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Expression and Regulation Properties of *Pseudomonas* L-Arabinose Utilization Plasmids

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EXPRESSION AND REGULATION PROPERTIES OF *PSEUDOMONAS* L-ARABINOSE UTILIZATION PLASMIDS

by

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Mary E. Barecki

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Western Michigan University, 1996

The L-arabinose utilization pathway in *Pseudomonas fluorescens* is a unique metabolic pathway. In *Pseudomonas*, L-arabinose is converted to the end product α-ketogluterate and does not involve phosphorylated intermediates. This differs from the well studied L-arabinose pathway found in *Escherichia coli* which contains phosphorylated intermediates.

The enzymes of the L-arabinose pathway have been shown to be induced in the presence of L-arabinose in *P. saccharophila.* The current study determined regulatory properties of previously cloned chromosomal fragments of *P. fluorescens* which confer an L-arabinose positive phenotype to the surrogate host, *Pseudomonas putida.* Cells of *P. putida* containing plasmids pPZ221, pPZ222, pPZ224, pPZ226, pPZ227, pPZ228, or pPZ229 were grown on various substrates which provided sole carbon and energy sources and crude cell-free extracts of these cells were assayed for the enzyme L-arabinose dehydrogenase. The specific activities (μmole/min/mg protein) for L-arabinose dehydrogenase indicated that this enzyme was inducible and also expressed at high constitutive levels under some, but not all, growth conditions.

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INTRODUCTION

The bacterium *Pseudomonas fluorescens* (ATCC 13525) is able to utilize several substrates, including L-arabinose, as sole carbon and energy sources. Closely related members of the aerobic fluorescent pseudomonads *P. putida* and *P. aeruginosa*, are unable to utilize L-arabinose. Chromosomal inserts from *P. fluorescens* were previously cloned into the cosmid vector pLA 2917 and plasmids containing the genetic elements necessary to support growth on L-arabinose were selected by transformation into the surrogate hosts *P. putida* and *P. aeruginosa* as described by Eisenberg and Phibbs (1988). Thus, non-sibling plasmids, designated pPZ221, pPZ222, pPZ224, pPZ226, pPZ227, pPZ228, and pPZ229, all have the properties of converting L-arabinose negative strains of *P. putida* and *P. aeruginosa* into L-arabinose positive strains (Eisenberg and Phibbs, 1988).

The current study was designed to gain information about the expression of the cloned *P. fluorescens* genes required for utilization of L-arabinose in the surrogate host, *P. putida* (ATCC 12633). Specifically, this study was intended to determine whether these cloned fragments of *P. fluorescens* contain regulatory elements for controlling expression of a putative L-arabinose operon in the *P. putida* surrogate. L-arabinose utilization is known to be induced in *P. fluorescens* only by growth in the presence of L-arabinose (Doudoroff, 1962).

To determine whether regulatory elements are present in the cloned fragments of *P. fluorescens*, the specific activity (µmoles L-arabinose oxidized/min/mg protein) of L-arabinose dehydrogenase was calculated for each of the non-sibling plasmids transformed into *P. putida*. L-arabinose dehydrogenase is the first enzyme in the L-arabinose pathway (Schimz et al., 1974) and is linked to the reduction of NAD. The specific activity of L-arabinose dehydrogenase was calculated using crude cell-free extracts of *P. putida* containing these various plasmids that were prepared from cells grown on a variety of carbon and energy sources.

From the L-arabinose dehydrogenase specific activities, the expression of the cloned *P. fluorescens* genes could be analyzed. By comparing the specific activities for growth on L-arabinose to the specific activities for growth on glucose, succinate or Luria broth, it could be determined whether the operon is induced (detectable activity only in the presence of L-arabinose) or that it is expressed constitutively (detectable activity in the presence of all substrates). The type of expression observed may then indicate the presence, or lack of, regulatory elements in the cloned fragments of *P. fluorescens*.

The results obtained in this study indicate that the L-arabinose operon is induced in the surrogate host *P. putida* when the cells are grown on L-arabinose. It was also observed that when the cells of *P. putida* containing the plasmids were grown on glucose, succinate, or Luria broth constitutive levels of L-arabinose dehydrogenase were detected in some, but not all, extracts.

REVIEW OF LITERATURE

Carbohydrate Utilization in *Pseudomonas*

Pseudomonas species are metabolically versatile in that these organisms are able to utilize an array of carbohydrates (Ornston, 1971; Clarke and Richmond, 1975). This metabolic diversity provides a basis for the study of unique pathways found within the pseudomonads. *Pseudomonas*, like most other aerobic Gram negative rods, use the Entner-Doudoroff pathway exclusively, or in addition to, other pathways for metabolism of hexoses and their derivatives (Hollmann, 1964). The Embden-Meyerhof-Parnas pathway (fermentative metabolism) appears to be incomplete in *Pseudomonas* because of a deficiency in 6-phosphofructokinase (Clarke and Richmond, 1975, Lessie and Phibbs, 1984; White, 1995).

The Entner-Doudoroff pathway was first described in *P. saccharophila* by Entner and Doudoroff in 1952 for the metabolism of glucose. This pathway is a non-glycolytic pathway, is the major route of glucose catabolism in pseudomonads (Lessie and Phibbs, 1984), and has been demonstrated to be the major route for catabolism of gluconate in enteric organisms such as *Escherichia coli* (Eisenberg and Dobrogsz, 1967; Fraenkel and Vinopal, 1973). The Entner-Doudoroff pathway utilizes two major enzymes which are unique to this pathway, 6-phosphogluconate dehydratase

and 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG) (Entner and Doudoroff, 1952; Lessie and Phibbs, 1984; Ornston, 1971; Weimberg and Doudoroff, 1955).

Examples of Entner-Doudoroff Pathway Utilization

The Entner-Doudoroff pathway is utilized by a variety of both Gram positive and Gram negative bacteria and not limited to pseudomonads (Lessie and Phibbs, 1967). Several variations on this pathway will be described to illustrate the ubiquity of this non-glycolytic mechanism of carbohydrate catabolism among a diverse group of bacteria.

Glucose Catabolism in *Pseudomonas*

The Entner-Doudoroff pathway is employed in *Pseudomonas* to oxidize glucose to pyruvate and glyceraldehyde-3-phosphate (De Ley, 1960; Entner and Doudoroff, 1951, Kovachevich and Wood, 1955). Studies on glucose catabolism in *P. saccharophila* provided for the original elucidation of this pathway by Entner and Doudoroff in 1952. The catabolism of glucose in *Pseudomonas* species begins with a periplasmic oxidative pathway (see Figure 1). Glucose is then oxidized to gluconate and 2-ketogluconate via membrane bound glucose and gluconate dehydrogenases. Both gluconate and 2-ketogluconate are transported into the cell and converted to 2-keto-6-phosphogluconate by ATP-dependent kinase activity. The 2-keto-6-phosphogluconate is then converted to 6-phosphogluconate which is in turn converted

to 2-keto-3-deoxy-6-phosphogluconate (KDPG) via 6-phosphogluconate dehydratase. A KDPG aldolase then cleaves the KDPG into pyruvate and glyceraldehyde-3-phosphate (De Ley, 1960; Entner and Doudoroff, 1952; Kovachevich and Wood, 1955; Weimberg and Doudoroff, 1955).

Gluconate Metabolism

Gluconate is the product of glucose dehydrogenase activity on glucose and accounts for the accumulation of extracellular gluconate from glucose in *Pseudomonas*. *E. coli*, *Salmonella typhimurium*, and *Enterococcus* (previously *Streptococcus*) *faecalis* are known to have a gluconate inducible Entner-Doudoroff pathway (Eisenberg et al., 1974; Fraenkel and Horecker, 1964; Sodatch and Gunsalus, 1957). Eisenberg and Dobrogosz (1967) found that the Entner-Doudoroff pathway is induced in *E. coli* (ML30) when grown on gluconate, and that *S. typhimurium* produces high levels of gluconate-induced Entner-Doudoroff enzymes when grown on gluconate. Tong et al. (1996) have also shown that the Entner-Doudoroff pathway in *E. coli* is induced by gluconate and allows for the entry of gluconate into its central glycolytic metabolism.

Galactose Metabolism in *Pseudomonas*

Galactose is catabolized to pyruvate and glyceraldehyde-3-phosphate via a variation on the Entner-Doudoroff pathway (De Ley and Doudoroff, 1957; Lessie and

Phibbs, 1984) in *Pseudomonas.* Galactose is converted to 2-keto-3-deoxygalactone before phosphorylation of intermediates occurs (De Ley and Doudoroff, 1951). *Pseudomonas* also contains an enzyme not found in *E. coli* for galactose metabolism, D-galactose dehydrogenase (Deacon and Cooper, 1977; Lessie and Phibbs, 1984). Galactose is metabolized by *Pseudomonas* by an initial oxidation of galactose to galactonate by a cytoplasmic NAD-dependent dehydrogenase. Galactonate is then converted to 2-keto-3-deoxygalactonate via dehydration. The 2-keto-3-deoxygalactonate is then phosphorylated to 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) by an ATP-dependent kinase. An aldolase specific for KDPGal then cleaves KDPGal into glyceraldehyde-3-phosphate and pyruvate (Clarke and Richmond, 1975; Deacon and Cooper, 1977; De Ley and Doudoroff, 1951; Lessie and Phibbs, 1985).

Uronic Acid Metabolism in *E. coli*

Uronic acid metabolism via an Entner-Doudoroff type pathway is widely distributed among the family Enterobacteriaceae (Hollmann, 1964). This pathway was first identified by Ashwell, Wahba, and Hickman in *E. coli* (1960). This pathway has also been demonstrated in *Erwinia carotova*, *Serratia marcescens*, and *Shigella flexneri* (Hollmann, 1964). According to Ashwell et al. (1960), this type of uronic acid metabolism occurs in *E. coli* with the substrates glucuronic acid and galacturonic acid. Glucuronic acid and galacturonic acid are isomerized to form their respective

keto-analogues, fructuronic acid and tagaturonic acid, by uronic acid isomerase. Fructuronic acid is reduced to D-mannonic acid and tagaturonic acid is reduced to D-altronic acid via NADH. Once D-mannonic acid and D-altronic acid are formed, the pathways come together to form 2-keto-3-deoxy-D-gluconic acid via a dehydration reaction. The 2-keto-3-deoxy-D-gluconic acid is then phosphorylated by ATP to produce KDPG. KDPG is then cleaved via KDPG aldolase to form pyruvic acid and glyceraldehyde-3-phosphate as in glucose catabolism in *Pseudomonas.*

Glycerol Metabolism in *Pseudomonas*

It has been established that growth of *P. aeruginosa* on glycerol, glycerol-3-phosphate, or glycerate results in the induction of glucose-6 phosphate dehydrogenase and the dehydratase and aldolase enzymes of the Entner-Doudoroff pathway (Heath and Gaudy, 1978; Hylemon and Phibbs, 1972; Lessie and Neidhart, 1967; Lessie and Phibbs, 1984). Glycerol metabolism occurs as described below. Once the glycerol is transported into *Pseudomonas*, the glycerol is phosphorylated via glycerol kinase to glycerol-3-phosphate. The glycerol-3-phosphate is then oxidized to glyceraldehyde-3-phosphate via glycerol-3-phosphate dehydrogenase (Lessie and Phibbs, 1984; Tsay et al., 1971). It was also found that *P. aeruginosa* mutants deficient in the Entner-Doudoroff dehydratase and aldolase enzymes grow poorly on glycerol, this suggests that glycerol catabolism occurs via the Entner-Doudoroff pathway (Blevins et al., 1981).

These pathways provide a basis for a variety of carbohydrates, such as galactose, glucose, and glycerol being converted to pyruvate without proceeding through the lower half of the Embden-Meyerhof-Parnas pathway (Lessie and Phibbs, 1984). Figures 1 and 2 summarize many of the events for carbohydrate metabolism which utilize the Entner-Doudoroff pathway (Wolff et al., 1991; Lessie and Phibbs, 1984).

L-arabinose Utilization in *Escherichia coli* and *Pseudomonas*

To appreciate the uniqueness of L-arabinose metabolism in *Pseudomonas*, it is instructive to understand how *E. coli* and related bacteria metabolize L-arabinose. L-arabinose utilization in *E. coli* has been studied extensively and the L-arabinose operon provides a basis for converting L-arabinose to a central metabolite (D-xylulose-5-phosphate) of carbohydrate metabolism in *E. coli* (Beverin et al., 1971; Englesberg, 1971, Englesberg et al., 1965, Hahn et al., 1984; Hendrickson et al., 1990; Horazdovsky and Hogg, 1989; Hou et al., 1988; Kolodrubetz and Schleif, 1981; Lee et al., 1981; Lobell and Schleif, 1990; Schleif, 1969; Sheppard, 1986; Singer and Englesberg, 1971; Stoner and Schleif, 1983; Wilcox, 1974; Wilcox et al., 1974). This L-arabinose operon is controlled by a regulatory gene (*araC*) whose product functions for both negative and positive control of the structural genes for L-arabinose catabolism. The transport systems for L-arabinose are coded for by the *araE* gene and the *araFGH* gene which are distal to the L-arabinose operon (*araBAD*).

