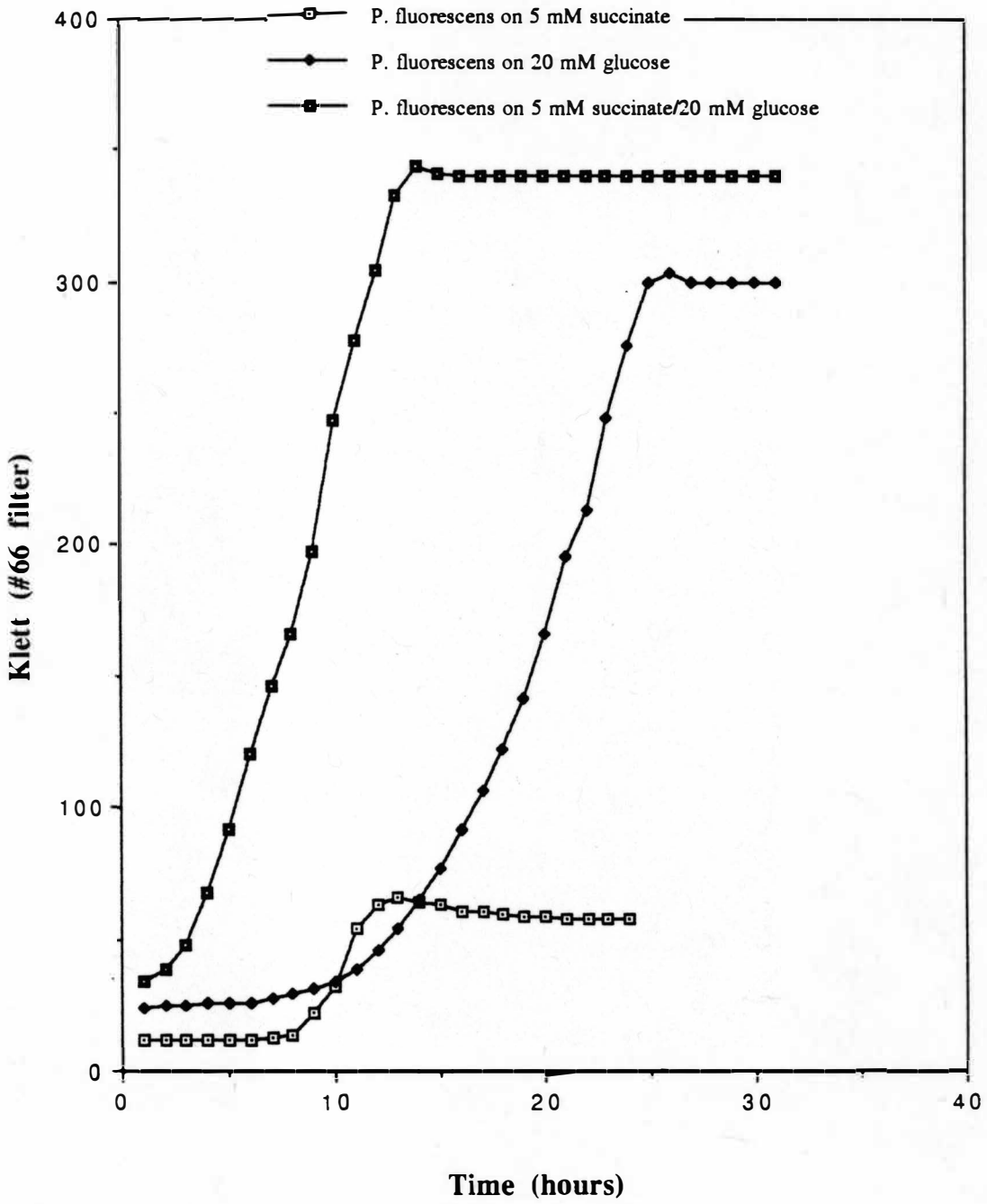

Figure 10. Growth Curve for *P. fluorescens* Grown on 5 mM Succinate, 20 mM Glucose, and a Mixture of 5 mM Succinate and 20 mM Glucose.

P. fluorescens were grown and doubling times calculated as described in Materials and Methods.

To examine the inducibility of LAD in *P. fluorescens* ATCC 13525, growth curves of *P. fluorescens* on 5 mM succinate as sole carbon and energy source and also on 20 mM L-arabinose were obtained (Figure 11). The growth curves shown in this figure were used to determine doubling times of *P. fluorescens* on these substrates. The doubling time for *P. fluorescens* was 92.7 minutes on 5 mM succinate and 361.2 minutes for 20 mM L-arabinose (Table 4). These times were then used as standards in order to determine if *P. fluorescens* was inducible when grown on media containing a mixture of 5 mM succinate and 20 mM L-arabinose.

Figure 11 also shows the results of an experiment where *P. fluorescens* was grown on a mixture of succinate and L-arabinose. The line shown in Figure 11 representing the growth curve of *P. fluorescens* when grown on a mixed growth medium of 5 mM succinate and 20 mM L-arabinose is a diauxic curve. These results clearly demonstrate that *P. fluorescens* uses succinate preferentially to L-arabinose.

Growth Curves for *Pseudomonas putida*

In previous literature, *P. putida* ATCC 12633 was shown to be unable to utilize L-arabinose (Stanier, 1966). This study confirmed this observation by inoculating *P. putida* into basal salts medium (BSM) containing 5 mM succinate and into BSM containing a mixture of 5 mM succinate and 20 mM L-arabinose and the resulting graph (Figure 12) was examined and the doubling times were calculated (Table 4).

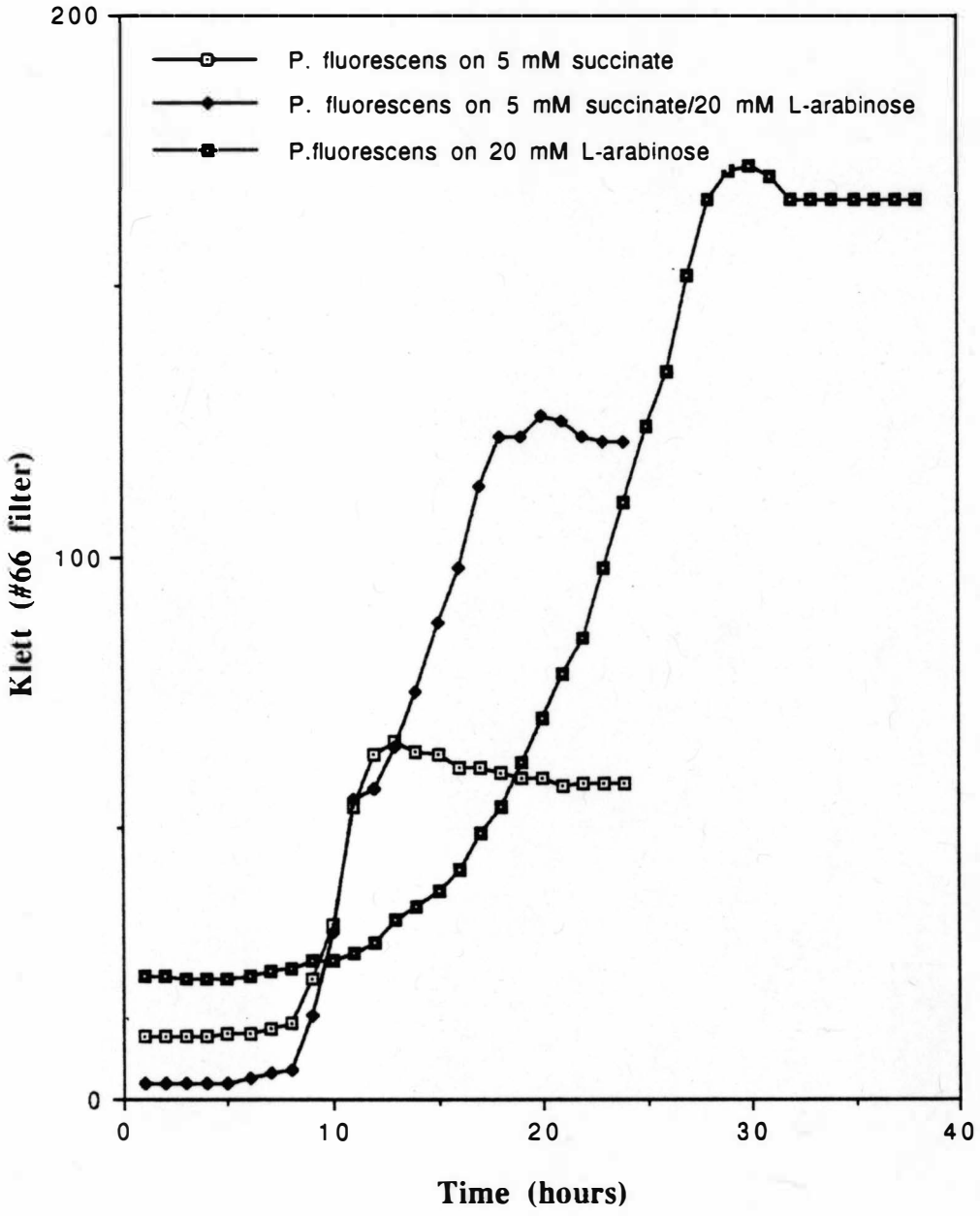

Figure 11. Growth Curve for *P. fluorescens* Grown on 5 mM Succinate, 20 mM L-arabinose, and a Mixture of 5 mM Succinate and 20 mM L-arabinose.

