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Title: The Influence of Phosphatase-Producing Bacteria on Phosphatase Activity and Available Phosphorus in Soil

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Phosphorus (P) is frequently a limiting soil nutrient because it is readily fixed by soil minerals and immobilized in organic compounds by soil organisms. As a result, often only small amounts of added fertilizer P are recovered in crops, and unavailable P accumulates in many agricultural soils.

Soils have been shown to have phosphatase activity, most of which originates from soil microorganisms. The role these organisms play in liberating orthophosphate (P_i) from organically-bound P (P_o), and the genetic controls involved in these processes are poorly understood.

Chapter 2 reports on a study involving a group of 17 soil bacteria, including members of the family *Enterobacteriaceae*, a group of pseudomonads, and *Flavobacterium* sp. The effects of P_i concentration, temperature, and pH on the phosphatase activities of these strains are reported. In addition, a study was made to determine which of them contained genomic DNA sequences that hybridize with the alkaline phosphatase structural gene, *phoA* of *Escherichia coli*. Of the 17 bacteria tested, detectable acid- and alkaline phosphatase activity was expressed by 14 and 12 strains, respectively when grown in low P_i medium. Under high P_i conditions, acid- and alkaline phosphatase activity were expressed by 11 and 9 of the strains, respectively. Phosphatase activity was low for most

strains, except for *Erwinia carotovora* subsp. *carotovora* W3C105, which gave high alkaline phosphatase activity under low P_i conditions. In general, the pseudomonads and *Flavobacterium* expressed higher acid- than alkaline phosphatase activity in both types of medium.

Southern blot hybridization studies using *E. coli phoA* gene as a probe with genomic DNA digests from the 17 soil bacteria resulted in hybridization with fragments of *E. carotovora* W3C105, *Enterobacter cloacae* E6, *E. coli* MPH69 ($PhoA^-$), and *E. coli* C600(P1). Of these, only *E. carotovora* exhibited high alkaline phosphatase activity. The *phoA* fragment did not hybridize with *Flavobacterium* MtCa7 nor with any of the pseudomonads.

The effects of incubation temperature and growth medium pH on phosphatase activity were examined for *E. carotovora* W3C105, *E. coli* MPH69 ($PhoA^-$), *E. coli* MPH43 ($PhoA^+$), and *Pseudomonas fluorescens* Pf-5, which expressed acid phosphatase activity in culture. All strains expressed lower acid- and alkaline phosphatase activity when grown at pH 6.5 than at pH 8.0, and no activity was detected for *E. carotovora* W3C105 at pH 6.5. When grown at 15, 20, 25, and 30° C, three of the strains expressed highest phosphatase activity near 25° C. The fourth strain, *E. coli* MPH69, gave little, if any, phosphatase activity under any of the pH or temperature conditions.

In Chapter 3, five bacterial strains previously studied, *E. carotovora* W3C105, *E. coli* MPH69 and MPH43, and *Pseudomonas fluorescens* strains Pf-5 and biotype F, were used to inoculate autoclaved soil samples limed with CaO. The samples were incubated for 7 d at 25° C, and the following were determined before and after incubation: bacterial plate counts; acid- and alkaline phosphatase activity; available ($NaHCO_3$-extractable) P; recently-fixed (H_2SO_4-extractable) P; and biomass P.

Recovered bacteria numbered approximately 10^8 cells g^{-1} soil. Samples inoculated with *P. fluorescens* Pf-5 had acid phosphatase activities 8 to 17 times greater than uninoculated control samples on Day 7. Samples containing *E. coli*

MPH43 ($PhoA^+$) and *E. carotovora* W3C105 had alkaline phosphatase activities significantly greater than those of control samples on Day 7. During incubation, levels of $NaHCO_3$-extractable P and H_2SO_4-extractable P both decreased for all samples. Biomass P, however, increased for all inoculated samples, but remained nearly the same for control samples. Increases in biomass P for all inoculated samples exceeded losses in $NaHCO_3$-extractable P, indicating that additional P had been mobilized, possibly from P_o in the soil. In uninoculated control samples, however, $NaHCO_3$-extractable P exceeded biomass P increases, indicating a net loss of available P, presumably by fixation.

Increases in bacterial counts and soil alkaline phosphatase activities related to final bacterial numbers appeared to be related to *in vitro* alkaline phosphatase activities for the *E. coli* and *E. carotovora* strains, which are members of the *Enterobacteriaceae*. Acid phosphatase activities of all strains and alkaline phosphatase activities of the pseudomonads appeared unrelated to *in vitro* phosphatase activities.

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The Influence of Phosphatase-Producing Bacteria on Phosphatase Activity and Available Phosphorus in Soil

INTRODUCTION

Phosphorus (P) is a nutrient essential for plant life because it is a component of RNA and DNA, vital for cell replication and protein synthesis (Ozanne, 1980). It is also needed by cells for energy storage and transfer through such compounds as ATP (Halstead and McKercher, 1975; Ozanne, 1980), and is a constituent of cell lipids (Halstead and McKercher, 1975).

Soil P originates from parent minerals, primarily calcium (Ca)-containing apatites, Ca phosphates, or iron (Fe)- and aluminum (Al)-containing minerals such as strengite and variscite (Brady, 1984). Most of these minerals are insoluble, and the soluble Ca phosphates readily revert to more insoluble forms, so that native soils may often contain large amounts of insoluble mineral phosphate (Tisdale and Nelson, 1975; Brady, 1984). Because of this low availability, P is usually the limiting nutrient in natural systems (Ozanne, 1980), and growers have routinely added phosphate fertilizers to soils to improve crop yields. The reactivity of P compounds, however, results in the immobilization of most of the added P. In the U.S., less than 15% of fertilizer P is taken up by crops during the year it is applied (Brady, 1984); consequently, P accumulates in these agricultural soils (Englestad and Terman, 1980; Brady, 1984).