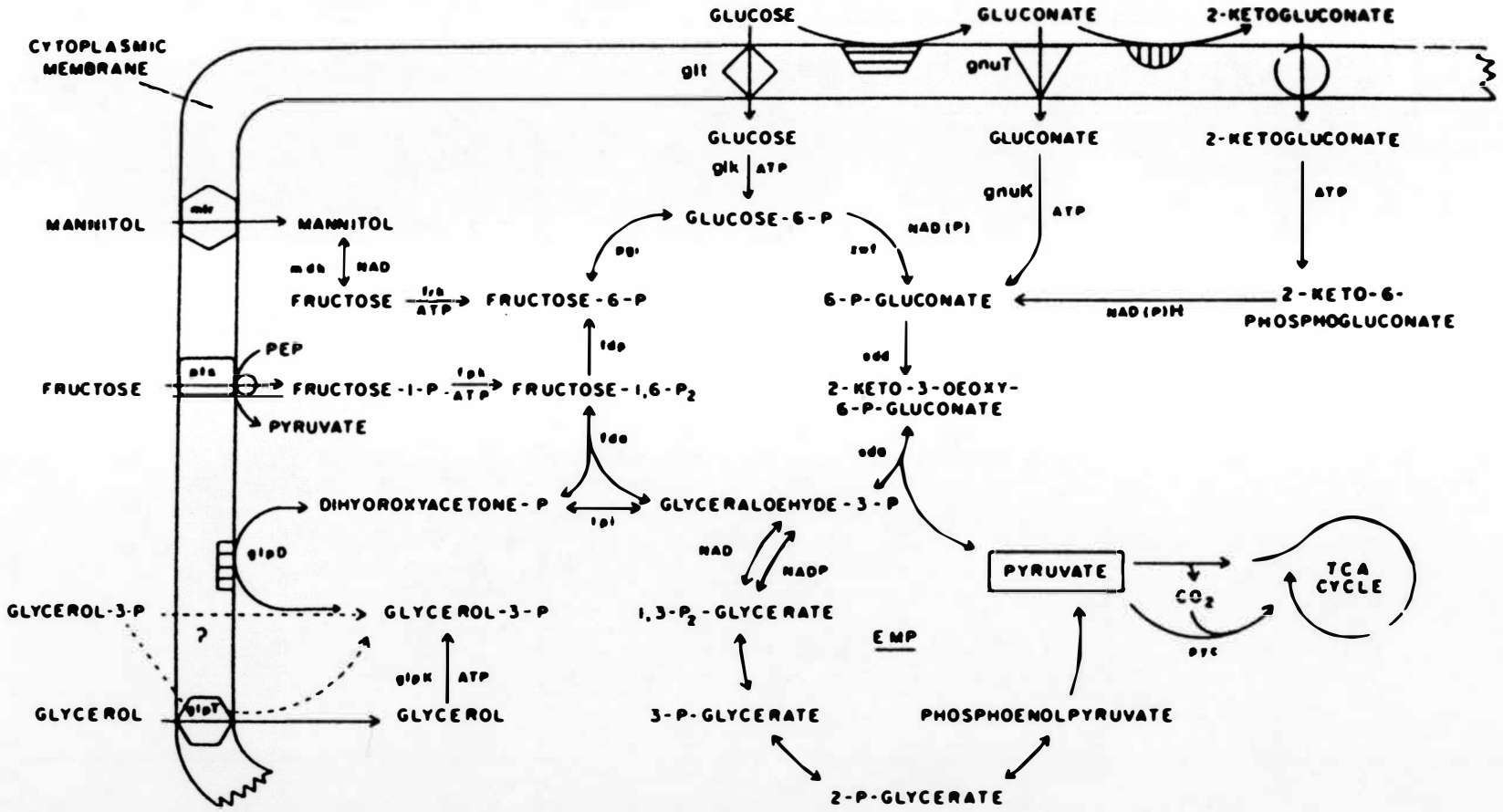

Figure 1. Carbohydrate Catabolism Utilizing the Entner-Doudoroff Pathway (Wolff et al., 1991).

This figure represents a summary of the catabolism of various carbohydrates which utilize the Entner-Doudoroff pathway to convert substrates to pyruvate and glyceraldehyde-3-phosphate.

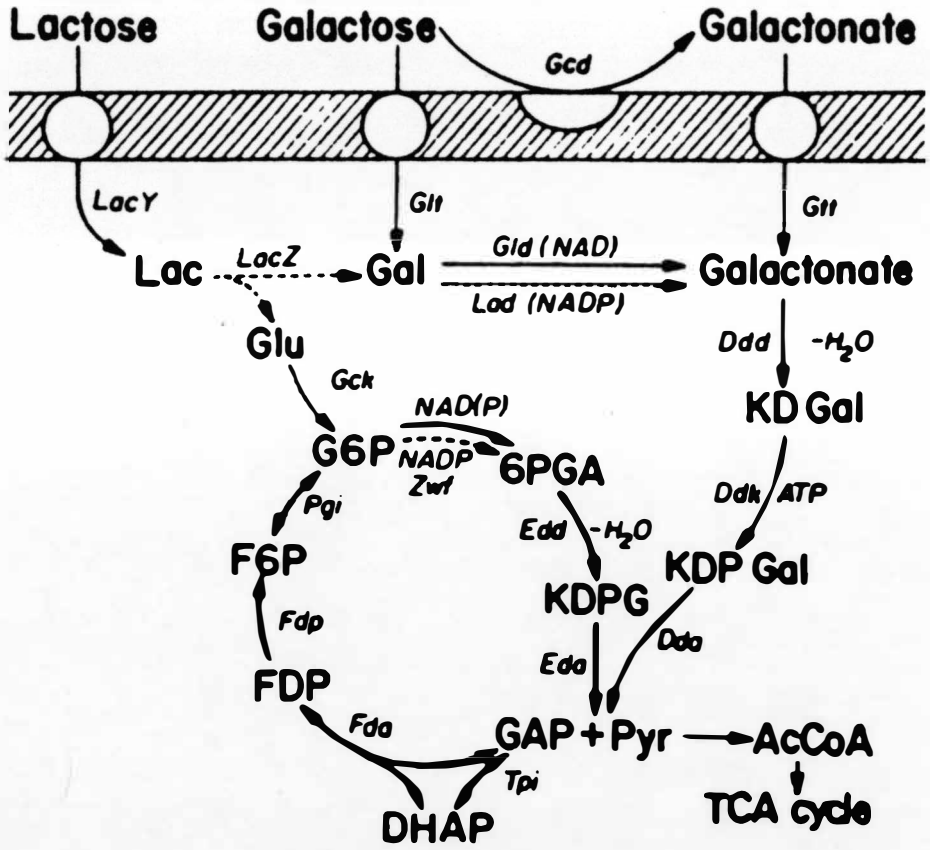

Figure 2. Galactose Catabolism in *Pseudomonas* (Lessie and Phibbs, 1984).

Galactose is converted to pyruvate and glyceraldehyde-3-phosphate via a variation on the Entner-Doudoroff pathway in *Pseudomonas*. Galactose is converted to 2-keto-3-deoxygalactone before phosphorylation of intermediates occur.

L-arabinose Pathway of *Escherichia coli*

E. coli catabolizes L-arabinose by the Embden-Meyerhof-Parnas pathway via conversion to hexose-phosphate by transketolase and transaldolase enzymes. This L-arabinose pathway is an autoregulatory pathway where the regulatory protein (AraC) is controlling its own synthesis (Brock and Madigan, 1991).

The events for L-arabinose catabolism in *E. coli* are summarized as described below. A permease system transports L-arabinose into the cell. Once inside, a series of reactions involving three enzymes converts L-arabinose to D-xylulose-5-phosphate (Englesberg, 1971; Englesberg et al., 1965; Lee, 1980; MacInnes et al., 1978; Moat and Foster, 1995). The three enzymes responsible for L-arabinose metabolism are L-arabinose isomerase, L-ribulokinase, and L-ribulokinase-5-phosphate which are coded for by the genes *araA*, *araB*, and *araD*, respectively (Englesberg, 1971; Englesberg et al., 1965; Lee, 1980; MacInnes et al., 1978). These genes, along with the initiator region (*araI*) and the operator region (*araO*), comprise the *araBAD* operon which is controlled by the *araC* gene product as seen in Figure 3 (Englesberg, 1971; Englesberg et al., 1965; Hahn et al., 1984; Hou et al., 1988; Koisba and Schleif, 1982; Kolodrubetz and Schleif, 1981; Lee, 1980; Lobell and Schleif, 1990, Ogden, 1980; Power and Irr, 1973; Sheppard, 1986; Stoner and Schleif, 1983; Wilcox et al., 1974). The genes in this operon are linked in the following order: *araD, araA, araB, araI, araO*, and *araC* is located between the genetic markers for threonine and leucine (Figure 3) (Englesberg, 1971).

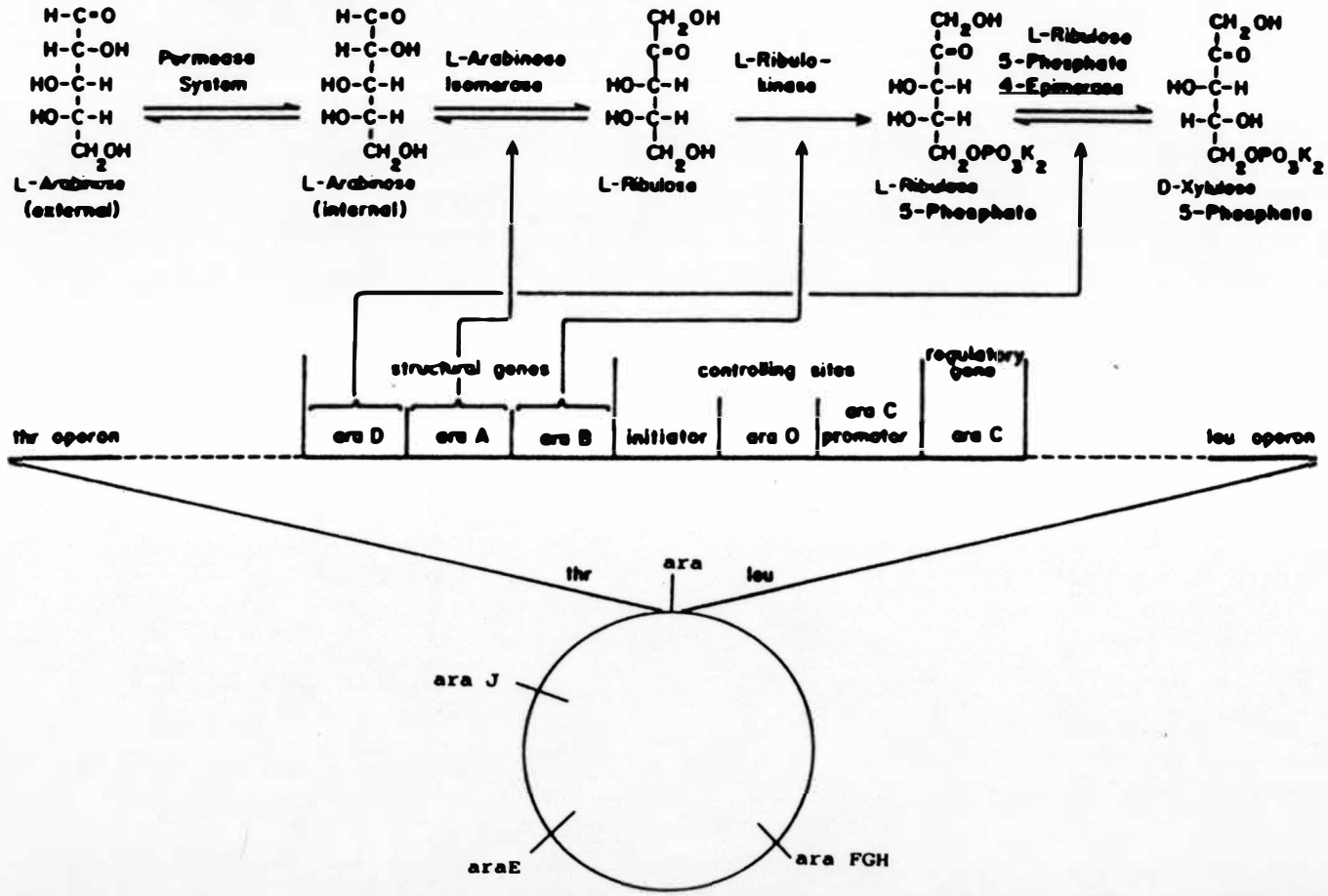

Figure 3. The Proposed Pathway for L-arabinose Catabolism in *Escherichia coli* (Englesberg, 1971; Lee, 1980; Neidhardt et al., 1996).