P. fluorescens were grown and doubling times calculated as described in the Materials and Methods.

Table 4

Doubling Times for Growth Curves From This Study

Organism	Substrate (mM)	Doubling time (minutes)[a]
Pseudomonas fluorescens	Succinate (5)	92.7
	L-Arabinose (20)	361.2
	Glucose (20)	286.9
	Succinate/L-arabinose Mix	73.9/36.1
	Succinate/Glucose Mix	199.3/332.2
Pseudomonas putida	Succinate (5)	145.5
	Glucose (20)	168.3
	L-arabinose (20)	did not grow
	Succinate/Glucose Mix	129/180.6
	Succinate/ L-arabinose Mix	120.5
Pseudomonas putida containing pPZ221	Succinate (5)	903.1
	Glucose (20)	662.3
	L-arabinose (20)	610.7
	Succinate/Glucose Mix	1083.7/321.1
	Succinate/L-arabinose Mix	903.1/459.8
Pseudomonas putida containing pLA2917	Succinate (5)	746.45
	Glucose (20)	288.9
	L-arabinose (20)	did not grow
	Succinate/Glucose Mix	505.7/412.
	Succinate/L-arabinose Mix	713.5

[a] Doubling Times were calculated from exponentially growing cultures using the equation:
$G_t = \log b/a \div 0.301\ t$
G_t = number of generations, a = O.D. at T_1, t = T_2 - T_1, b = O.D. at T_2

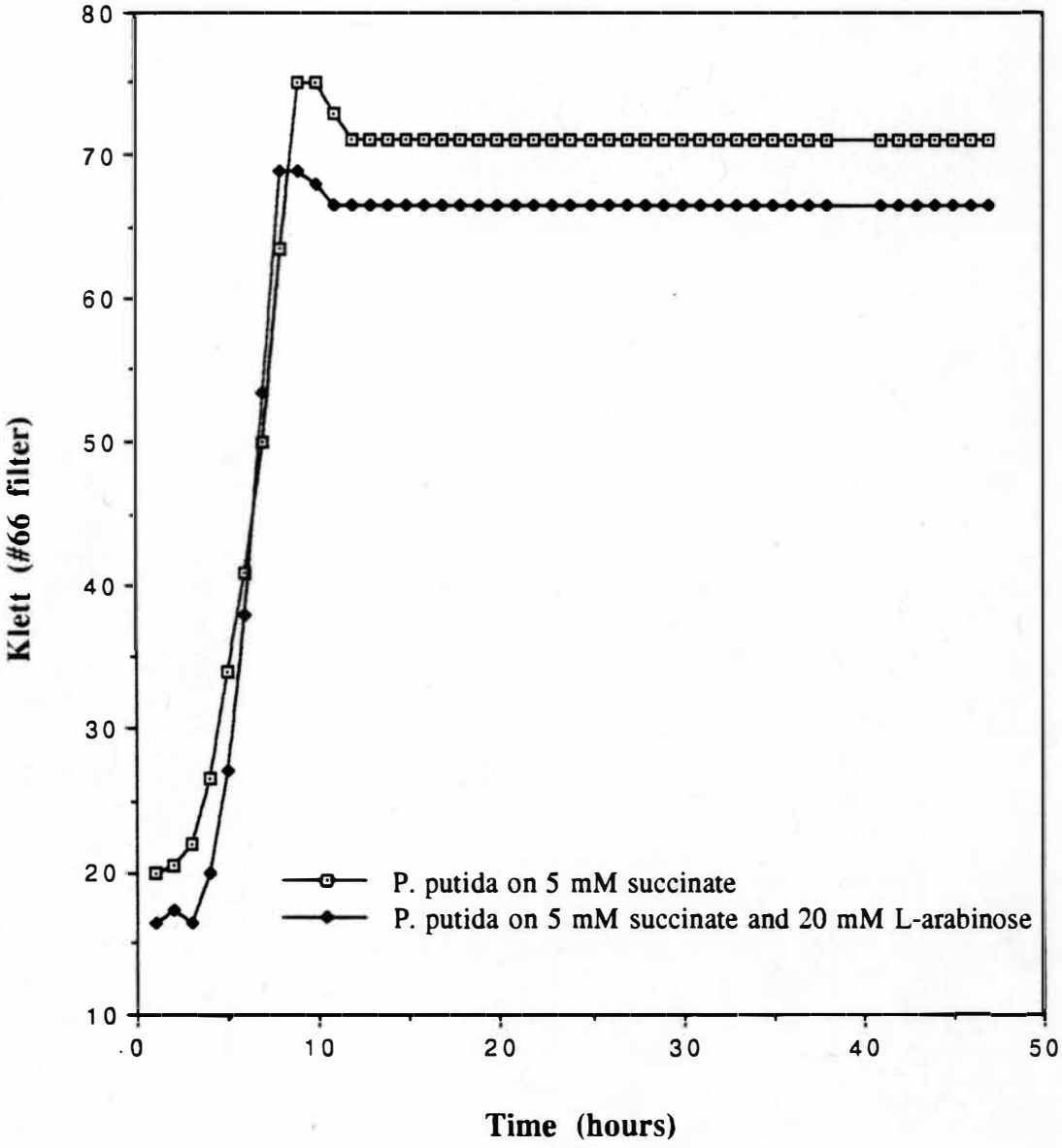

Figure 12. Growth Curve for *P. putida* Grown on 5 mM Succinate and a Mixture of 5 mM Succinate and 20 mM L-arabinose.

P. putida were grown and doubling times were calculated as described in Materials and Methods.

When the growth curve for the bacteria grown on the mixture was compared to that of 5 mM succinate grown *P. putida* ATCC 12633, we could see that the curves were approximately the same (Figure 12). Upon examining the doubling times for these two growth curves (Table 4), it was seen that the doubling time for *P. putida*, when grown on 5 mM succinate alone, are very similar to that for *P. putida* when grown on a mixture of 5 mM succinate and 20 mM L-arabinose. These results confirmed the earlier observations that *P. putida* ATCC 12633 is unable to utilize L-arabinose as a sole carbon and energy source.

To determine if *P. putida* ATCC 12633 was inducible for other types of carbohydrate utilization, this microorganism was grown on 20 mM glucose, or 5 mM succinate, or on a mixture of 5 mM succinate and 20 mM glucose (Figure 13). Figure 12 shows a slight shift of the Klett values between 10 and 12 hours when *P. putida* was grown on the two carbon and energy sources. The resulting doubling times (Table 4) indicated that *P. putida* ATCC 12633 preferentially utilized succinate but was inducible for glucose utilization.

pLA2917 and pPZ221 contain genes which code for tetracycline resistance. The concentration of tetracycline necessary to kill *P. putida* ATCC 12633 was determined so that later assays dealing with either *P. putida* containing pLA2917 or *P. putida* containing pPZ221 were not contaminated with *P. putida* ATCC 12633. A growth curve for *P. putida* ATCC 12633 grown on varying concentrations of tetracycline was run using this organism prior to transformation with pLA2917 or pPZ221(Figure 14). From this figure,

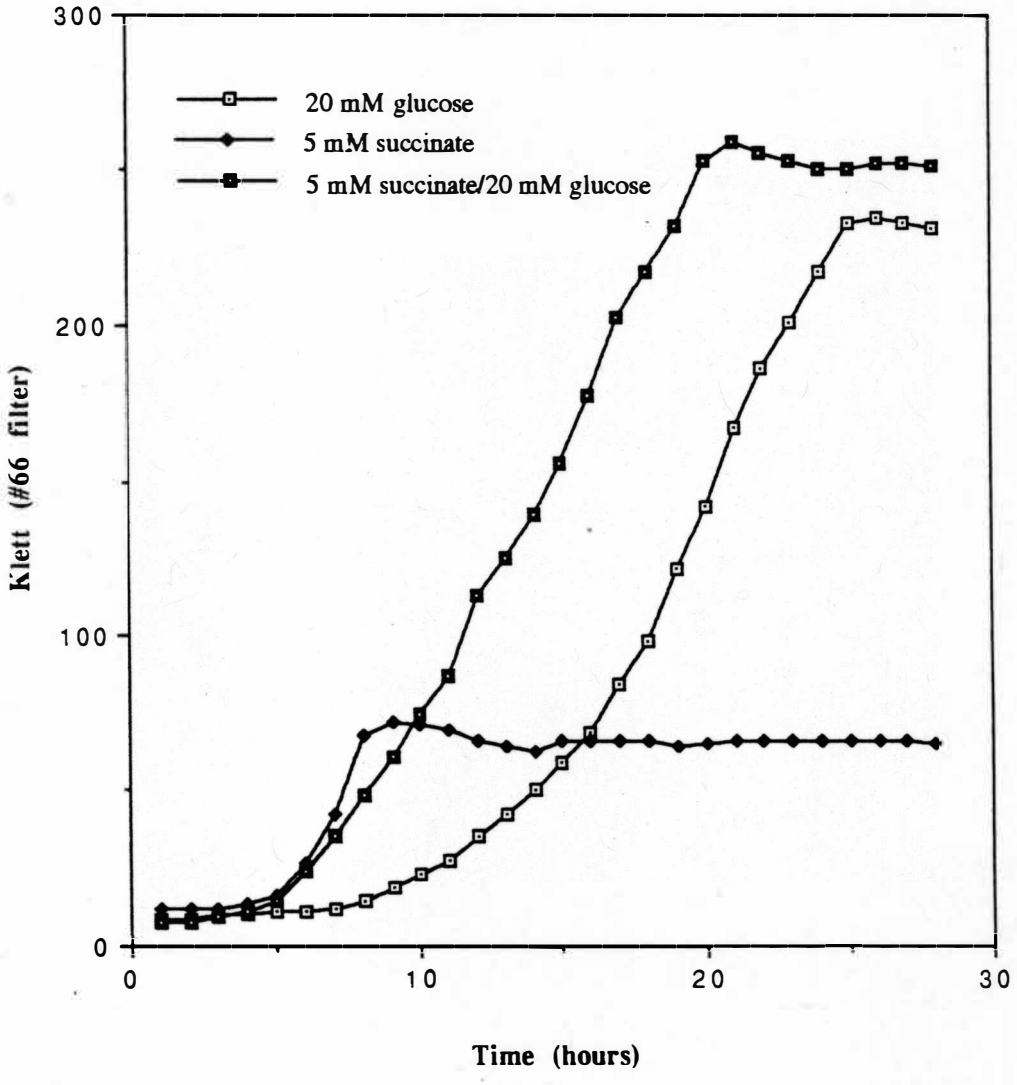

Figure 13. Growth Curve for *P. putida* ATCC 12633 Grown on 5 mM Succinate, 20 mM Glucose and a Mixture of 5 mM Succinate and 20 mM Glucose.

P. putida were grown and doubling times calculated as described in Materials and Methods.

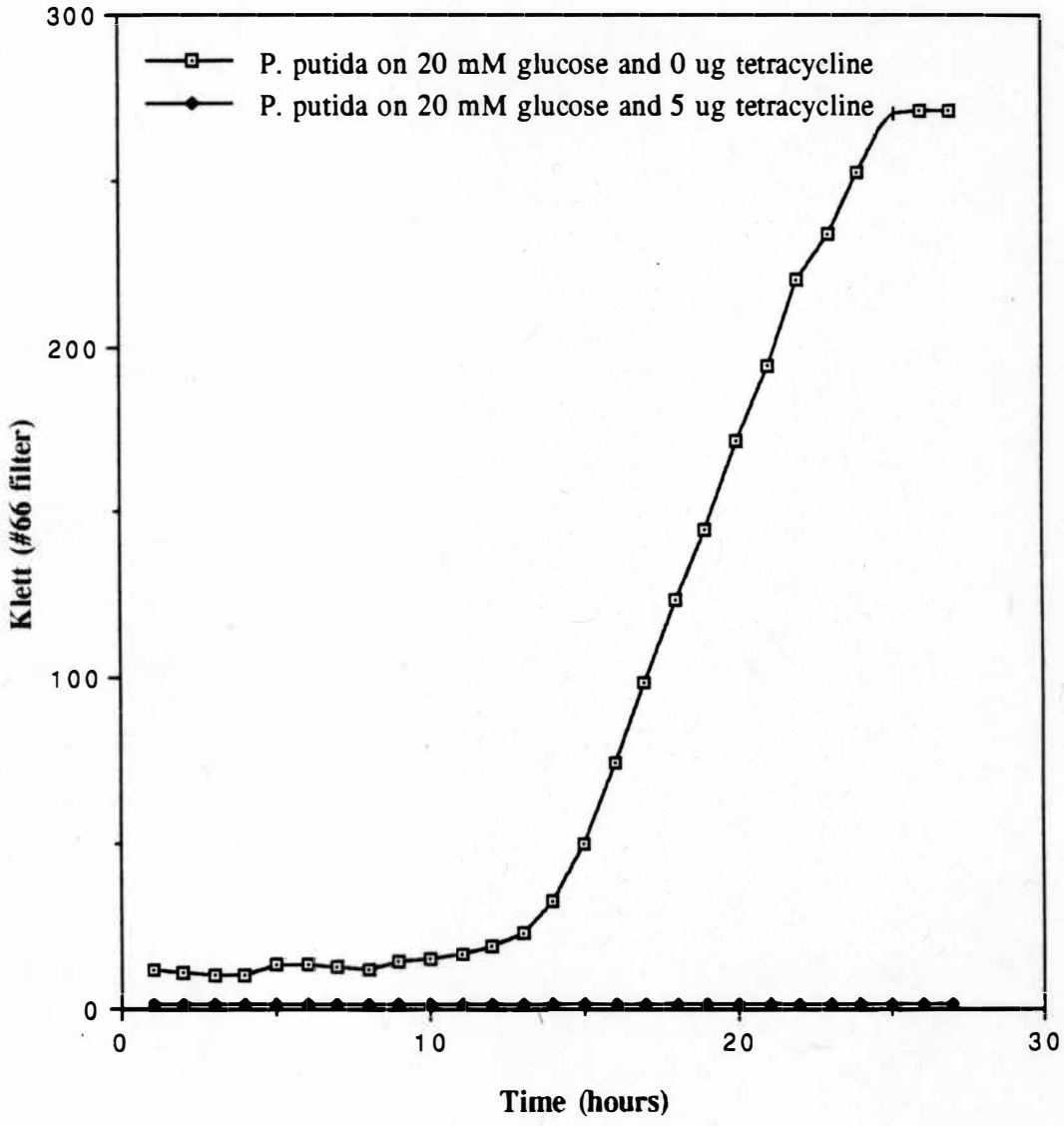

Figure 14. Growth Curve for *P. putida* ATCC 12633 Grown on 20 mM Glucose and Varying Amounts of Tetracycline(μg/ ml).

P. putida were grown and doubling times calculated as described in Materials and Methods.

it can be seen that *P. putida* is unable to grow in the presence of a minimal of 5 μg/ml of Tetracycline.

Growth Curves for *P. putida* Containing *pLA2917*

Growth curves for *Pseudomonas putida* containing the wide-host-range cosmid, pLA2917, were obtained in order to serve as a comparison for pPZ221. When *P. putida* containing pLA2917 was grown on either 5 mM succinate, 20 mM L-arabinose, or a mixture of these two carbon sources in the same concentrations, the results presented in Figure 15 were seen. This figure also shows that the insertion of the cosmid did not change the bacteria's inability to utilize L-arabinose. The doubling time for glucose is the same as above. When L-arabinose was the only sugar available to this organism, the organism did not grow.

The growth curves for *P. putida* containing pLA2917 grown on 5 mM succinate, 20 mM glucose, and a mixture of 5 mM succinate and 20 mM glucose can be seen in Figure 16. From this figure, it can be determined that the insertion of pLA2917 into *P. putida* has not altered this organism's ability to grow on succinate and glucose. When pLA2917 was grown on a mixture of these two carbon and energy sources, the growth curve indicated that the insertion of the cosmid had not altered the preferential utilization of succinate over glucose.

The doubling times for *P. putida* containing pLA2917 was calculated to be 746.45 minutes when the organism was grown on 5 mM succinate (Table 4). When *P. putida*