This is a classical pathway which is induced in the presence of L-arabinose. A permease system transports L-arabinose into the cell where it is converted to D-xylulose-5-phosphate. The relative positions of the L-arabinose utilization genes are also shown here. Note that the transport genes (*araE* and *araFGH*) are not linked to the *araCBAD* operon.

L-arabinose Transport in *Escherichia coli*

Two different permease systems provide for the transport of L-arabinose into the cell (Englesberg, 1971; Horazdovsky and Hogg, 1989; Horazdovsky and Hogg, 1987; Kolodrubetz and Schleif, 1981; Lee, 1980; Reeder and Schleif, 1991; Scripture et al., 1987; Stoner and Schleif, 1983). The first transport system identified was the low-affinity transport system. The *araE* locus codes for the structural gene of this system (Kolodrubetz and Schleif, 1981; Lee, 1980; Novotny and Englesberg, 1966). Singer and Englesberg (1971) showed this system to be inducible in the presence of L-arabinose. It was also shown that in the presence of L-arabinose, the *araC* gene product activates the L-arabinose transport system (Englesberg, 1971; Englesberg et al., 1965).

The second transport system of L-arabinose in *E. coli* was characterized as a high- affinity system (Schleif, 1969; Singer and Englesberg, 1971). This high-affinity system is encoded by an operon which includes the genes *araF*, *araG*, and *araH* (Kolodrubetz and Schleif, 1969; Horazdovdky and Hogg, 1987). This high-affinity system functions in the initial recognition of L-arabinose (Scripture et al., 1987). Characterization of this operon shows that *araF* codes for the L-arabinose binding protein (Horazdovsky and Hogg, 1989; Scripture et al., 1987). This protein is soluble and hydrophilic, is located in the periplasm and is released when the cell is osmotically shocked, therefore further confirming the properties of a binding protein (Englesberg, 1971; Horazdovsky and Hogg, 1989; Schleif, 1969; Scripture et al.,

1987). The genes *araG* and *araH* code for a membrane associated complex for L-arabinose transport (Horazdovsky and Hogg, 1987). This complex is induced by the presence of L-arabinose (Hendrickson et al., 1990) and aids in the translocation of the substrate, L-arabinose (Horazdovsky and Hogg, 1987).

The *araJ* promoter was the fourth promoter in *E. coli* which was found to be regulated by L-arabinose (Hendrickson et al., 1990). An attempt was made by Reeder and Schleif (1991) to determine the function of *araJ* since no function had been identified as of yet. This was accomplished by mapping and sequencing the gene and also creating mutants in the *araJ* region. Reeder and Schleif (1991) found that *araJ* is not necessary for either the low-affinity or the high-affinity transport systems of L-arabinose, nor was it found to be involved in the regulation of L-arabinose metabolism in the assay system employed in their study. Reeder and Schleif proposed a possible function of *ara*J as being one of transporting or processing L-arabinose (1991). This theory is supported by the fact that *araJ* possesses a probable signal peptide for the *araJ* gene product to cross the inner membrane and function as an outer membrane porin for diffusion of L-arabinose into the periplasmic space (Reeder and Schleif, 1991).

Regulation of the L-arabinose Pathway in *Escherichia coli*

The L-arabinose operon along with the genes encoding the transport systems for L-arabinose are regulated by the *araC* gene product. The AraC protein is a unique

regulatory protein since this protein has the ability to function as both a repressor and an activator of the L-arabinose operon (Wilcox et al., 1974). The *araC* gene product regulates its own expression in the presence or absence of L-arabinose, therefore giving the operon an autoregulatory property (Moat and Foster, 1995). AraC has also been shown to regulate all L-arabinose utilization operons at the transcriptional level (Englesberg, 1971; Hahn et al., 1984; Koisba and Schleif, 1982; Sheppard, 1986).

Hou et al. (1988) and Lobell and Schleif (1990) have proposed the current model for the mechanism of regulation of the L-arabinose operon in *E. coli*. The proposed mechanism shows that in the presence of L-arabinose, the AraC protein is bound at the *araI* site (initiator) which stimulates the transcription of the *araBAD* genes. In the absence of L-arabinose this protein represses the mRNA synthesis from the *araBAD* promoter by the formation of a DNA loop, as seen in Figure 4 (Hou et al., 1988; Lobell and Schleif, 1990). At one end of this DNA loop, the AraC protein is bound at the $araO_2$ site. It has been suggested by Hahn et al. (1984), that the other end of the DNA loop is bound to the *araI* site. The AraC protein is also able to repress its own synthesis by binding to the operator $araO_1$ which blocks the entry of RNA polymerase to the *araC* gene promoter (Hou et al., 1988).

According to Huo et al. (1988), when L-arabinose is added to a repressed L-arabinose operon the L-arabinose binds to the AraC protein and the $araO_2$-*araI* loop is opened. Once the loop opens, most of the AraC protein molecules which were bound at the $araO_2$ site are released. At the same time, most of the AraC protein

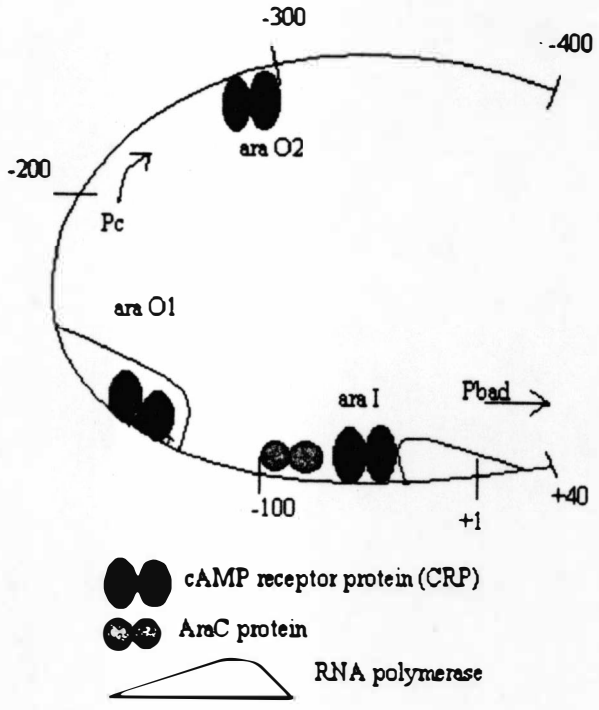

Figure 4. The Proposed Binding Sites for CRP and AraC Protein on the *araCBAD* Operon During the Formation of the Repression Loop in *E. coli* (Hou et al., 1988; Lobell and Schleif, 1990).

In the presence of L-arabinose, the AraC protein, bound at the *araI* site, stimulates transciption of *araBAD* genes. In the absence of L-arabinose, the AraC protein represses RNA synthesis from the *araBAD* promoter by forming a DNA loop. At one end the AraC protein is bound to the $araO_2$ site while the other end is bound to the *araI* site. AraC can also repress its own synthesis by binding to the operator $araO_1$.

molecules bound to the *araI* site remain bound. The absence of the DNA looping along with the presence of bound L-arabinose induces a conformational change of the AraC protein molecules bound to *araI* into an induced state which allows for the induction of the *araBAD* promoter (Hou et al., 1988).

The transport systems for L-arabinose, *araE* and *araFGH* are also regulated by the AraC protein (Englesberg, 1971; Koisba and Schleif, 1982; Kolodrubetz and Schleif, 1981; Stoner and Schleif, 1983). In *araE*, the binding sites for AraC are homologous to the binding sites of *araBAD* (Koisba and Schleif, 1982; Stoner and Schleif, 1983). The binding sites for AraC in *araFGH* are different than those found in *araE* and *araBAD*, but is similar to *araE* in that both of these transport systems require CRP for full induction (Englesberg, 1971; Koisba and Schleif, 1982; Kolodrubetz and Schleif, 1981; Stoner and Schleif, 1983).

The cAMP receptor protein (CRP) and cAMP have been shown to be involved in regulation of the L-arabinose operon (Englesberg, 1971; Lichenstein et al., 1987; Koisba and Schleif, 1982; Ogden et al., 1980; Stoner and Schleif, 1983). The complex formed by both of these molecules is involved in the activation of the L-arabinose operon (Englesberg, 1971; Koisba and Schleif, 1982; Ogden et al., 1980) by binding to the active site which is located between the *ara*B and both of the *ara*O regions on the DNA (Englesberg, 1971; Huo et al., 1988).

In summary, the metabolism of L-arabinose in *E. coli* begins with L-arabinose transport into the cell via either the high or low affinity permease system. Once inside

the cell, the L-arabinose is isomerized to L-ribulose via L-arabinose isomerase. L-ribulose is converted to L-ribulose-5-phosphate by L-ribulokinase. The enzyme, L-ribulose-5-phosphate-4-epimerase then converts L-ribulose-5-phosphate to D-xylulose-5-phosphate as seen in Figure 3 (Englesberg, 1971). Finally, D-xylulose-5-phosphate enters the central pathways of carbohydrate catabolism in the organism.

L-arabinose Pathway of *Pseudomonas*

When comparing L-arabinose metabolism in *Pseudomonas* to that of *E. coli* it is obvious that *Pseudomonas* possesses a unique pathway for catabolism of the pentose sugar, L-arabinose. In 1955, Weimberg and Doudoroff reported that *P. saccharophila* converts L-arabinose to α-ketogluterate via reactions that do not involve the Kreb's Tricarboxylic Acid Cycle (TCA) cycle nor phosphorylated intermediates. The end-product of this metabolic pathway is unique because α-ketogluterate is a TCA intermediate and most TCA intermediates function to repress enzyme synthesis for carbohydrate metabolism in *Pseudomonas* (Clarke and Richmond, 1975; Lessie and Phibbs, 1984; Paigen and Williams, 1970). Weimberg and Doudoroff (1955) reported that L-arabinose is oxidized to L-arabinolactone with NAD-specific L-arabinose dehydrogenase. L-arabinolactone is metabolized to L-arabonate which is then catabolized to an unstable intermediate and then converted to α-ketogluterate. It was suggested that NAD or NADP acts as the electron acceptor for this metabolic pathway (Weimberg and Doudoroff, 1955). Weimberg showed, at

a later date, that *Pseudomonas fragi* also metabolized pentose sugars through a similar pathway (1961). In 1962, Doudoroff demonstrated that *P. saccharophila* requires NAD to convert L-arabinose to L-arabinolactone in cell-free extracts.

Further studies by Dagley and Trudgill (1965) showed that the cell-free extracts of *P. saccharophila* converted L-arabonate into 2-ketogluterate semialdehyde without the presence of NAD. When NAD was added to the assay, L-arabonate was metabolized to 2-ketogluterate. In 1974, Schimz et al. proposed the entire pathway for L-arabinose metabolism in *P. fluorescens* (Figure 5). As with the previous studies, L-arabinose is broken down into L-arabino-1,5-lactone by the enzyme L-arabinose dehydrogenase. This is then converted to L-arabonate which in turn is degraded by a dehydratase enzyme, to 2-keto-3-L-arabonate. 2-keto-3-L-arabonate is then converted to α-ketogluterate semialdehyde via an aldolase reaction which is reduced to α-ketogluterate.

A transport system for L-arabinose in *Pseudomonas* has yet to be characterized. However, other transport systems for carbohydrates in *Pseudomonas* have been studied. Eisenberg and Phibbs (1982) were able to characterize a binding protein which was inducible in the presence of mannitol. They also confirmed that osmotic shock eliminated glucose transport in cells of *P. aeruginosa* (Eisenberg and Phibbs, 1982).