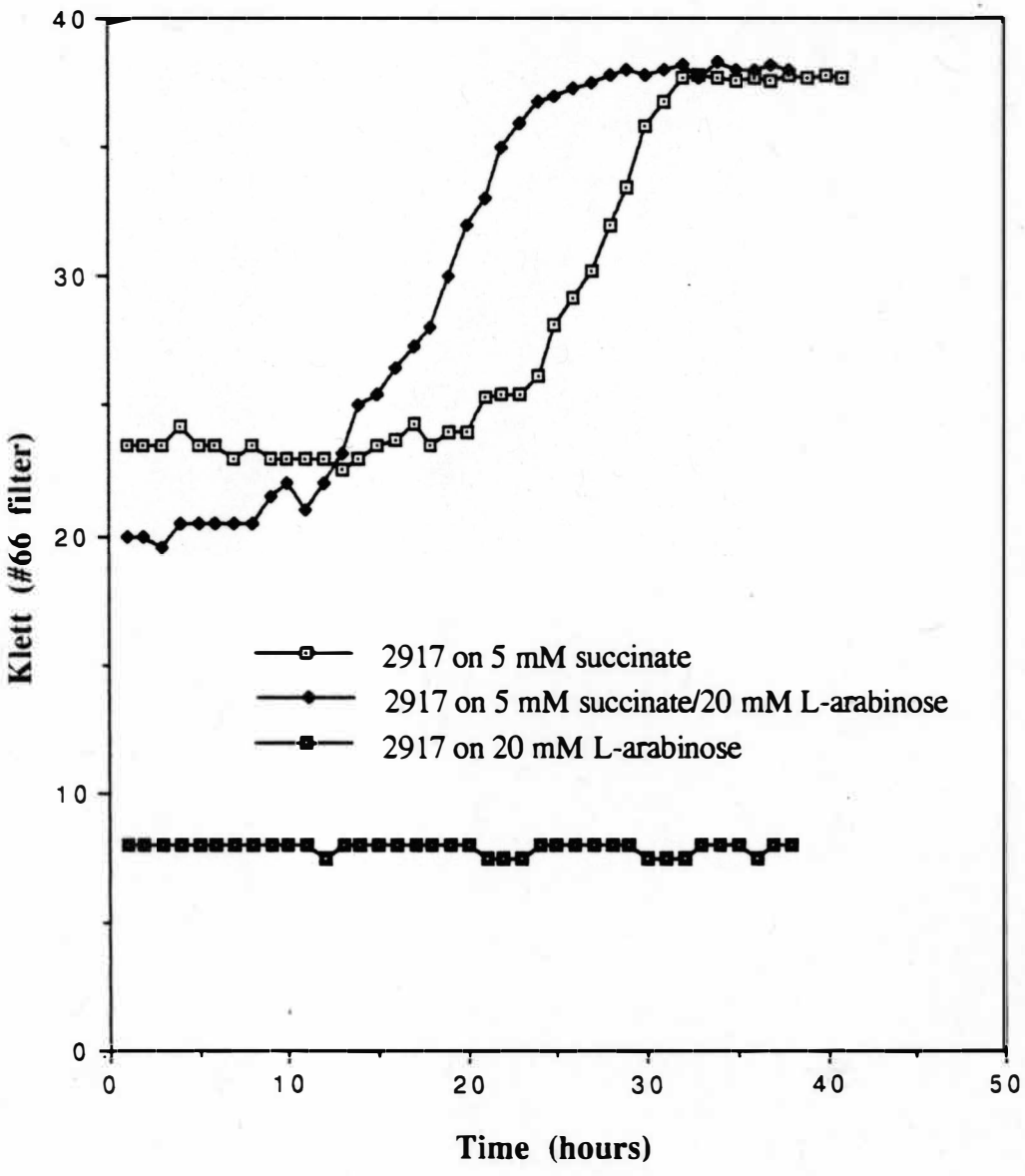

Figure 15. Growth Curve for *P. putida* Containing pLA2917 on 5 mM Succinate, 20 mM L-arabinose and a Mixture of 5 mM Succinate and 20 mM L-arabinose.

P. putida containing pLA2917 were grown and doubling times calculated as described as in Materials and Methods.

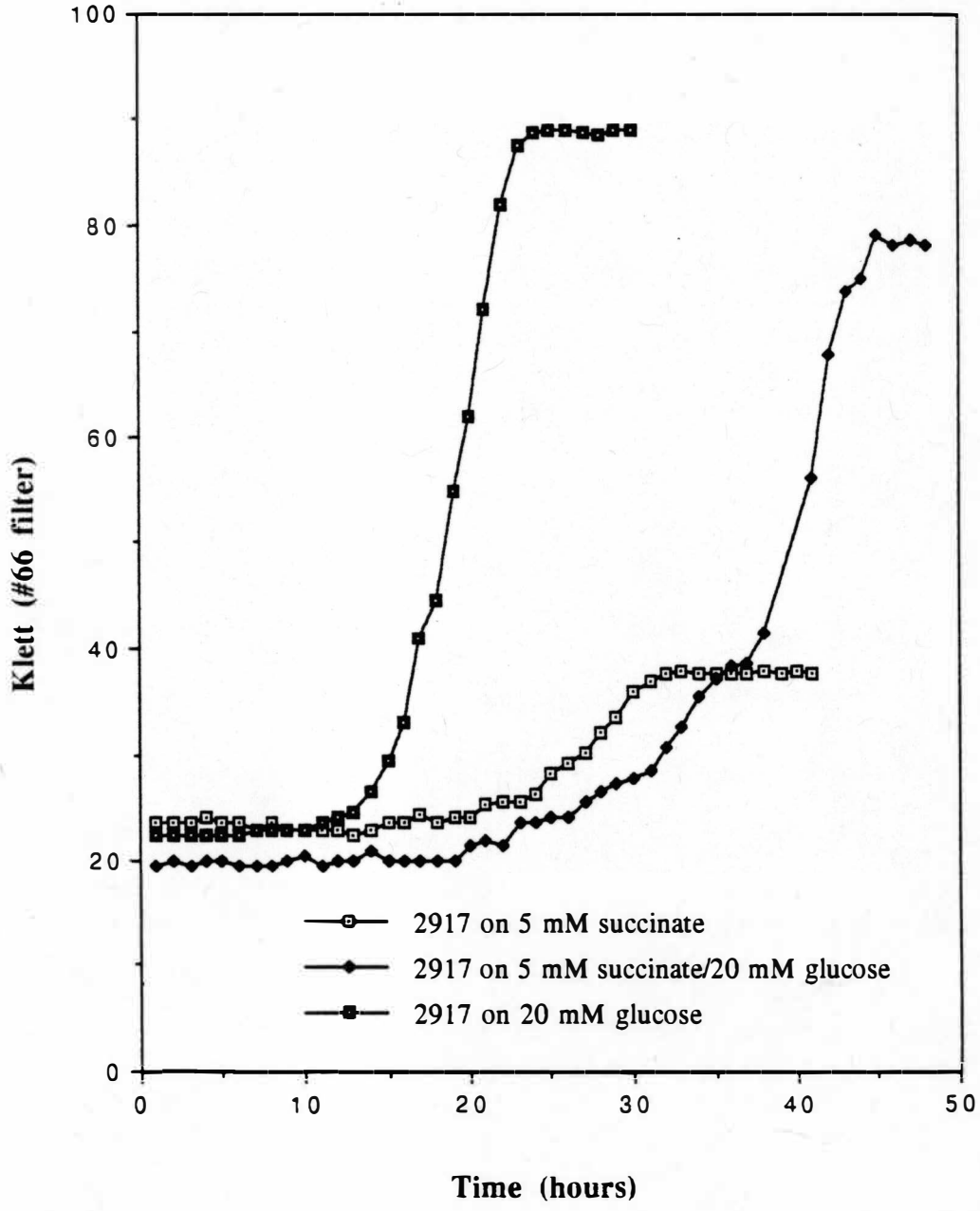

Figure 16. Growth Curve for *P. putida* Containing pLA2917 on 5 mM Succinate, 20 mM Glucose, and a Mixture of 5 mM Succinate and 20 mM Glucose.

P. putida containing pLA2917 were grown and doubling times calculated as described as in Materials and Methods.

containing pLA2917 was grown on BSM with glucose as the sole carbon and energy source, the doubling time was calculated to be 288.9 minutes (Table 4).

Growth Curves for *P. putida* Containing pPZ221

Figure 17 illustrates a typical growth curve for *P. putida* containing pPZ221 grown on either 20 mM glucose or 20 mM succinate as the sole carbon and energy source. The doubling times of *P. putida* containing pPZ221 on these substrates were calculated. The doubling time for *P. putida* containing pPZ221 on 20 mM glucose was 662.3 minutes and 903.1 minutes on 5 mM succinate (Table 4). Figure 17 also shows the results of an experiment where *P. putida* containing pPZ221 was grown on a mixture of succinate (5 mM) and glucose (20 mM). The growth curves in Figure 17 were examined to determine if *P. putida* containing pPZ221 preferentially utilizes succinate over glucose and it appears that *P. putida* containing pPZ221 does preferentially use succinate over glucose. This experiment demonstrates that the insertion of pPZ221 has not influenced the preferential utilization of succinate over glucose.

The growth of *P. putida* containing pPZ221 in succinate and L-arabinose is shown in Figure 18. To determine if this organism is inducible on these sugars, *P. putida* containing pPZ221 was either grown on 5 mM succinate, 20 mM L-arabinose, or on a mixture of these two sugars. The doubling times were determined from these growth curves. The doubling times for 5 mM succinate was 903.1 minutes. For 20 mM L-arabinose, the doubling time was 610.7 minutes (Table 4). There is no clear evidence for

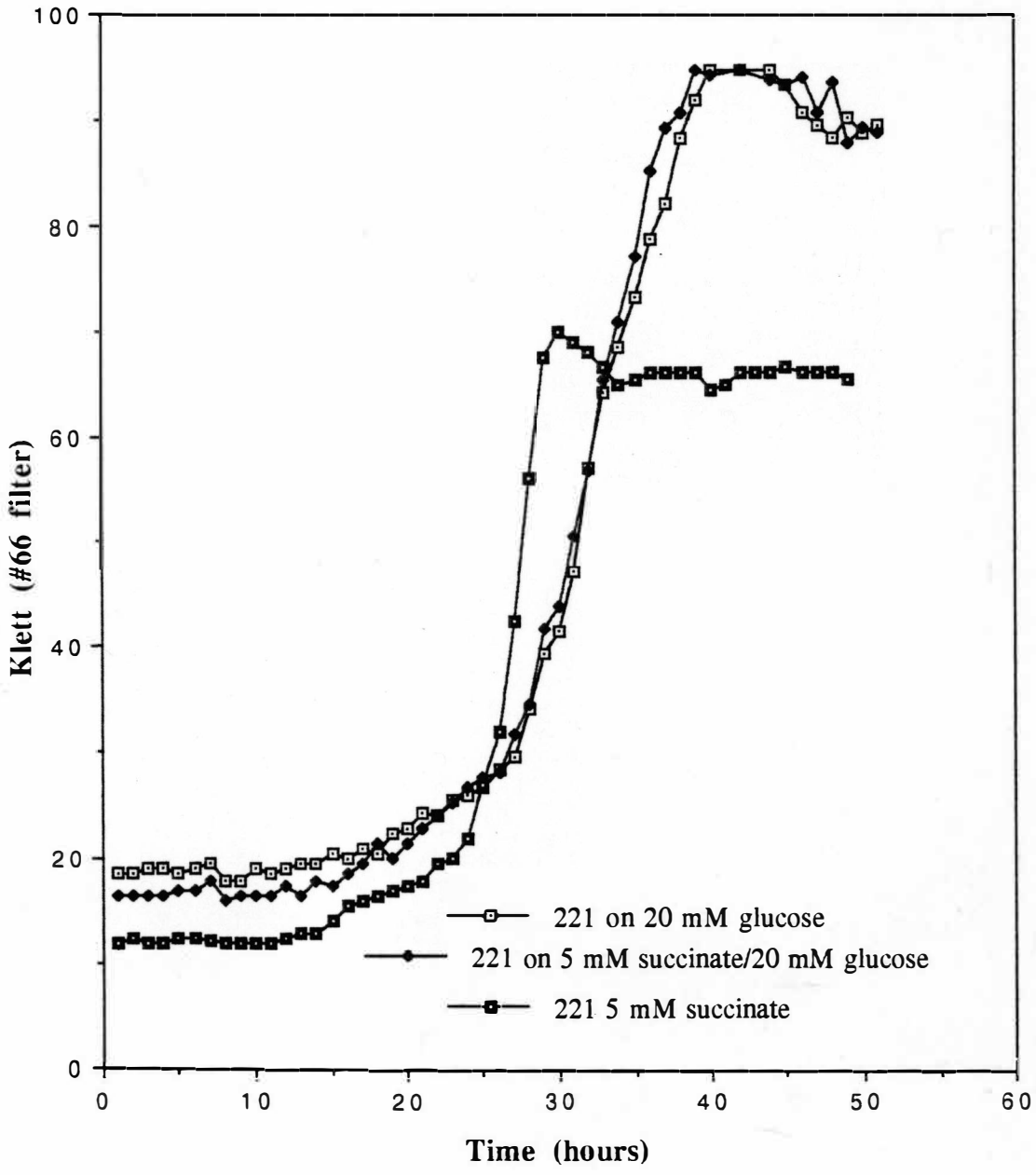

Figure 17. Growth Curve for *P. putida* Containing pPZ221 on 20 mM Glucose, 5 mM Succinate, and a Mixture of 5 mM Succinate and 20 mM Glucose.

P. putida containing *pPZ*221 were grown and doubling times calculated as described as in Materials and Methods.

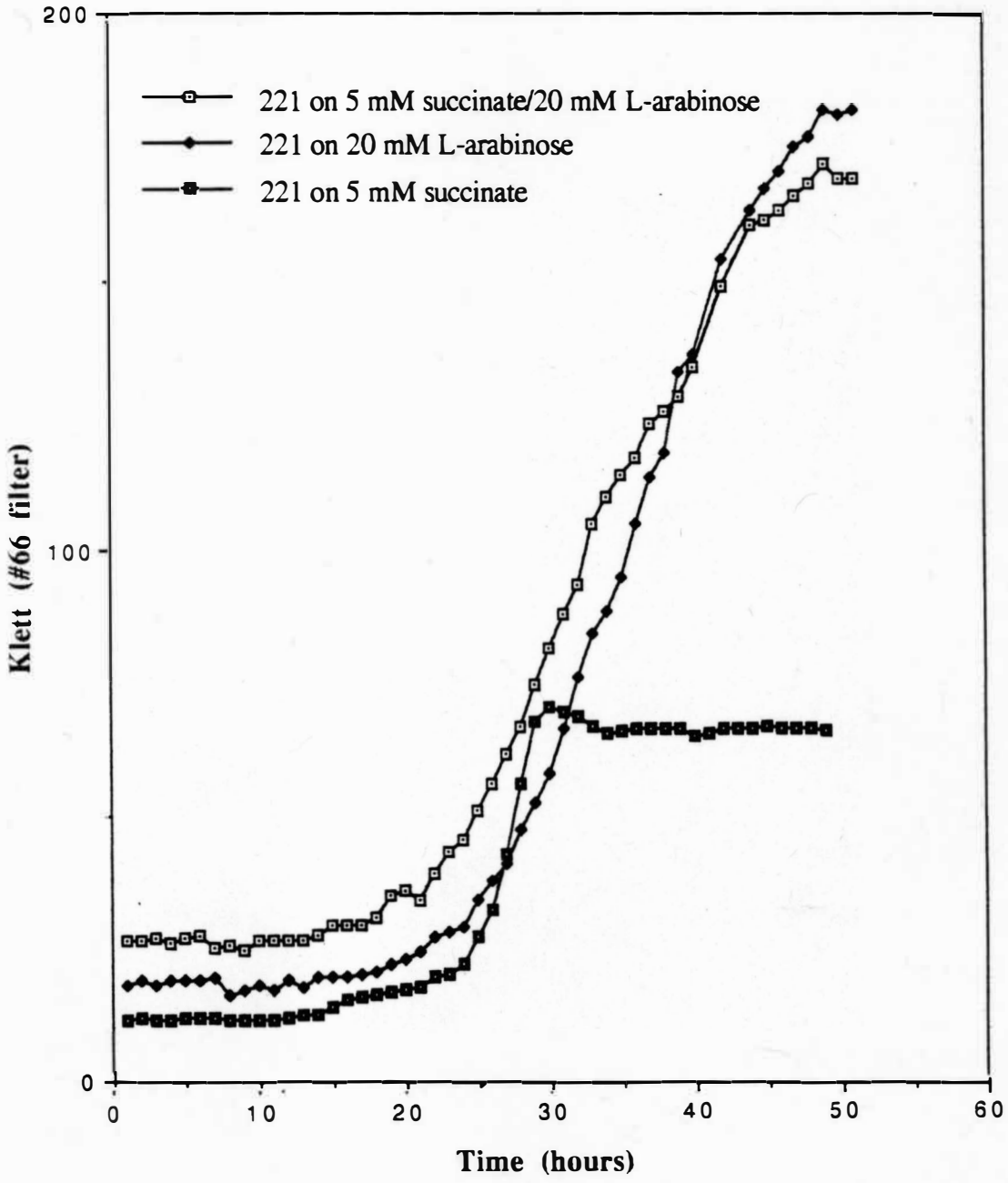

Figure 18. Growth Curve for *P. putida* Containing pPZ221 on 5 mM Succinate, 20 mM L-arabinose, and a Mixture of 5 mM Succinate and 20 mM L-arabinose.

P. putida containing pPZ221 were grown and doubling times calculated as described as in Materials and Methods.

diauxic growth in *P. putida* containing pPZ221 when grown in the presence of both succinate and L-arabinose.

Induction of LAD in *P. putida* Containing pPZ221

In order to determine if all the regulatory features were included in *P. putida* containing pPZ221, the specific activity for *P. fluorescens* on a number of different carbohydrate sources was determined as a control (Table 5). These activities were then compared to those in extracts of *P. putida* containing pPZ221 under similar growth conditions. When the specific activities for glucose-grown *P. fluorescens* assayed on L-arabinose were compares with the activities for glucose-grown *P. putida* containing pPZ221, it was seen that the specific activity levels were approximately the same. This also held true for the same cell-free extracts assayed on galactose.

Cell-free extracts from *P. putida* containing pPZ221 and *P. fluorescens* grown on L-arabinose were also compared. From this it was found that the specific activity levels were roughly ten times higher in the *P. putida* containing pPZ221 cell-free extracts when the extract was assayed with L-arabinose or with galactose (Table 5).

P. putida containing pPZ221 cell-free extract was assayed using L-arabinose, galactose, or a mixture of these two carbohydrates, resulting in the specific activities which are shown in Table 3. By examining the specific activities for *P. putida* containing pPZ221 grown on L-arabinose in light of the activities for *P. putida* containing pPZ221 grown on glucose, it can be seen that *P. putida* containing pPZ221 has approximately 17-fold more

Table 5

Specific Activities of Cell-Free Extracts From *Pseudomonas fluorescens* and *Pseudomonas putida* Grown on and Assayed With Various Carbohydrates

Organism	Grown on	Assayed with	Specific Activity
P. fluorescens	glucose	L-arabinose (L-ara)	0.002
		galactose (gal)	0.001
		L-ara / gal	0.004
P. fluorescens	L-arabinose	L-arabinose (L-ara)	0.010
		galactose (gal)	0.003
		L-ara / gal	0.008
P. fluorescens	galactsoe	L-arabinose (L-ara)	0.012
		galactose (gal)	0.059
		L-ara / gal	0.014
P. putida containing pPZ221	glucose	L-arabinose (L-ara)	0.009
		galactose (gal)	<0.001
		L-ara / gal	0.008
P. putida containing pPZ221	L-arabinose	L-arabinose (L-ara)	0.154
		galactose (gal)	0.064
		L-ara / gal	0.129

LAD activity in the L-arabinose-grown cell-free extract than in glucose-grown cell-free extract (Table 5).