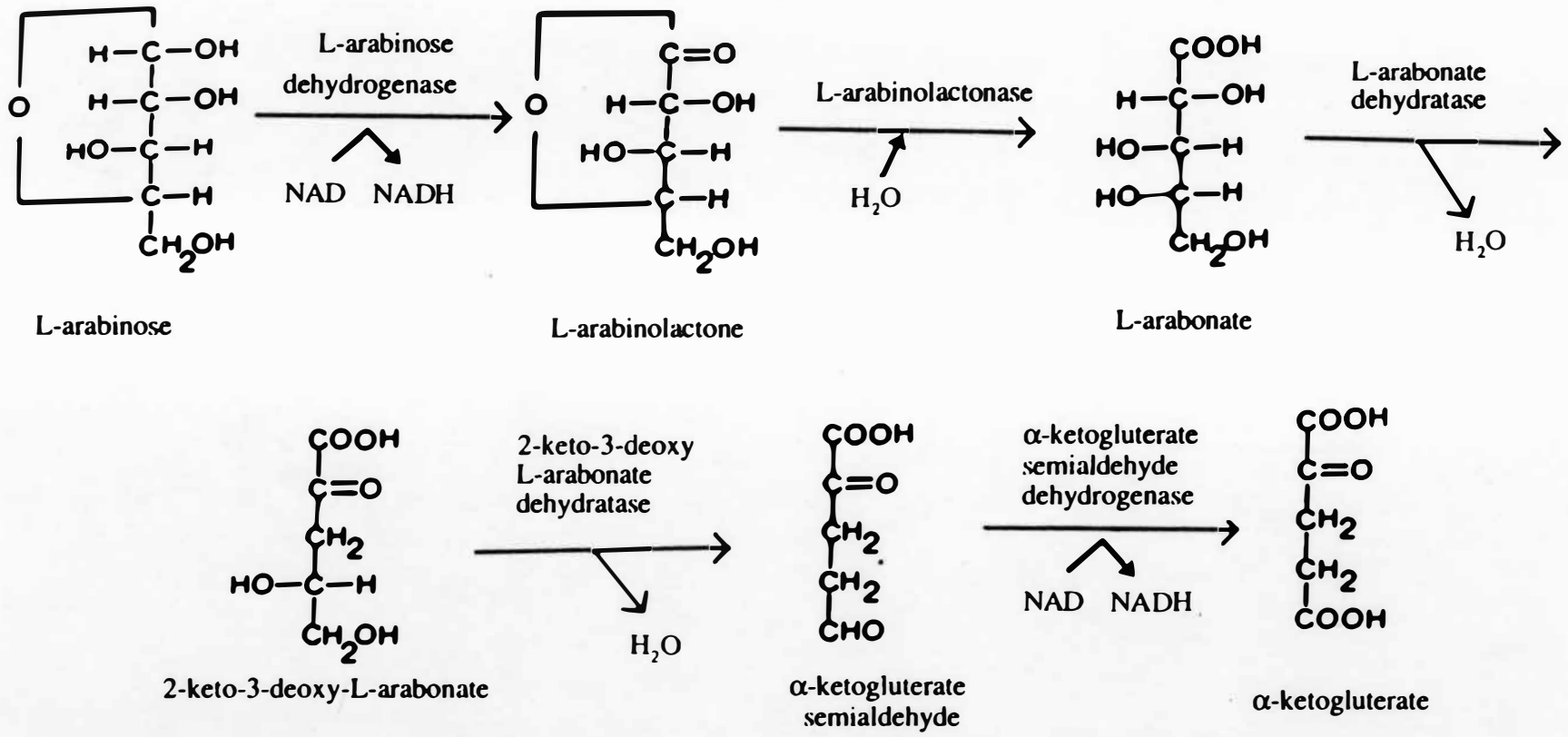

Figure 5. Proposed Pathway for L-arabinose Catabolism in *Pseudomonas fluorescens* (Schimz et al., 1974).

Study of Expression of L-arabinose Utilization in *Pseudomonas*

To study the expression of the L-arabinose pathway in *P. putida* the cosmid vector pLA2917 was used. A cosmid vector is a plasmid which contains cloned DNA with a ligated lambda cos site (cohesive ends) (Brock and Madigan, 1988). This cosmid vector was constructed by Allen and Hanson (1985). It is a 21 kb broad-host range vector which contains a tetracycline resistance marker and a kanamycin resistance marker (Figure 6). According to Allen and Hanson (1985), pLA2917 contains six unique restriction enzyme sites: *Bgl*II (in the kanamycin resistance gene), *BstE*II, *Hin*dIII, *Hpa*I, *Pst*I (in the kanamycin resistance gene), and *Sal*I (in the tetracycline resistance gene).

Plasmids Used in this Study

In order to study the expression of this unique L-arabinose pathway found in *P. fluorescens*, DNA fragments from *P. fluorescens* were inserted into the cosmid vector pLA2917 into the *Bgl*II site and transformed into the surrogate host, *P. putida* (Eisenberg and Phibbs, 1988). Seven non-sibling plasmids were constructed. These non-sibling plasmids were designated pPZ221, pPZ222, pPZ224, pPZ226, pPZ227, pPZ228, and pPZ229. These plasmids confer the ability to convert L-arabinose negative strains of *P. putida* and *P. aeruginosa* into L-arabinose positive strains (Eisenberg and Phibbs, 1988).

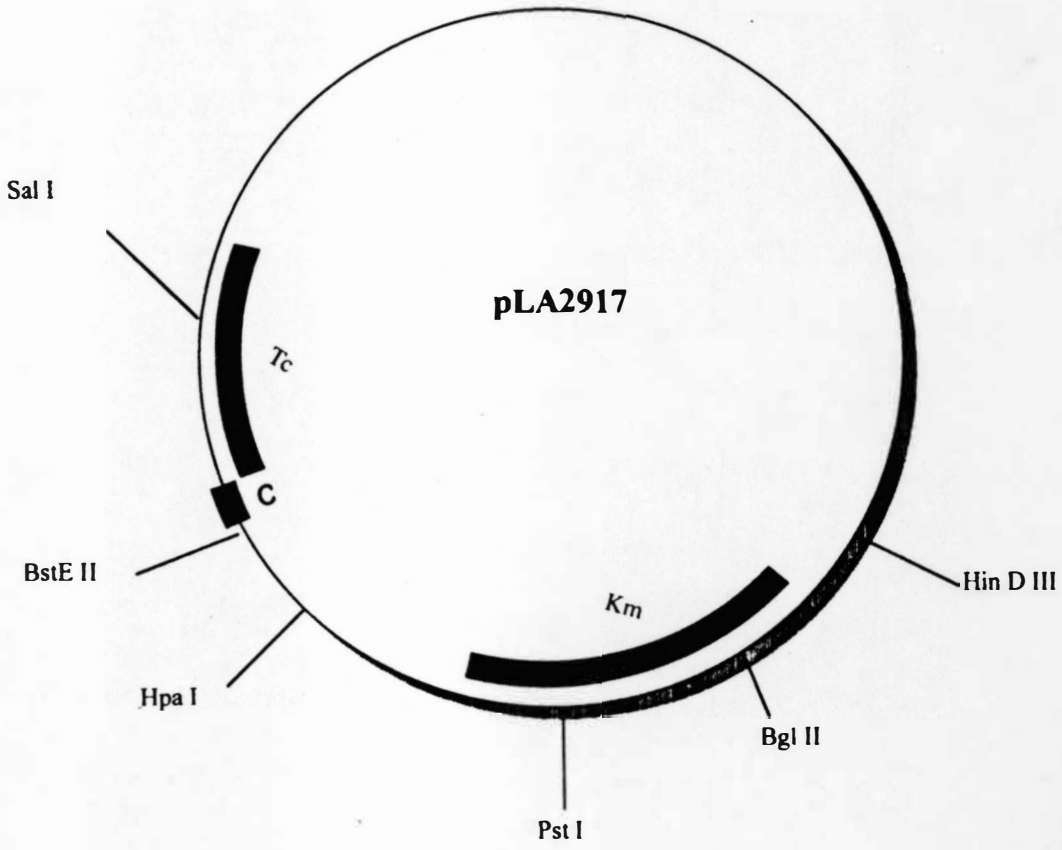

Figure 6. The Cosmid Vector pLA2917 (Allen and Hanson, 1985).

The 21 kb vector, pLA2917, contains tetracycline and kanamycin resistance markers. The chromosomal fragments from *Pseudomonas fluorescens* were ligated into the *Bgl*II site.

Importance of This Study

The expression of the L-arabinose operon in *P. fluorescens* is of interest due to the fact that unique regulatory properties may exist because of the formation of the end-product α-ketogluterate which is a TCA cycle intermediate and is fed directly into the TCA cycle. The TCA cycle is the central catabolic pathway in aerobic utilization of nutrients in *Pseudomonas*. Another point of interest is the fact that L-arabinose metabolism in *Pseudomonas* is quite different than the metabolism of L-arabinose in *E. coli*. However, the product of the L-arabinose pathway in *E. coli*, D-xylulose-5-phosphate, is an intermediate of the central pathways for sugar fermentation in *E. coli* and must be converted further through the pentose phosphate pathway from which it will enter into glycolysis.

MATERIALS AND METHODS

Growth of Organisms

Stock cultures were maintained on Luria broth culture medium (LB) and basal salts medium (BSM). LB contained (grams/liter): Bacto-tryptone (Difco), 10 grams; Bacto-yeast extract (Difco), 5 grams; sodium chloride, 10 grams; pH 7.5. BSM contained (per 100 ml): 10 ml of 0.5M potassium phosphate pH 7.0; 0.5 ml of 3.0M $(NH_4)_2SO_4$; 0.1 ml of 1.0M $MgCl_2$; 0.1 ml of 2mM $FeSO_4$. Stock solutions of carbon sources were sterilized by filtration and added to sterile BSM to the final concentrations of 30mM L-arabinose, 20mM glucose, and 20mM succinate. Antibiotics were added, when necessary, to the media in the following concentrations: tetracycline 10 µg/ml, kanamycin 25 µg/ml (Boehringer Mannheim). Cultures of *Pseudomonas* were incubated at 28°C and cultures of *E. coli* were incubated at 37°C and transferred weekly. Table 1 indicates all strains, vectors and plasmids used throughout this study. All chemicals were obtained commercially through Sigma Company unless otherwise indicated.

Preparation of Cell-Free Extracts

Bacterial cultures were inoculated into a 50 ml Fernbach flask containing 10 ml of LB or BSM with appropriate carbon source and antibiotic added. This was

Table 1

Strains and Plasmids

Strain or Plasmid	Phenotype	Characteristics	Source
P. fluorescens ATCC 13525	Ara^+, Tc^s	Parent Neotype Strain	ATCC, Rockville, MD
P. putida ATCC 12633	Ara^-, Tc^s	Surrogate Host	ATCC, Rockville, MD
Plasmids			
pLA2917	Tc^r, Km^r	Cosmid Vector	Allen & Hanson (1985)
pPZ221 pPZ222 pPZ224 pPZ226 pPZ227 pPZ228 pPZ229	Ara^+, Tc^r, Km^s	Cosmids containing *P. fluorescens* DNA inserts in the *Bgl*II site	Eisenberg & Phibbs (1988)

allowed to grow overnight at 27°C on a New Brunswick Controlled Environment Gyrator at 100 rpm. This culture was then used to inoculate a 2.8 L Fernbach flask containing 500 ml of appropriate medium and antibiotic and allowed to grow to midlog stage at 27°C. The cells were then washed three times with 0.025M potassium phosphate buffer, pH 7.0, with 0.1% 2-mercaptoethanol (2-ME) and pelleted by centrifugation in a Sorvall Superspeed RC2-B Automatic Refrigerated

Centrifuge using a Sorvall GSA rotor at 7500 rpm for 15 minutes at 4°C. The final cell pellet was then harvested, stored in a plastic vial and frozen at -20°C for at least 24 hours.

The frozen cell pellet was thawed at room temperature and resuspended in a minimum of 5 ml of 0.025M potassium phosphate buffer, pH7.0 containing 0.1% 2-ME. The bacterial cells were disrupted using a Branson model W185 Sonifier at 50 watts with three ten second sonication periods. A ten second break between each sonication period was carried out to allow the cell suspension to cool in stainless steal sonication tubes maintained in an ice bath during sonication. The broken cell suspension was placed in a Corex tube and the cell-free extract was then obtained as the supernatant after centrifugation in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge at 13,000 rpm using a Sorvall SS-34 rotor for 10 minutes (4°C). Cell-free extracts were retained in an ice bath before assays were made and samples were frozen at -20°C prior to protein concentration estimation.

L-arabinose Dehydrogenase Assay

L-arabinose dehydrogenase (LAD) activity was measured by observing NAD reduction on a Gilford 2000 Recording Spectrophotometer at a wavelength of 340 nm. The reaction mixture contained (per 1.0 ml): 0.5 ml of 0.5M potassium phosphate buffer, pH 9.0, with 0.1% 2-mercaptoethanol; 1.5mM NAD; 1.3mM L-

arabinose; and crude cell-free extract. The reaction was observed at 25°C for one minute immediately after the addition of L-arabinose.

Protein Assay

Protein concentrations were determined utilizing the Lowry method (Lowry et al., 1951). A standard curve was established using a 3mg/10ml stock solution of Bovine Serum Albumin (BSA). A titration of the sample was run by adding the cell-free extract with deionized water to total 0.5 ml. After vortexing, 5 ml protein reagent was added (50 ml of 2% Na_2CO_3 in 0.1N NaOH, 0.5 ml 2% Sodium tartrate, 0.5 ml 1% $CuSo_4 \bullet 5H_2O$), and vortexed. The solution was incubated at 37°C for 30 minutes. After incubation, 0.5 ml of Folin reagent (1:3 dilution of 2N Folin-Ciocalteu reagent with deionized water) was added and the solution was vortexed. This was then allowed to incubate at 25°C for 20 minutes. The absorbance was then read on a Klett-Summerson Photoelectric Colorimeter using a #66 filter with a spectral range of 640-700 millimicrons (mμ).