DISCUSSION

The pathway for utilization of L-arabinose by *Pseudomonas fluorescens* involves the production of the Krebs cycle intermediate, α-ketoglutarate (Schmiz *et al.*, 1974). In many bacteria, the presence of an intermediate of the Krebs cycle inhibits the utilization of the carbohydrate (Clarke & Richmond, 1975; Lessie & Phibbs, 1984; Paigen & Williams, 1970). The presence of this Krebs cycle intermediate in this pathway may involve unique regulatory and expression features for utilization of L-arabinose. In order to examine the regulatory and expression features of the *P. fluorescens* genes for L-arabinose utilization, we decided to examine the production of L-arabinose dehydrogenase (LAD) in a putative cloned L-arabinose operon expressed in a surrogate host (*Pseudomonas putida* containing pPZ221). LAD is the first enzyme involved in the degradative pathway for this carbohydrate and can serve as a regulatable marker for this pathway (Figure 4) (Schmiz *et al.*, 1974). To examine this enzyme, development of a reliable assay was necessary. A similar assay had been developed previously by M. Doudoroff to study the LAD in *Pseudomonas saccrophila* (1962).

Our results for the saturation kinetics for NAD, L-arabinose, and the pH optima were similar to those obtained by Doudoroff (1962). We reported the optimal pH to be between 9.0 and 10.0 (Figure 7), as did Doudoroff. Like Doudoroff, we found that LAD oxidizes L-arabinose or D-galactose as a substrate (Figure 8 & Figure 9) and that crude

more studies, we examined the enzyme that is required for the degradation of L-arabinose and galactose. We did this by assaying *P. putida* containing pPZ221 cell-free extract from cells that were grown on L-arabinose or glucose. If two enzymes were involved in the utilization of these sugars, we predicted that the specific activities would be additive. Table 3 shows that the specific activities were not additive. This suggests that only one enzyme is involved in the utilization of L-arabinose and galactose. There is a possibility that there could be more than one enzyme and it is inhibited by the pH or the potassium phosphate buffer that was used in the assay.

The saturation kinetics that we reported are similar to Doudoroff's results (1962). and energy sources are different enzymes. We reported that the apparent Km for NAD was 1.63×10^{-5} M, in comparison to Doudoroff's apparent Km of 9.2×10^{-4} M (Figure 5). The apparent Km for L-arabinose reported in this study was 6.65×10^{-7} M, in comparison to the apparent Km of 1.3×10^{-4} M that Doudoroff reported (Figure 6). The differences between the kinetics from this experiment and Doudoroff's study may be due to the fact that Doudoroff used a pH 8.6 and 30°, while we used pH 9.0 and 22-25°, or that this study did not use purified proteins while Doudoroff's study did.

Another similarity between these two studies is that LAD is an inducible enzyme (Table 1). To further examine the inducibility of these bacteria, growth curves were obtained for *P. fluorescens*. Figure 10 and Figure 11 show the growth curve for *P. fluorescens* on succinate and glucose or succinate and L-arabinose, respectively. Figure 10 illustrates that *P. fluorescens* is able to utilize both succinate and glucose. Another growth

curve would have to be obtained in order to determine the inducibility relationships between these two sugars. From Figure 11, we can see that this organism is able to utilize either succinate or L-arabinose as a single carbon and energy source. When *P. fluorescens* is grown on a mixture of succinate and L-arabinose, with the amount of succinate being limited, we can see that this organism preferentially utilizes succinate but is inducible for L-arabinose. From the doubling times determined from these curves (Table 4) and by examining the actual graph of the Klett values versus the time (hours), the inducibility of the organism for the carbon and energy source in question can be determined.

For *P. fluorescens*, the growth curve for growth solely on 5 mM succinate or L-arabinose was a typical curve, starting in a lag phase, reaching a linear growth phase, and then reaching the plateau of stationary growth. The doubling time for this organism grown on 5 mM succinate was 92.7 minutes. On 20 mM L-arabinose, the doubling time was 361.2 minutes. When *P. fluorescens* was grown on a mixture of 5 mM succinate and 20 mM L-arabinose, the growth curve was that of a diauxic curve. This curve starts out in a lag phase and then goes into a period of linear growth. The doubling time for this part of the curve is similar to that for growth on 5 mM succinate alone. The growth curve briefly plateaus where the growth on succinate alone plateaus into stationary growth. The growth curve then shows that *P. fluorescens* then started growing on L-arabinose which is indicated by another period of linear growth, which has a doubling time which is similar to the doubling time for *P. fluorescens* grown with L-arabinose, as the sole carbon and energy source.

Since we wished to examine the expression of genes from *P. fluorescens* in the surrogate host, *P. putida*, the next step was to obtain growth curves for *P. putida* on various carbohydrates and tetracycline (Figures 12, 13, & 14). The genes for L-arabinose utilization in *P. fluorescens* were placed into a cosmid which contains tetracycline as the selective marker. *P. putida* is unable to grow in the presence of 5 μg/ml tetracycline (Figure 14) which indicates that when *P. putida* containing pLA2917 was able to grow in the presence of tetracycline, it was able to grow as a result of the transformation process.

Growth curves for *P. putida* containing the cosmid vector pLA2917 were obtained. Figure 15 shows that the insertion of pLA2917 did not change *P. putida*'s inability to utilize L-arabinose as a sole carbon and energy source as is shown in Figure 12. Figure 16 shows the *P. putida* is able to grow on succinate and glucose as sole carbon and energy sources. This figure also shows that the inserted cosmid did not alter the organism's preferential usage of succinate over glucose nor did it alter the inducibility of the glucose utilization enzymes which was demonstrated in Figure 13.

The cosmid that contained the *P. fluorescens* genes for L-arabinose utilization was designated pPZ221. The growth curve for *P. putida* containing pPZ221 shows that the addition of the *P. fluorescens* genes did not effect the ability of *P. putida* to use succinate over glucose (Figure 17). When L-arabinose was the sole carbon and energy source in the growth curve, it was seen that *P. putida* containing pPZ221 was now able to utilize the sugar (Figure 18). When the specific activities of the cell-free extracts of *P. putida*

containing pPZ221 and *P. fluorescens* were compared for the induction of LAD, the trends that were seen in the specific activities were similar.

The specific activities from the results showed that L-arabinose operon contained in the pPZ221 vector is inducible (Table 3 & Table 5). The data in Table 3 show that the specific activity for LAD is 0.154 for *P. putida* containing pPZ221 and grown on L-arabinose. For *P. putida* containing pPZ221 and grown on glucose, the specific activity for LAD was 0.009. This is a 17-fold increase in LAD expression when the organism is grown on L-arabinose. These results are similar to *P. fluorescens* LAD data where the specific activities were 0.002 and 0.010 for glucose grown *P. fluorescens* and L-arabinose grown *P. fluorescens* assayed on L-arabinose, respectively. The increase for *P. fluorescens* is a 5-fold increase. The difference between these two organisms may be due to the high-copy number cosmid, or a number of other factors, such as the amount of protein in the reaction, the size of the initial pellet, the destruction of LAD activity during sonification. Both of the *P. fluorescens* cell-free extracts discussed above were assayed using L-arabinose in the reaction mixture. Thus, when the specific activity values for the cell-free extracts from glucose-grown *P. fluorescens* and *P. putida* containing pPZ221 which were assayed with L-arabinose in the reaction mixture were compared, it was found that the specific activities were approximately the same. Another example that indicated that the plasmid LAD was still inducible was the assay involving the cell-free extracts from *P. putida* containing pPZ221 and *P. fluorescens* which were grown on L-arabinose and then assayed in L-arabinose or galactose. When the specific activities from these assays were compared, we

saw that the cell-free extract from *P. putida* containing pPZ221 grown on L-arabinose had a specific activity that was 15x that of the cell-free extract from *P. fluorescens* grown on L-arabinose (Table 5) when L-arabinose was in the reaction mixture. An increase of 21x was observed when the same cell-free extracts were used with galactose as the sugar in the reaction mixture. The most simple explanation for this is that the genes inserted into the high-copy number cosmid contain the features necessary for regulation of L-arabinose utilization. Other possible reasons have been mentioned previously.

Another observation regarding the regulatory features in the inserted genes involved *P. putida* containing pPZ221 grown on either L-arabinose or glucose and the cell-free extract assayed with L-arabinose as the carbohydrate in the reaction mixture. When the specific activity from the cell-free extract from *P. putida* containing pPZ221 grown on L-arabinose was compared to those of the same organism grown on glucose, an increase in LAD of approximately 17-fold was observed when assayed with L-arabinose (Table 3). This shows that the cloned piece of DNA contained in the pPZ221 is expressed at a higher specific activity when the organism is grown on L-arabinose. This result demonstrates the L-arabinose utilization genes in this plasmid were induced in the presence of L-arabinose but were repressed by growth in the absence of L-arabinose.

These results suggest that the insert piece of *P. fluorescens* chromosomal DNA in pPZ221 contains a regulatory element, a promoter, an operator, a transport mechanism, and all the structural genes for L-arabinose utilization.

BIBLIOGRAPHY

Alam, A. (1992). A model for formulation of protein assay. Analytical Biochemistry, 203, 121-126.

Allen, L. N. & Hanson, R. S. (1985). Construction of broad-host range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. Journal of Bacteriology, 161, 955-962.