Miniprep Plasmid DNA Extraction

Plasmid DNA was extracted utilizing the following protocol from Maniatis et al. (1982). The plasmid containing host strain was inoculated into 10 ml LB with appropriate antibiotic and grown overnight. The cells were centrifuged at 3000 rpm

(SS-34 rotor), 4°C, for 15 minutes in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge. The supernatant was removed and 100 µl of Solution I (50mM glucose, 25mM TRIS:HCl pH8, 10mM EDTA) was added. This was vortexed to resuspend the pellet and transferred to microcentrifuge tubes containing 10 µl lysozyme (Boehringer Mannheim Lysozyme, 50 milligrams dissolved in one milliliter of Solution I). This was vortexed and incubated at room temperature (25°C) for 5 minutes. Two hundred microliters of Solution II (0.5 ml 5M NaOH, 0.5 ml 20% SDS, 9.0 ml deionized water) were added. This was mixed gently and placed on ice for 5 minutes. One hundred fifty microliters of Solution III (5M Potassium Acetate, pH4.8) were added, mixed by tapping, and placed on ice for 5 minutes. This was centrifuged for 7 to 10 minutes using a Fotodyne microcentrifuge at 10,000 rpm. The supernatant was placed into new microcentrifuge tubes and an equal amount of 1:1 phenol:chloroform was added and mixed by inversion. This was then centrifuged for 5 minutes at 10,000 rpm in a Fotodyne microcentrifuge. The supernatant was placed into sterile microcentrifuge tubes with 800 µl of ice cold 100% ethanol added. This was mixed by gently shaking the tubes and then placed at -20°C for 30 minutes. The tubes were then centrifuged at 10,000 rpm, 4°C, for 45 minutes in a Fisher Microcentrifuge Model 235A. The supernatant was discarded and the pellet was washed with 1.0 ml of 70% ethanol. This was centrifuged for 3 to 4 minutes at 25°C using a Fotodyne microcentrifuge. The pellet was allowed to dry in a 37°C heating

block (Laboratory Supplies Co., Inc.). Once dry, the pellet was then resuspended in 100 µl of TE buffer (20mM TRIS, pH 7.4; 1mM EDTA). All solutions except Solution II were kept at 4°C, Solution II was kept at 25°C.

Transformation of *Pseudomonas*

Transformation for *Pseudomonas* (Durham et al., 1984) was accomplished by allowing twenty-five milliliters of cells to grow to midlog phase then placing on ice for 20 minutes. The chilled cells were centrifuged at 5,000 rpm (SS-34 rotor) in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 25 milliliters of 100mM $MgCl_2$. The pellet was resuspended using 12.5 ml of 100mM $CaCl_2$ and 50mM $MgCl_2$ and placed on ice for one hour. The cells were then centrifuged again at 5000 rpm, the supernatant was discarded and the pellet was resuspended in 0.5 ml of 100mM $CaCl_2$/50mM $MgCl_2$. The cells were then chilled on ice for one hour. Aliquots of 0.1 ml of the cells were then placed into microcentrifuge tubes and 1 to 5 micrograms of plasmid DNA was added. This was allowed to chill on ice for 25 minutes. All aliquots were then heat shocked for 3 minutes in a 42°C water bath and placed directly into an ice bath. One milliliter of LB was added and the cells were allowed to incubate at 30°C for 90 minutes. The microcentrifuge tubes were centrifuged for 10 minutes at 10,000 rpm using a Fotodyne microcentrifuge, and the

supernatant was discarded. The pellet was resuspended in 0.5 ml of 50 mM potassium phosphate buffer pH 7.0 and aliquoted onto selective media plates.

Plasmid DNA Quantitation

To determine the amount of plasmid DNA recovered after a plasmid extraction, the absorbance was read at 260 nm using a Gilford 2000 recording spectrophotometer. The quantity of DNA was determined by comparing the absorbance at OD_{260} and assuming an OD_{260} of 1.0 corresponds to 50 μg/ml of doublestranded DNA. (Maniatis et al., 1982).

RESULTS

The questions we pose in this study are (1) are the genes for the L-arabinose pathway encoded in the chromosome fragments cloned from *P. fluorescens* expressed constitutively when they are transcribed into a *P. putida* surrogate host and (2) if not, are they regulated in the same inducible manner as they are in *P. fluorescens*.

In order to answer these questions, we selected the L-arabinose dehydrogenase enzyme as a marker to illustrate the relative levels of all the L-arabinose pathway enzymes (Figure 5) encoded within our plasmid constructs which can convert *P. putida* from an L-arabinose negative phenotype to an L-arabinose positive phenotype. L-arabinose dehydrogenase activity was chosen as a marker for expression of this pathway because this is a simple and convenient assay which relies on a spectrophotometric estimate of the concentration of NADH.

Development of an L-arabinose Dependent NAD Reduction Assay

Reproducible assays for L-arabinose dehydrogenase activity from extracts of *P. putida* must be a prerequisite when using crude cell-free extracts which contain a variety of potentially competing reactions. Therefore, the development of this enzyme assay was intentionally done using crude extracts of *P. fluorescens* and not with partially purified preparations. This approach should preclude unnecessary

difficulties with interpreting results when crude extracts of *P. putida,* grown under a variety of conditions, are used for estimating the relative concentration (specific activity) of L-arabinose dehydrogenase.

Results shown in Figure 7 illustrate the approach used in these studies for obtaining the L-arabinose dependent reduction of NAD to NADH in crude extracts (L-arabinose dehydrogenase activity). This experiment (Figure 7) was constructed by using a reaction mixture containing all the components (buffer and cell-free extract) for oxidation of L-arabinose by NAD except that neither NAD nor L-arabinose were present when the absorbance measurements were begun. Therefore, there was no change in absorbance at 340 nm during the first part of the measurements. Addition of NAD (electron acceptor) may or may not result in the reduction of NAD depending on whether there were other potential oxidizable substrates present in the crude extract. In this experiment, there was no increase in absorbance after NAD was added to the cell extract reaction mixture (Figure 7, NAD addition). Addition of L-arabinose resulted in an immediate and linear increase in absorption (production of NADH) which confirms the presence of an L-arabinose dependent dehydrogenase activity.

This lack of NAD reduction in these extracts before addition of L-arabinose was not always consistent with the results shown in Figure 8. There was an increase in absorbance in some extracts when NAD was added but before the addition of L-arabinose. The results in Figure 8 illustrate this type of L-arabinose independent

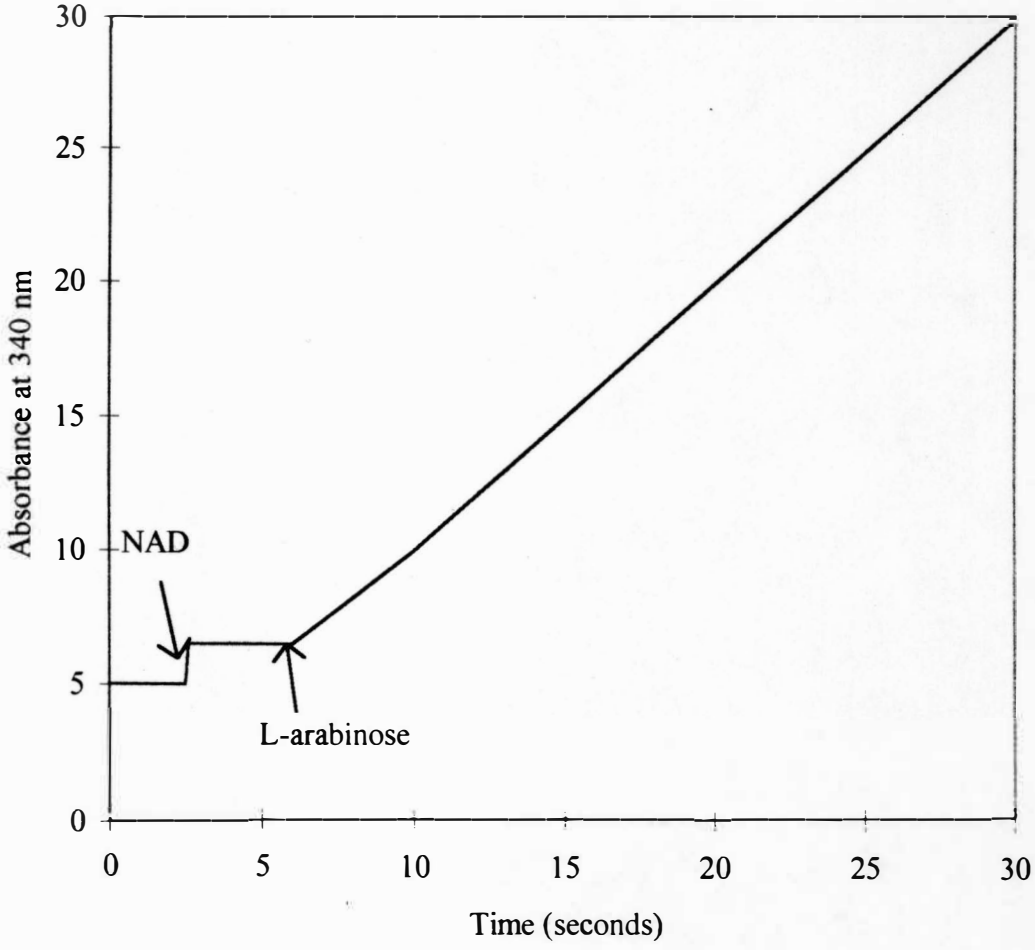

Figure 7. L-arabinose Dependent NAD Reduction.

This graph shows an actual data trace. The initial reading (buffer and crude cell-free extract) shows no change in absorbance at 340 nm. Addition of NAD caused no increase in absorbance. Addition of L-arabinose resulted in an immediate and linear increase in absorbance (production of NADH).

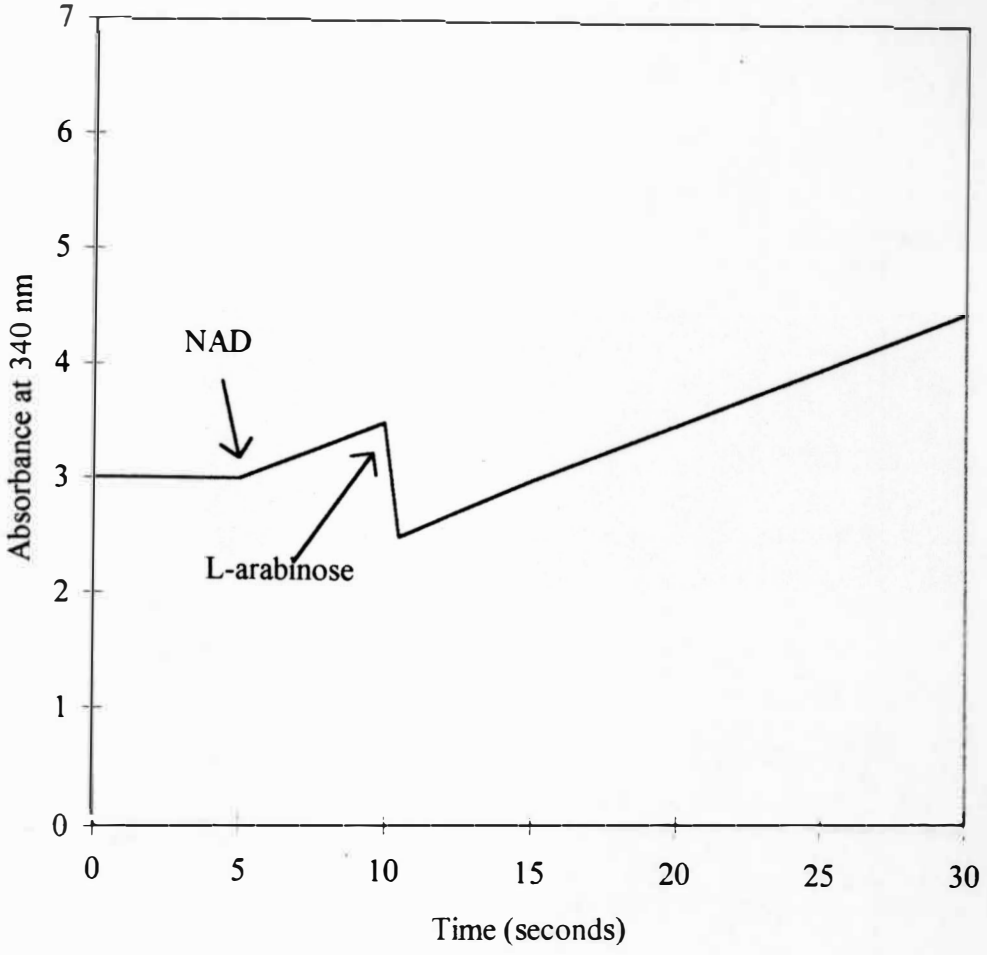

Figure 8. L-arabinose NAD Dependent Reduction in the Presence of an Endogenous NAD Reduction.

This is an actual data trace showing L-arabinose independent NAD reduction. The addition of NAD gave a linear increase in absorbance and the addition of L-arabinose gave an additional linear increase in absorbance. The L-arabinose dependent NAD reduction was estimated by subtracting the rate of the L-arabinose independent NAD reduction rate from L-arabinose dependent reduction rate.

NAD reduction. In these experiments, illustrated in Figure 8, addition of NAD gave a linear increase in absorbance and addition of L-arabinose (see Figure 8) lead to an additional linear increase of absorbance. We then estimated the L-arabinose dependent NAD reduction by subtracting the rate of L-arabinose independent NAD reduction from the rate obtained after addition of L-arabinose (L-arabinose dehydrogenase activity). Therefore, all L-arabinose dehydrogenase activities reported in this study represent L-arabinose dependent NAD reduction (L-arabinose dehydrogenase activity) independent of an endogenous NAD reduction.

The presence or absence of an endogenous NAD reduction in these extracts occurred in extracts of both *P. fluorescens* and *P. putida*. Therefore, this potential artifact was not due to a difference in these two organisms. Our experience was that these differences were due to the period of time between collecting the extracts and performing the assays. However, all extracts were retained on ice until the assays were performed and they all were done within 30 to 45 minutes after extract collection. In those few experiments (not shown here) where the extracts showed continuing endogenous NAD reduction, these extracts were retained for an additional 10 to15 minutes until the endogenous NAD reduction ceased. No difference in the activity of L-arabinose dehydrogenase activity was observed after this time period. Therefore, these results provided the basis for estimating L-arabinose dehydrogenase activities in these crude cell-free extracts independent of whether there was an endogenous NAD reduction activity present.

Development of an Optimal pH for L-arabinose Dehydrogenase Assay

Using the assay conditions described above, the optimal pH for L-arabinose dehydrogenase activity in these cell-free crude extracts was estimated. The results of these experiments are presented in Figure 9. Separate experiments (not shown) also confirmed the maximal L-arabinose dependent NAD reduction (L-arabinose dehydrogenase) activity occurred at pH 9.0. It should be noted that all assays at the various pH values indicated in Figure 9 were done in the presence of saturating levels of the substrates for this enzyme activity (both L-arabinose and NAD).

Estimation of Saturation Kinetics for NAD

In order to assure that all assays for L-arabinose dehydrogenase activity represented conditions required for establishing a linear relationship between the amount of enzyme present and the activity observed, the saturating concentrations of NAD for L-arabinose dehydrogenase activity were estimated. The results from these experiments are presented in Figure 10. All values reported represent the initial rate immediately after addition of L-arabinose; this was always less than five seconds. These results clearly demonstrate that an NAD concentration of 1.5 mM provided for zero order kinetics of L-arabinose dehydrogenase. It should be noted that these assays were conducted at saturating concentrations of L-arabinose (1.3 mM) (see Figure 12). A Lineweaver-Burk plot of the data from Figure 10 is presented in Figure 11. These

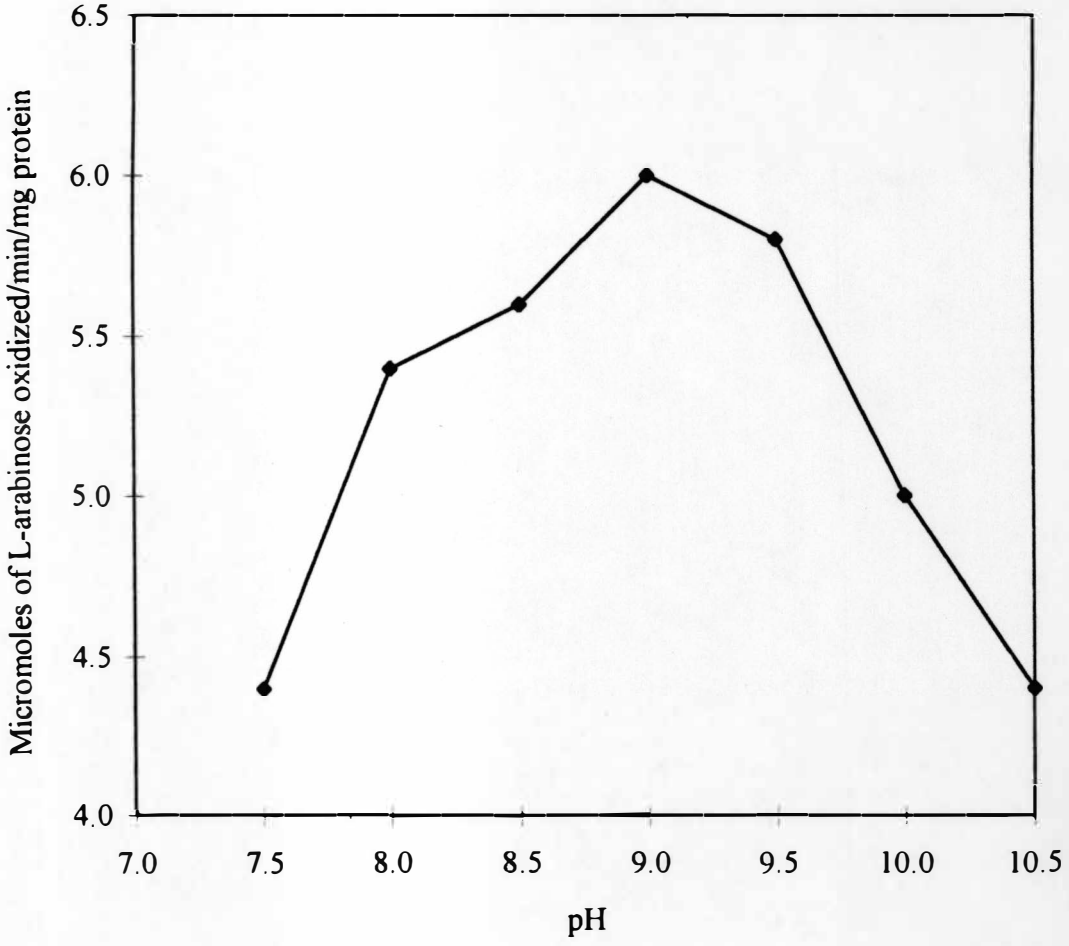

Figure 9. Determination of Optimum pH.

The pH of the reaction was varied to determine L-arabinose dehydrogenase activity as a function of pH. A pH value of 9.0 for L-arabinose dependent NAD reduction activity appeared to be optimal.

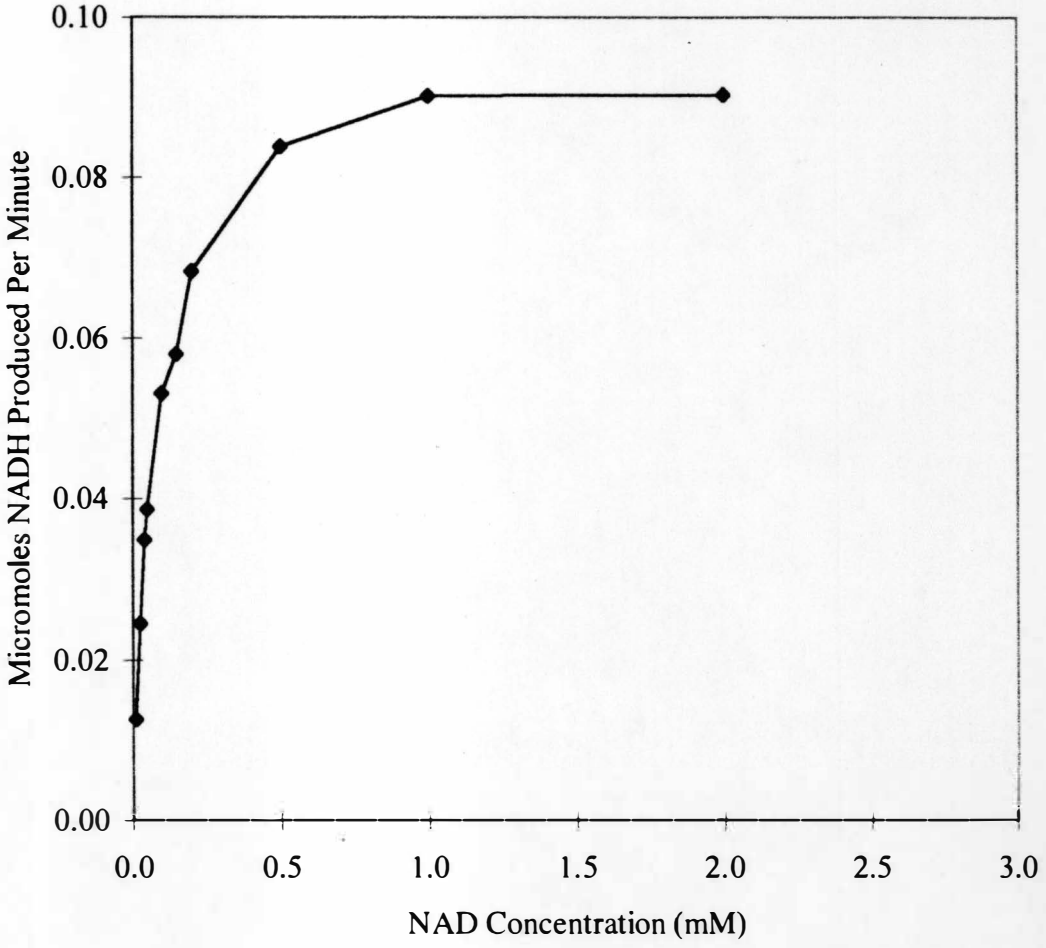

Figure 10. Saturation Kinetics for NAD Concentration.

The activity of L-arabinose dehydrogenase was estimated as a function of NAD concentration. The concentrations which provided for zero-order kinetics were found to be in the range of 1.0 to 2.0 mM. A concentration of 1.5 mM was used in all subsequent assays.

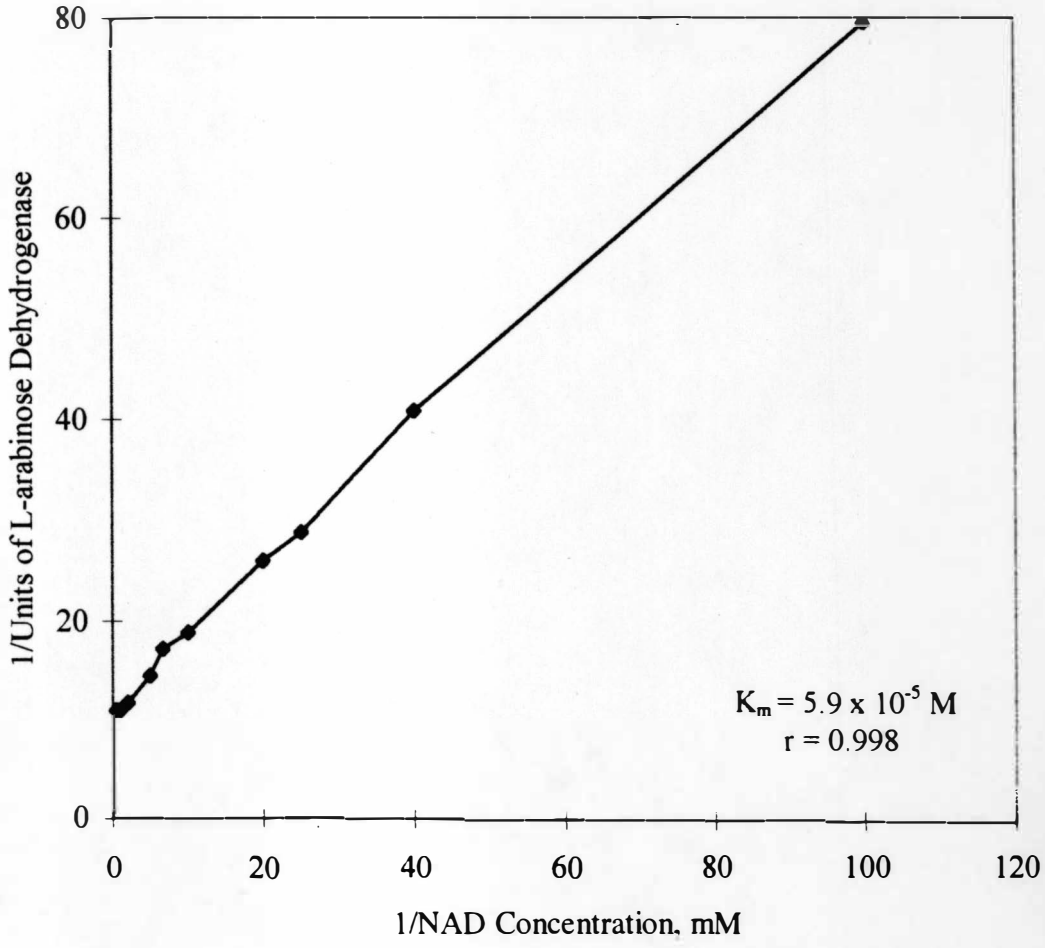

Figure 11. A Lineweaver-Burk Plot to Determine K_m Value for NAD Concentration.