Beverin, S., Sheppard, D. E., & Park, S. S. (1971). D-Fructose as a gratuitous inducer of the L-arabinose operon in strains of *Escherichia coli* B/r mutant in gene *araC*. Journal of Bacteriology, 107, 79-86.

Brickman, E., Soll, L., & Beckwith, J. (1973). Genetic Characterization of mutation which affect catabolite-sensitive operons in *Escherichia coli* , including deletions of the gene for adenyl cyclase. Journal of Bacteriology, 116, 582-587.

Brock, T. D. & Madigan, M. T. (1988). Biology of Microorganisms. Englewoods Cliffs, NJ: Prentice Hall.

Bustos, S. A. & Schleif, R. F. (1993). Functional domains of the AraC protein. Proceedings of the National Academy of Science USA, 90, 5638-5642.

Clarke, P. H. & Ornston, L. N. (1975). Metabolic pathways and regulation. In P. H. Clarke & m. H. Richmond(Ed.), Genetics and Biochemistry of *Pseudomonas* (pp 215-221, 240-247). NY: John Wiley & Sons.

Cohn, M. & Horibata, K. (1959). Inhibition by glucose of the induced synthesis of the β-galactoside-enzyme system of *Escherichia coli*. Analysis of maintenance. Journal of Bacteriology, 78, 601-612.

Consevage, S. M., Porter, R. D., & Phillips, A. T. (1985). Cloning and expression in *Escherichia coli* of histidine utilization genes from *Pseudomonas putida*. Journal of Bacteriology, 162, 138-146.

Cuskey, S.M., Wolff, J. A., Phibbs Jr., J. A., & Olsen, R. H. (1985). Cloning of genes specifying carbohydrate catabolism in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Journal of Bacteriology, 162, 865-871.

Dagley, S. & Trudgill, P. W. (1965). The metabolism of galactarate, D-glucarate and various pentoses by species of *Pseudomonas*. Biochemical Journal, 95, 48-57.

de Crombrugghe, B., Busby, S. & Buc, H. (1984). Cyclic AMP receptor protein: role in transcription activation. Science, 224, 831-838.

de Crombrugghe B., Chen, B., Anderson, W. A., Gottesman, M. E., Perlman, R. L., & Pastan, I. (1971). Role of cyclic adenosine 3', 5'-monophosphate and the cyclic adenosine 3', 5'-monophosphate receptor protein in the initiation of *lac* transcription. The Journal of Biological Chemistry, 246, 7343-7348.

de Crombrugghe, B., & Pastan, I. (1980). cyclic AMP, the cyclic AMP receptor protein, and their dual control of the galactose operon. In J. H. Miller & W. S. Reznikoff (Ed.), The Operon (pp 303 - 320). Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

De Reuse, H. & Danchin, A. (1991). Positive regulation off the *pts* operon of *Escherichia coli:* genetic evidence for a signal transduction mechanism. Journal of Bacteriology, 173, 727-733.

Dills, S., Apperson, A., Schmidt, M. R., & Saier Jr., M. H. (1980). Carbohydrate transport in bacteria. Microbiological Reviews, 44, 385-418.

Doudoroff, M. (1962). L-Arabinose dehydrogenase of *Pseudomonas saccharophilia*. Methods in Enzymology, 5, 342-344.

Durham, D. R., Roehl, R. A., Flickinger, J. L., Gilford, J. E., & Wattam, R. E. (1984). Genetic engineering in *Pseudomonas*: cloning and expression of catabolite genes. In C. H. Nash & L. A. Underkofler (Ed.), Developments in Industrial Microbiology: a publication of the Society for Industrial Microbiology, 25, (pp 163-174). Victor Graphics, Inc., Baltimore, Maryland.

Eisenberg, R. C. & Phibbs, Jr., P. V. (1982). Characterization of an inducible mannitol-binding protein from *Pseudomonas aeruginosa*. Current Microbiology, 7, 229-234.

Eisenberg, R. C. & Phibbs, Jr., P. V. (1988). Cloning of *Pseudomonas fluorescens* L-arabinose utilization genes. Abstracts of the Annual Meeting of the American Society for Microbiology, p226, K118.

Englesberg, E. (1971). Regulation in the L-Arabinose system. In H. J. Vogel (Ed.), Metabolic Pathways, 5, (pp 257-297). Academic Press, NY.

Englesberg, E, , Irr, J., Power, J., & Lee, N. (1965). Positive control of enzyme synthesis by gene C in the L-arabinose system. Journal of Bacteriology, 90, 946-957.

Epstein, W. & Beckwith, J. R. (1968). Regulation of gene expression. Annual Review of Biochemistry, 37, 411-436.

Eschenlauer, A. C., & Reznikoff, W. S. (1991). *Escherichia coli* catabolite gene activator protein mutants defective in positive control of *lac* operon transcription. Journal of Bacteriology, 173, 5024-5029.

Garges, S. & Adhya, S. (1988). Cyclic AMP-induced conformational change of cyclic AMP receptor protein(CRP): intragenic suppressers of cyclic AMP-independent CRP mutations. Journal of Bacteriology, 170, 1417-1422.

Gottesman, S. (1984). Bacterial regulation: global regulatory networks. Annual Review of Genetics, 18, 415-441.

Haggerty, D. M. & Schleif, R. F.(1975). Kinetics of the onset of catabolic repression in *Escherichia coli* as determined by *lac* messenger ribonucleic acid initiations and intracellular cyclic adenosine 3', 5'- monphosphate levels. Journal of Bacteriology, 123, 946-953

Hahn, S., Dunn, T, & Schleif, R. (1984). Upstream repression and CRP stimulation of the *Escherichia coli* L-arabinose operon. Journal of Molecular Biology, 180,61-72.

Hamilton, W. A. & Dawes, E. A. (1959). A diauxic effect with *Pseudomonas aeruginosa*. Biochemical Journal, 71, 25P-26P.

Hamilton, W. A. & Dawes, E. A. (1960). The nature of the diauxic effect with glucose and organic acids in *Pseudomonas aeruginosa*. Biochemical Journal, 76, 70P.

Hartl, D. L. (1991) Basic Genetics. Boston, MA: Jones & Bartlett Publishers, Inc.

Heffernan, L. & Wilcox, G. (1976). Effect of *araC* gene product on catabolite repression in the L-arabinose regulon. Journal of Bacteriology, 126, 1132-1135.

Hendrickson, W., Stoner, C. & Schleif, R. (1990). Characterization of the *Escherichia coli araFGH* and *araJ* promoters. Journal of Molecular Biology, 215, 497-510.

Hogg, R. W. & Englesberg, E. (1969). L-arabinose binding protein from *Escherichia coli* B/r. Journal of Bacteriology, 100, 423-432.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., & Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology. Ninth Edition. Baltimore, MD.: Williams & Wilkins

Horazdovsky, B. F. & Hogg, R. W. (1987). High-affinity L-arabinose transport operon. Gene Expression and mRNAs. Journal of Molecular Biology, 197, 27-35.

Horazdovsky, B. F. & Hogg, R. W. (1989). Genetic reconstitution of the high-affinity L-arabinose transport system. Journal of Bacteriology, 171, 3053-3059.

Hou, L., Martin, K. J., & Schleif, R. (1988). Alternative DNA loops regulate the arabinose operon in *Escherichia coli*. Proceedings of the National Academy of Science USA, 85, 5444-5448.

Hylemon, P. B. & Phibbs, Jr., P. V. (1972). Independent regulation of hexose catabolizing enzymes and glucose transport activity in *Pseudomonas aeruginosa*. Biochemical and Biophysical Research Communications, 48, 1041-1048.

Jacob, F. & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. Journal of Molecular Biology, 3, 318-356.

Kalb, V. F. & Bernlohr, R. W. (1977). A new spectrophotometeric assay for protein in cell extracts. Analytical Biochemistry, 82, 362-371.

Koisba, B. E. & Schleif, R. (1982). Arabinose-inducible promoter from *Escherichia coli*. Journal of Molecular Biology, 156, 53-66.

Kolodrubetz, D., & Schleif, R. (1981a). L-Arabinose transport systems in *Escherichia coli* K-12. Journal of Bacteriology, 148, 472-479

Kolodrubetz, D., & Schleif, R. (1981b). Regulation of the L-arabinose transport operons in *Escherichia coli*. Journal of Molecular Biology, 152, 215-227.

Kundig, W., Ghosh, S. & Roseman, S. (1964). Phosphate bound to histidine in a protein as an intermediate in a novel phosphotransferase system. Proceedings of the National Academy of Science USA, 52, 1067-1074.

Lee, N. (1980). Molecular aspects of *ara* regulation. In J. H. Miller & W. S. Reznikoff (Ed.), The Operon (pp 389-405). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Lee, N. L., Gielow, W. O. & Wallace, R. G. (1981). Mechanism of *araC* autoregulation and the domains of two Overlapping Promoters, P_C and P_{BAD}, in the L-arabinose regulatory region of *Escherichia coli*. Proceedings of the National Academy of Science USA, 78, 752-756.

Lessie, T. G., & Phibbs Jr., P. V. (1984). Alternative pathways of carbohydrate utilization on Pseudomonads. Annual Review of Microbiology, 38, 359-387.