A K_m value of 5.9×10^{-5} M was determined for NAD. A regression coefficient of 0.998 was obtained for these results. A unit of L-arabinose dehydrogenase is equal to the µmoles of NADH produced per minute.

results indicate a K_m of 5.9 x 10^{-5} M for NAD. The regression coefficient of 0.998 give a high degree of confidence for this estimate of the affinity constant of NAD for L-arabinose dehydrogenase in these crude extracts.

We chose to use a concentration of 1.5 mM in all the assays to be used in this study because, as shown in Figure 10, small errors in the actual concentration of NAD in these assays would have a negligible effect on the observed activity of L-arabinose dehydrogenase.

Estimation of Saturation Kinetics for L-arabinose

The saturating concentrations of L-arabinose for L-arabinose dehydrogenase activity were also determined to insure a linear relationship between the enzyme concentration and the activity observed. Figure 12 represents the results from these experiments. These results illustrate that an L-arabinose concentration of 1.3 mM will allow for zero order kinetics of L-arabinose dehydrogenase. These assays were performed at saturating conditions of NAD (1.5 mM, see Figure 10) and a pH of 9.0 (Figure 9).

As for NAD, a Lineweaver-Burk plot was performed for the L-arabinose data in Figure 12. The results in Figure 13 indicate a K_m of 2.5 x10^{-4} M for L-arabinose. The regression coefficient of 0.999 gives a high degree of confidence for this estimate of the affinity constant of L-arabinose for L-arabinose dehydrogenase activity.

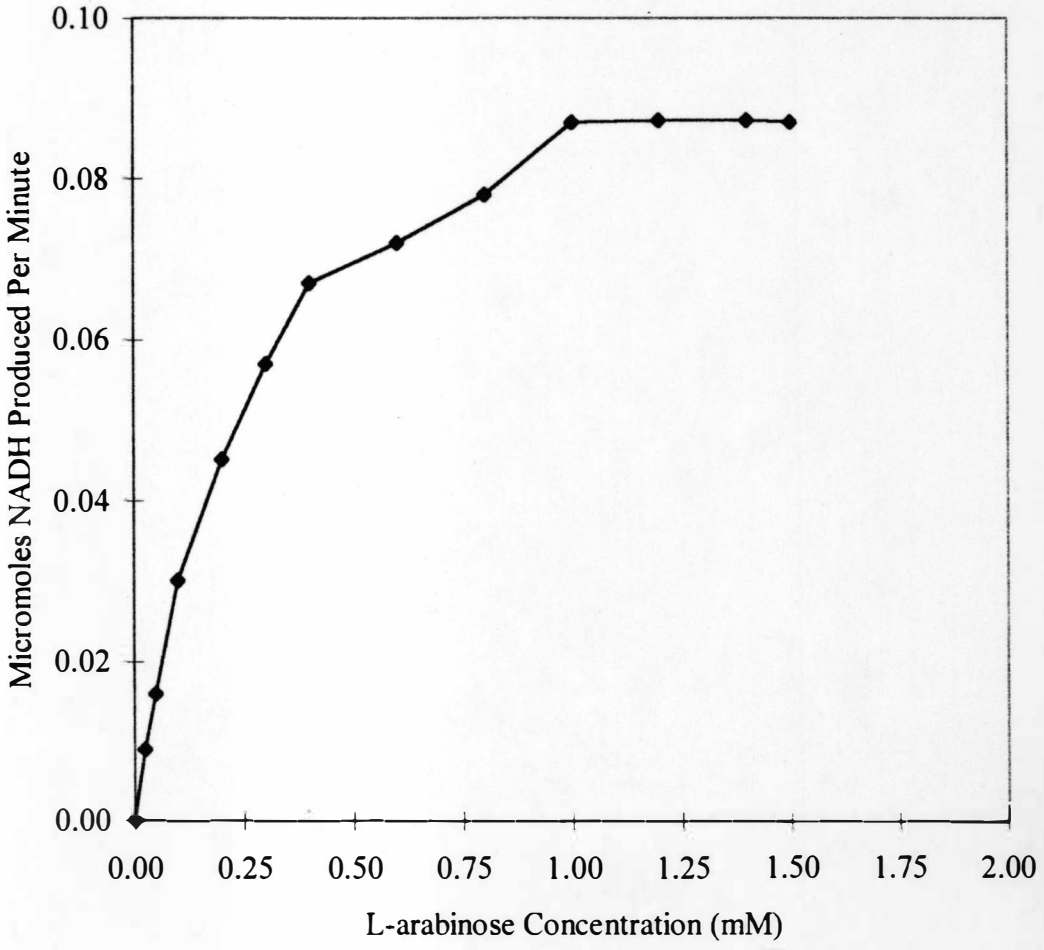

Figure 12. Saturation Kinetics for L-arabinose Concentration.

The activity of L-arabinose dehydrogenase was estimated as a function of L-arabinose concentration. The concentrations which provided for zero-order kinetics were found to be in the range of 1.0 to 1.5 mM. A concentration of 1.3 mM L-arabinose was used in all subsequent assays.

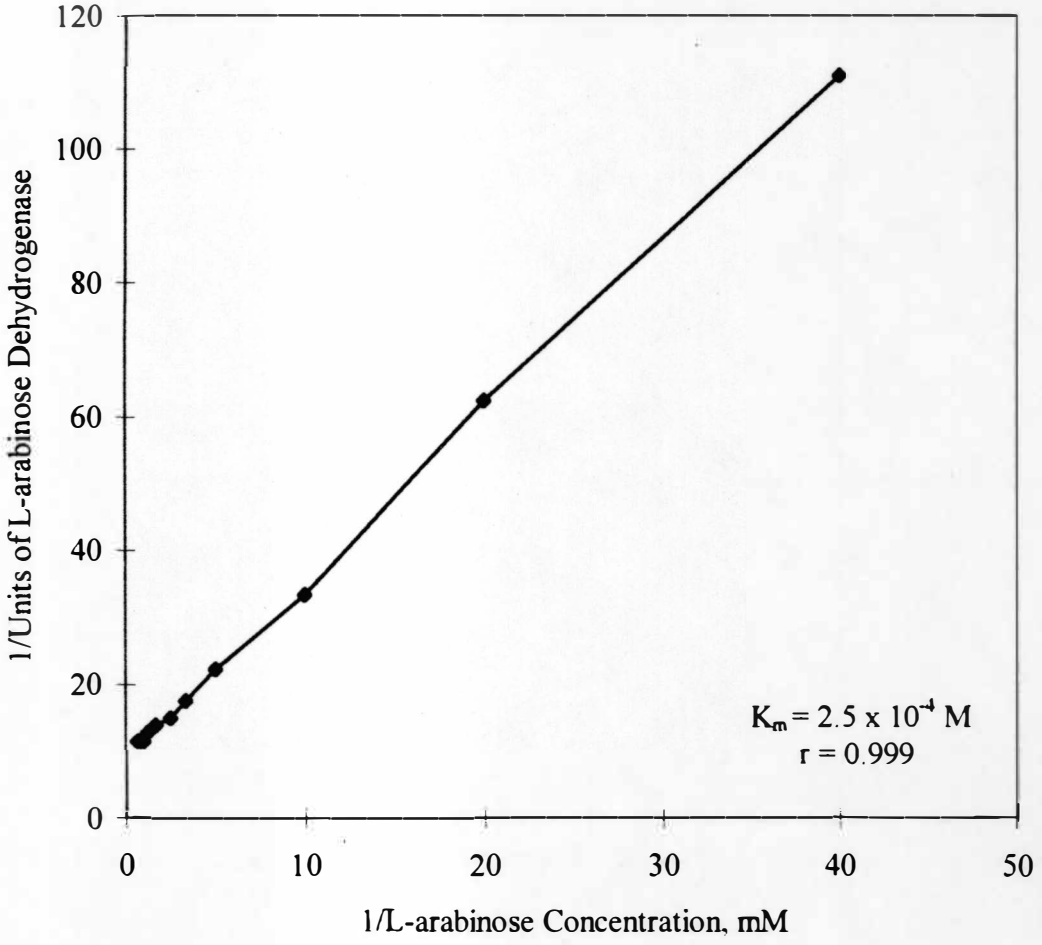

Figure 13. A Lineweaver-Burk Plot to Determine K_m Value for L-arabinose Concentration.

A K_m value of 2.5×10^{-4} M was determined for L-arabinose. A regression coefficient of 0.999 was obtained for these results. A unit of L-arabinose dehydrogenase is equal to the µmoles of NADH produced per minute.

Estimates of L-arabinose Dehydrogenase Activity in Cell-Free Extracts

The objectives of this study included the question as to whether L-arabinose dehydrogenase is expressed in an inducible or constitutive fashion in the surrogate host, *P. putida*. In order to address this issue, it was first important to confirm the inducible nature of this enzyme in crude cell-free extracts in *P. fluorescens* using our assay system. The results from experiments designed to test this assay in *P. fluorescens* extracts are presented in Table 2. These data clearly show that L-arabinose dehydrogenase is inducible in *P. fluorescens* since this enzyme is present in crude extracts from cells grown on L-arabinose but not in crude extracts grown on succinate or glucose. Also, growth of *P. fluorescens* in a complex medium deficient in L-arabinose failed to stimulate induction of L-arabinose dehydrogenase. Therefore, our assay system for crude cell-free extracts is clearly suitable for estimating the inducible nature of L-arabinose dehydrogenase.

Another important control for addressing the question of expression of chromosomal inserts from *P. fluorescens* cloned in the cosmid vector pLA2917 is whether there is a contribution of the vector to regulation of the enzyme. This issue is also addressed in the experimental results presented in Table 2. *P. fluorescens* containing the cosmid vector pLA2917 (and grown in the presence of tetracycline) extracts also reflect the same pattern observed for *P. fluorescens* grown in the absence of the cosmid vector and tetracycline. Therefore, these controls demonstrate that neither the vector for the chromosomal inserts nor tetracycline

influence the induction of L-arabinose dehydrogenase in *P. fluorescens*. Growth of these cells in the presence of tetracycline was required in order to assure that the cosmid (which contains a tetracycline resistance marker) was maintained by using a selective growth environment.

The results presented in Table 2 for extracts prepared from *P. putida*, grown with and without the cosmid vector, also demonstrate that L-arabinose dehydrogenase is not inducible by growth on glucose, succinate or Luria broth. *P. putida* does not grow on L-arabinose as a sole source of carbon and energy (Stanier et al., 1966). These results (Table 2) lead us to the conclusions that (a) our assay system functions to identify the inducible nature of L-arabinose dehydrogenase in *P. fluorescens* crude cell-free extracts, and (b) the cosmid vector does not contribute to the induction or repression of L-arabinose dehydrogenase in either *P. fluorescens* or *P. putida*.

Results from experiments designed to test expression of L-arabinose dehydrogenase, encoded by the *P. fluorescens* chromosomal inserts, by the surrogate host *P. putida*, are presented in Table 3. These results demonstrate that all of the plasmids containing chromosomal inserts of *P. fluorescens* tested, confer the ability to convert *P. putida* from an L-arabinose negative to an L-arabinose positive phenotype. Also, no significant difference was observed for induction of L-arabinose dehydrogenase in either *P. putida* (Table 3) or *P. fluorescens* (Table 2) when these cells were grown in the presence or absence of tetracycline. Addition of this antibiotic to the growth medium was necessary in order to maintain the plasmids

Table 2

Specific Activity of L-arabinose Dehydrogenase in Control Cell-Free Extracts

Organism	Growth Substrate	L-arabinose Dehydrogenase[1] (μmole/min/mg protein)
P. fluorescens ATCC 13525	30mM L-arabinose	3.14
	20mM Glucose	not detectable[2]
	20mM Succinate	not detectable
	Luria Broth	not detectable
P. fluorescens containing pLA2917	30mM L-arabinose + Tc^{10}	2.68
	20mM Glucose + Tc^{10}	not detectable
	20mM Succinate + Tc^{10}	not detectable
	Luria Broth + Tc^{10}	not detectable
P. putida ATCC 12633	30mM L-arabinose	(no growth)
	20mM Glucose	not detectable
	20mM Succinate	not detectable
	Luria Broth	not detectable
P. putida containing pLA2917	30mM L-arabinose	(no growth)
	30mM L-arabinose + Tc^{10}	(no growth)
	20mM Glucose + Tc^{10}	not detectable
	20mM Succinate + Tc^{10}	not detectable
	Luria Broth + Tc^{10}	not detectable

[1]Values given as an average of two values.
[2]Not detectable indicates that a change in optical density could not be detected.

(which contain a tetracycline resistance marker) when cells were grown in an environment that does not (absence of L-arabinose) select for the plasmids. This

observation that the presence or absence of tetracycline has no significant effect on expression of the L-arabinose operon is important since some of the controls (see below) for this study requires growing cells in the presence of tetracycline.

As can be seen in Table 3, crude cell-free extracts of *P. putida* pPZ221 has detectable and significant levels of L-arabinose dehydrogenase activity when grown in the absence of L-arabinose (growth on succinate, glucose or Luria broth). When *P. putida* pPZ221 was grown on glucose, the levels of this enzyme was twice that found when cells were grown in succinate or Luria broth. The levels of L-arabinose dehydrogenase in *P. putida* pPZ221 grown on glucose, however, were only about 20% of that found in cells grown on L-arabinose. Therefore, these results, taken together, demonstrate that L-arabinose dehydrogenase in *P. putida* pPZ221 is inducible by growth on L-arabinose and there is a high constitutive level of this enzyme in these cells when grown in the absence of L-arabinose (glucose, succinate, or Luria broth).

With the exception of low, barely detectable, levels of enzyme in cells from Luria broth, the same pattern for induction was found for *P. putida* pPZ222. *P. putida* containing plasmids pPZ224, pPZ226, pPZ227, pPZ228, and pPZ229 all were induced from 10 to 50 fold by growth on L-arabinose compared to growth on glucose (Table 3). High constitutive levels of this enzyme was found in pPZ224, pPZ227, and pPZ228 grown in succinate and barely detectable, low constitutive levels, were found

Table 3

Comparison of Specific Activity for *P. putida* Containing the Plasmids for L-arabinose Utilization

Organism	Growth Substrate	L-arabinose Dehydrogenase[1] (μmole/min/mg protein)
P. putida containing pPZ221	30mM L-arabinose	2.88
	30mM L-arabinose + Tc^{10}	2.82
	20mM Glucose + Tc^{10}	0.52
	20mM Succinate + Tc^{10}	0.22
	Luria Broth + Tc^{10}	0.28
P. putida containing pPZ222	30mM L-arabinose	2.29
	30mM L-arabinose + Tc^{10}	2.20
	20mM Glucose + Tc^{10}	0.18
	20mM Succinate + Tc^{10}	0.18
	Luria Broth + Tc^{10}	0.04
P. putida containing pPZ224	30mM L-arabinose	3.85
	30mM L-arabinose + Tc^{10}	2.91
	20mM Glucose + Tc^{10}	0.25
	20mM Succinate + Tc^{10}	0.46
	Luria Broth + Tc^{10}	not detectable[2]
P. putida containing pPZ226	30mM L-arabinose	5.23
	30mM L-arabinose + Tc^{10}	5.58
	20mM Glucose + Tc^{10}	0.11
	20mM Succinate + Tc^{10}	0.07, not detectable
	Luria Broth + Tc^{10}	0.06
P. putida containing pPZ227	30mM L-arabinose	5.00
	30mM L-arabinose + Tc^{10}	7.11
	20mM Glucose + Tc^{10}	0.46
	20mM Succinate + Tc^{10}	1.64
	Luria Broth + Tc^{10}	0.36

Table 3-Continued

Organism	Growth Substrate	L-arabinose Dehydrogenase[1] (μmole/min/mg protein)
P. putida containing pPZ228	30mM L-arabinose	2.92
	30mM L-arabinose + Tc^{10}	4.28
	20mM Glucose + Tc^{10}	0.26
	20mM Succinate + Tc^{10}	0.22
	Luria Broth + Tc^{10}	0.12
P. putida containing pPZ229	30mM L-arabinose	2.25
	30mM L-arabinose + Tc^{10}	2.30
	20mM Glucose + Tc^{10}	0.22
	20mM Succinate + Tc^{10}	0.08
	Luria Broth + Tc^{10}	0.51

[1]Values given as an average of two values.
[2]Not detectable indicates that a change in optical density could not be detected

in pPZ226 and pPZ229. However, high constitutive levels were found in pPZ227, pPZ228, and pPZ229 grown in Luria broth compared to low constitutive levels in pPZ224 and pPZ226 grown in Luria broth.

Table 4 presents the results obtained from control experiments designed to test the contribution(s) of plasmids pPZ221, pPZ226, and pPZ227 to L-arabinose dehydrogenase levels when the parent *P. fluorescens* strain was grown in these various media. Again, these results (Table 4) confirm that growth in the presence of tetracycline had no major effect on the induction of this enzyme present in these cells and the specific activities of L-arabinose dehydrogenase compare favorably with that

Table 4

Specific Activity of L-arabinose Dehydrogenase in Cell-Free Extracts for *P. fluorescens* Containing the Plasmids for L-arabinose Utilization

Organism	Growth Substrate	L-arabinose Dehydrogenase[1] (μmole/min/mg protein)
P. fluorescens containing pPZ221	30mM L-arabinose	2.47
	30mM L-arabinose + Tc^{10}	2.34
	20mM Glucose + Tc^{10}	not detectable[2]
	20mM Succinate + Tc^{10}	0.52
	Luria Broth + Tc^{10}	not detectable
P. fluorescens containing pPZ226	30mM L-arabinose + Tc^{10}	2.68
	20mM Glucose + Tc^{10}	not detectable
	20mM Succinate + Tc^{10}	not detectable
	Luria Broth + Tc^{10}	not detectable
P. fluorescens containing pPZ227	30mM L-arabinose + Tc^{10}	1.84
	20mM Glucose + Tc^{10}	not detectable
	20mM Succinate + Tc^{10}	0.66
	Luria Broth + Tc^{10}	not detectable

[1]Values given as an average of two values.
[2]Not detectable indicates that a change in optical density could not be detected

found in *P. fluorescens* containing the cosmid vector alone when these cells were grown on L-arabinose (Table 2).

However, the presence of pPZ221 and pPZ227 did cause expression of a high constitutive level of L-arabinose dehydrogenase in *P. fluorescens* when cells were grown in succinate medium. This is a significant difference from the results shown in

Table 2 where the growth of *P. fluorescens*, with or without the cosmid vector, had no detectable levels of this marker enzyme for the L-arabinose pathway when those cells were grown on succinate. *P. fluorescens* grown on glucose or Luria broth did not contain detectable levels of L-arabinose dehydrogenase in the presence or absence of the cosmid vector (Table 2) or any of the plasmids tested (Table 4). Both of these plasmids, pPZ221 and pPZ227, also caused production of high constitutive levels of this marker enzyme in *P. putida*. However, both of these plasmids produced high constitutive levels of this enzyme when *P. putida* was grown on glucose or Luria broth; this is in contrast to the absence of any detectable levels of this enzyme in *P. fluorescens* (Table 4).

DISCUSSION

To insure that the assay used to estimate activity of L-arabinose dehydrogenase would allow for the acquisition of reproducible results, optimal conditions for the reaction mixture pH and the substrates were determined. As reported in the results section, the optimal pH (Figure 9) for L-arabinose dehydrogenase activity was a value of 9.0. This concurs with the optimal pH value range of 9.0 to 10.0 determined by Doudoroff (1962) in the study of L-arabinose dehydrogenase activity in *P. saccharophila*. The apparent K_m values obtained for NAD and L-arabinose are also similar to those obtained by Doudoroff (1962). We estimated the K_m value for NAD to be 5.9×10^{-5} M (Figure 11). Doudoroff reported an apparent K_m value of 1.3×10^{-4} M for NAD. The apparent K_m determined in this study for L-arabinose was 2.5×10^{-4} M (Figure 13). A K_m value of 9.2×10^{-4} M for L-arabinose was estimated by Doudoroff. Although these estimated K_m values for both L-arabinose and NAD are similar to those obtained by Doudoroff, the data show slight differences. These differences could be due to the fact that even though Doudoroff (1962) reported an optimal pH range of 9.0 to 10.0, a pH of 8.6 at 30°C was utilized for his studies. In this study, a pH of 9.0 at 22-25°C was used. Doudoroff (1962) also used purified proteins to test for the activity of L-arabinose dehydrogenase, while crude cell-free extracts were used in our experiments.

The two questions posed in this study were (1) are the plasmid genes for L-arabinose utilization, encoded in the chromosomal fragments from *P. fluorescens*, expressed constitutively in the surrogate host *P. putida*; or (2) are these genes regulated in the same inducible manner as in *P. fluorescens*?

The results obtained in these studies demonstrate that high inducible levels of L-arabinose dehydrogenase are found in all *P. putida* strains containing these plasmids. Therefore, the levels of this enzyme are similar to those found in *P. fluorescens* when grown in the presence of L-arabinose compared to levels found in *P. fluorescens* grown on succinate, glucose, or Luria broth. However, there was a significant level of L-arabinose dehydrogenase present in *P. putida* plasmid strains grown on succinate, glucose, or Luria broth compared to the low, undetectable levels of this enzyme in *P. fluorescens* grown on these substrates (Tables 2 and 3).

Control experiments (Table 2) demonstrated that the cosmid vector alone did not contribute to synthesis of L-arabinose dehydrogenase when *P. putida* was grown on the permissive substrates; glucose, succinate, and Luria broth. Also, the cosmid vector pLA2917 did not influence synthesis (induction) of this enzyme in *P. fluorescens* grown on these substrates (Table 2). These controls rule out any contribution of the cosmid vector to the presence of L-arabinose dehydrogenase in these experiments. Therefore, all results for the production of this enzyme in *P. putida* strains carrying these plasmids are due to the presence of the chromosomal inserts from *P. fluorescens*.

As pointed out in the Results sections, the high constitutive levels of L-arabinose dehydrogenase in *P. putida* pPZ221 and pPZ227 strains grown on succinate are also found in the control *P. fluorescens* pPZ221 and pPZ227 strains. Also, it is interesting to note that the low, barely detectable levels of this enzyme found in *P. putida* pPZ226 are also low, undetectable levels in the control *P. fluorescens* pPZ226. These consistent results strongly suggest that there are significant differences between the presence of regulatory elements in these plasmids or that they may represent two different classes of chromosomal inserts.

A different pattern appears for these plasmids when *P. putida* was grown on glucose. Therefore, pPZ221, pPZ224, pPZ227, pPZ228, and pPZ229 all confer high constitutive levels (specific activities of 0.22 to 0.52) in *P. putida*. Plasmids pPZ221 and pPZ227 in the *P. fluorescens* controls did not elicit detectable levels of enzyme when cells were grown on glucose. The presence of tetracycline in these experiments does not contribute to the expression of L-arabinose dehydrogenase because this control was established from the results presented in Table 2.

The results described above identify that high constitutive levels of the L-arabinose pathway occurs when cells contain some of these plasmids, but not all. And that there is a difference between some of these plasmids as a function of the growth substrate. Not all of the plasmids tested in *P. putida* were also tested in *P. fluorescens*. Therefore, the lack of information about the properties of pPZ222, pPZ224, pPZ228, and pPZ229 in *P. fluorescens* precludes the ability to systematically

group all of these plasmids for their ability to confer high constitutive levels of the L-arabinose pathway in the surrogate host *P. putida* and the control *P. fluorescens*.

Nevertheless, the results obtained in this study clearly suggest that the normal regulatory properties of a putative L-arabinose operon in *P. fluorescens* is not complete when some of the chromosomal inserts of *P. fluorescens* cloned into pLA2917 are expressed in the surrogate host *P. putida* and parent strain *P. fluorescens*. These conclusions strongly infer that there is more than one simple regulatory mechanism operating for controlling expression of the L-arabinose operon in *P. fluorescens*.

One interpretation for the relatively high constitutive concentration of L-arabinose dehydrogenase in succinate grown *P., putida* and *P. fluorescens* both containing plasmids pPZ221 and pPZ227 is a high copy number of the plasmid. However, the original plasmids (RK2 and pRK290, Ditta et al., 1980) used by Allen and Hanson (1985) to construct pLA2917, are low copy number plasmids. Furthermore, if a high copy number of a putative L-arabinose operon causes the high constitutive level of L-arabinose dehydrogenase, then it is difficult to interpret the normal levels of this enzyme in *P. fluorescens* pPZ221 and pPZ227 grown on L-arabinose (compare the specific activity of this enzyme under these conditions, 2.47 and 1.84, respectively, (Table 4) to the specific activity, 3.14, found in the control *P. fluorescens* without the plasmid (Table 2)). These data again suggest that there are

significant differences in regulatory properties for expression of L-arabinose dehydrogenase between these plasmids.

The L-arabinose operon in *E. coli* contains only the structural genes for cytoplasmic catabolic enzymes for converting L-arabinose to D-xylulose-5-phosphate (Englesberg, 1971). The transport systems for L-arabinose permease are encoded by the genes *araE* and *araFGH* which are located on a chromosomal region far removed from the L-arabinose operon (Figure 3). The ability of the plasmids used in this study to confer L-arabinose utilization in *P. putida* strongly imply that the genes for L-arabinose transport in *P. fluorescens* are either contained within the putative L-arabinose operon or are very closely linked to the genes for catabolism of L-arabinose. Another possibility is that *P. putida* may be able to transport L-arabinose into the cell.

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