Lichenstein, H. S., Hamilton, E. P., & Lee, N. (1987). Repression and catabolite gene activation in the *araBAD* operon. Journal of Bacteriology, 169, 811- 822.

Lis, J. T. & Schleif, R. (1973). Different cyclic AMP requirements for induction of the arabinose and lactose operons of *Eschericha coli*. Journal of Molecular Biology, 79, 149-162.

Lobell, R. B. & Schleif, R. F. (1990). DNA looping and unlooping by AraC protein. Science, 250, 528-532.

Lowry, O. H., Rosebough, N. J., Farr, A. L., & Randall, R. J. (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 173, 7204-7212.

MacGregor, C.H., Wolff, J. A., Arora, S. K., & Phibbs Jr., P. V. (1991). Cloning of a Catabolite Repression Control (*crc*) Gene from *Pseudomonas aeruginosa*, Expression of the Gene on *Escherichia coli*, and Identification of the Gene Product in *Pseudomonas aeruginosa*. Journal of Bacteriology, 173, 7204-7212.

MacInnes, K. R., Sheppard, D. E. & Falgout, B. (1978). Regulatory properties of $araC^c$ mutants in the L-arabinose operon of *Escherichia coli* B/r. Journal of Bacteriology, 133, 178-184.

Magasanik, B. (1961). Catabolite repression. Cold Spring Harbor Symposium on Quantitative Biology, 26, 249-256.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982). Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

McGinnis, J. F. & Paigen, K. (1969). Catabolite inhibition: a general phenomenon in the control of carbohydrate utilization. Journal of Bacteriology, 100, 902-913.

Meadow, N. D., Fox, D. K., & Roseman, S. (1990). The bacterial phosphoenolpyruvate: glucose phosphotransferase system. Annual Review of Biochemistry, 59, 497-542.

Novotny, C. P. & Englesberg, E. (1966). The L-arabinose permease system in *Escherichia coli* B/r. Biochim. Biophys. Acta, 177, 217-230.

Ogden, S., Haggerty, D., Stoner, C. M., Kolodrubetz, D., & Schleif, R. (1980). The *Escherichia coli* L-Arabinose operon: binding sites of the regulatory proteins and a mechanism of positive and negative regulation. Proceedings of the National Academy of Science, 77, 3364-3350.

Ornston, L. N. (1971). Regulation of catabolite pathways in *Pseudomonas*. Bacteriological Reviews, 35, 87-116.

Paigen, K. & Williams, B. (1970). Catabolite repression and other control mechanisms in carbohydrate utilization. In A. H. Rose & J. F. Wilkinson (Ed.), Advances in Microbial Physiology (pp 251-324). NY: Academic Press.

Pastan. I. & Adhya, S. (1976). Cyclic adenosine 5'-monophosphate in *Escherichia coli*. Bacteriological Reviews, 40, 527-551.

Perlman, R., Chen, B., de Crombrugghe, B., Emmer, M., Gottesman, M., Varmus, H. & Pastan, I. (1970). The regulation of *lac* operon transcription by cyclic 3', 5'-monophosphate. Cold Spring Harbor Symposium on Quantitative Biology, 35, 419-423.

Perlman, R. L., de Crombrugghe, B., & Pastan, I. (1969). Cyclic AMP regulates catabolite and transient repression in *E. coli*. Nature, 223, 810-812.

Perlman, R. L. & Pastan, I. (1969). Pleotropic deficiency of carbohydrate utilization in an adenyl cyclase deficient mutant of *Escherichia coli*. Biochemical and Biophysical Research Communications, 37, 151-157.

Phillips, A. T. & Mulfinger, L. M. (1981). Cyclic adenosine 3', 5'-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. Journal of Bacteriology, 145, 1286-1292.

Postma, P.W., & Lengler, J. W. (1985). Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiological Review, 49, 232-269.

Postma, P.W., Lengler, J. W. & Jacobson, G. R. (1993). Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiological Review, 57, 543-594.

Power, J. & Irr, J. (1973). Regulatory gene control of transcription of the L-arabinose operon in *Escherichia coli*. The Journal of Biological Chemistry, 248, 7806-7810.

Rafferty, J & Norling, R. (1986). Cricket Graph version 1.3.2. Cricket Software. Malvern, PA

Reeder, T. & Schleif, R. (1991). Mapping, sequence, and apparent lack of function of *araJ*, a gene of the *Escherichia coli* arabinose regulon. Journal of Bacteriology, 173, 7765-7771.

Reznikoff, W. (1992). Catabolite gene activator protein activation of *lac* transcription. Journal of Bacteriology, 174, 655-658.

Saier, M. H., Feucht, B. U. & McCaman, M. T. (1975). Regulation of intracellular adenosine cyclic 3':5'-monophosphate levels in *Escherichia coli* and *Salmonella typhimurium*. The Journal of Biological Chemistry, 250, 7593-7601.

Saier, M. H. (1977). Bacterial phosphoenolpyruvate : sugar phosphotransferase systems: structural, functional, and evolutionary interrelationships. Bacteriological Reviews, 41, 856-871.

Schleif, R. (1969). An L-arabinose binding protein and arabinose permeation in *Escherichia coli*. Journal of Molecular Biology, 46, 185-196.

Schmiz, K. L., Lessman, D., & Kurz, G. (1974). Aspects of regulation of the pathway for D-glucose 6-phosphate, D-galactose, and L-arabinose in *Pseudomonas fluorescens*. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie, 355, 1249.

Schwartz, D. & Beckwith, J. R. (1970). Mutants missing a factor necessary for the expression of catabolite-sensitive operons in *E. coli*. In J. R. Beckwith & D. Zipser (Ed.), Lactose Operon (pp 417-422). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Scripture, J. B., Voelker, C., Miller, S., O'Donnell, R. T., Polgar, L., Rade, J., Horazdovsky, B. F., & Hogg, R. W. (1987). High-affinity L-arabinose transport operon. Nucleotide Sequence and Analysis of Gene Products. Journal of Molecular Biology, 197, 37-46.

Sheppard, D. E. (1986) Dominance relationships among mutant alleles of regulatory gene *araC* in the *Escherichia coli* B/r L-arabinose operon. Journal of Bacteriology, 168, 999-1001.

Sheppard, D. E. & Eleuterio, M. (1976). Hypersensitivity to catabolite repression in the L-arabinose operon of *Escherichia coli* B/r Is *trans* acting. Journal of Bacteriology, 126, 1014-1016.

Singer, J. & Englesberg, E. (1971). Arabinose transport in *araC*- strains of *Escherichia* B/r. Biochimica et Biophysica Acta, 249, 498-505.

Stanier, R. Y., Palleroni, N. J., & Doudoroff, M. (1966). The aerobic pseudomonads: a taxonomic study. Journal of General Microbiology, 43, 159-271.

Stenesh, J. (1984). Experimental Biochemistry. Toronto: Allyn and Bacon, Inc.

Stoner, C. & Schleif, R. (1983). The *araE* low-affinity L-Arabinose transport promoter - cloning, sequence, transcription start site and DNA binding sites of regulatory proteins. Journal of Molecular Biology, 171, 369-381.

Temple, L., Cuskey, S. M., Perkins, R. E. , Bass, R. C., Morales, N. M., Christie, G. E., Olsen, R. H., & Phibbs, Jr., P. V. (1990). Analysis of cloned structural and regulatory genes for carbohydrate utilization in *Pseudomonas aeruginosa PAO*. Journal of Bacteriology, 172, 6396-6402.

Temple, L., Sage, A., Christie, G. E., & Phibbs Jr., P. V. (1994). Two genes for carbohydrate catabolism are divergently transcribed from a region of DNA containing the *hexC* locus in *Pseudomonas aeruginosa* PAO1. Journal of Bacteriology, 176, 4700-4709.

Tiwari, N. P. & Campbell, J. J. R. (1969). Enzymatic control of the metabolic activity of *Pseudomonas aeruginosa* grown in glucose or succinate media. Biochimica et Biophysica Acta, 192, 395-401.

Weimberg, R. (1961). Pentose oxidation by *Pseudomonas fragi*. The Journal of Biological Chemistry, 236, 629-635.

Weimberg, R. & Doudoroff, M. (1955). The oxidation of L-Arabinose by *Pseudomonas saccharophilia*. The Journal of Biological Chemistry, 217, 606-624.

Wilcox, G. (1974). The interaction of L-arabinose and D-fucose with *AraC* protein. The Journal of Biological Chemistry, 249, 6892-6894.

Wilcox, G., Clemetson, K. J., Clearly, P., & Englesberg, E. (1974). Interaction of the regulatory gene product with the operator site in the L-arabinose operon of *Escherichia coli*. Journal of Molecular Biology, 85, 589-602.

Wolff, J. A., MacGregor, C. H., Eisenberg, R. C., & Phibbs, Jr., P. V. (1991). Isolation and characterization of catabolite repression control mutants of *Pseudomonas aeruginosa PAO*. Journal of Bacteriology, 173, 4700-4706.

Zubay, G., Schwartz, D., & Beckwith, J. (1970). Mechanisms of activation of catabolite-sensitive genes: a positive control system. Proceedings of the National Academy of Sciences USA, 66, 104-110.

Zylstra, G. J., Olsen, R. H., & Ballou, D. P. (1989). Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. Journal of Bacteriology, 171, 5907-5914.