The world's supply of phosphate fertilizers comes from non-renewable mineral deposits, nearly all of which contain one of the apatite group of minerals (Cathcart, 1980). Estimates of world phosphate reserves vary widely, depending on how much information is available and which types of resources are considered economically useful by the estimator. In 1971, the Institute of Ecology extimated that known P reserves, containing approximately 3.4 billion tons of P, could be exhausted in 90 to 130 years (Cathcart, 1980). These figures are

considered to be erroneously low (Emigh, 1972; Cathcart, 1980), but the 1,298,000 million tons of phosphate rock estimated by Emigh (1972) is likely far too great, given the low grade and inaccessibility of much of it (Cathcart, 1980). Long-range predictions are made more difficult due to the cyclic nature of the P market. As prices rise, growers may choose to "mine" excess applied P from their soils and add more fertilizer when prices fall again (Harre and Isherwood, 1980). In addition, environmental regulations and restrictions on the mining of phosphate rock are causing the number of low-cost reserves to dwindle at a rate that was not anticipated earlier in this century (Cathcart, 1980). This uncertainty of future P fertilizer availability in the face of increasing world demands makes it imperative to continue studying both P cycling in the soil and possible means of releasing fixed P and of retarding fixation of added P fertilizers (Brady, 1984).

Soil P occurs in three major fractions: insoluble inorganic P; soluble orthophosphate (P_i), a small, variable fraction; and soil organic P (P_o), which may contain as much as 50% of total soil P (Ozanne, 1980). To date, substantial research has been done on the chemical transformations undergone by the insoluble inorganic P components and their relationships to the amount of P_i in soil solution; very little is understood, however, about transformations of P_o in the soil and the part played by soil organisms in these processes.

The majority of P_o compounds in soil are mono- and diesters of orthophosphoric acid. Soil phosphatases are accorded a major role in cleaving the phosphate ester bonds of these molecules to release P_i (Speir and Ross, 1978). Plant roots, protozoa, fungi, earthworms, yeasts, and bacteria are all known to produce phosphatases (Casida, 1959; Estermann and McLaren, 1961; Hollander, 1971; Speir and Ross, 1978; Gould et al., 1979). Of these, however, the microorganisms are thought to contribute the largest amounts of phosphatase due to their high biomass and metabolic activities and short lifetimes (Skujins, 1978). *Escherichia coli* has been shown to produce a number of phosphatases in culture, which allow it to hydrolyze P esters to yield P_i (Garen and Levinthal,

1960; Rogers and Reithel, 1960; von Hofsten, 1961; Neu and Heppel, 1965; Dvorak et al., 1967; Hafckenscheid, 1968; Dassa et al., 1980; Pradel and Boquet, 1988).

Because bacteria grown in culture frequently do not behave as they would in natural environments, it is important to study known phosphatase-producing soil bacteria in soil to discover what effects they may have on accumulated soil phosphatase levels and available P. In addition, if these bacteria contain a common gene for phosphatase production, their presence in soil could be detected using DNA hybridization techniques (Fredrickson et al., 1988; Holben et al., 1988).

A study of a group of soil bacteria will be discussed in Chapter 2, including their phosphatase activities in culture as affected by P_i concentration; determination of which ones possess genes which hybridize with the *phoA* gene of *E. coli*; and temperature and pH conditions required by four of them for optimal phosphatase activity. The growth of five of the bacteria in autoclaved soil will be addressed in Chapter III, including resulting changes in soil phosphatase activities, readily available P, recently-fixed P, and biomass P of the soil samples.

CHAPTER 1

LITERATURE REVIEW

PHOSPHORUS MOBILITY IN SOILS

Phosphorus in soil solution

All of the soil macronutrients except for P commonly occur at concentrations of 0.1 to 1.0 m*M* in soil solution (Ozanne, 1980; Marschner, 1986), whereas P is frequently present at 0.0016 m*M* and rarely above 0.0096 m*M* except in heavily fertilized soils (Fried and Shapiro, 1961; Barber et al., 1963; Tisdale et al., 1985). Commonly, therefore, only 1/1500 to 1/2000 of soil P is in solution at any time (Fried and Brosehart, 1967). In addition, the diffusion coefficient for P in soil solution is less than 10^{-2} that in water (Olsen et al., 1962), so that even under optimum conditions, P diffuses slowly in the soil. Under adverse conditions such as low soil moisture and low temperatures early in the growing season, P diffusion is negligible (Tisdale et al., 1985); in general, therefore, P is nearly immobile in the soil in the absence of mass flow (Fried and Brosehart, 1967), and only about 1% of the P requirement of plants is met by mass flow (Tisdale et al., 1985).

Fixation of P by soil inorganic constituents

The mobility of P in soil is also hampered by the chemical characteristics of P itself. Unlike other anions such as SO_4^{2-} and NO_3^-, P interacts strongly with surface-active oxihydrates and sesquioxides of clay minerals (Marschner, 1986) and is rapidly fixed by reactions with these and other inorganic soil constituents when it is added to the soil (Sample et al., 1980; Brady, 1984). When thus immobilized, the P in some of the newly formed compounds remains relatively available; over time, however, these compounds change to more insoluble ones (Sample et al., 1980) and become inaccessible to plants.

Fixation and precipitation of soil P by inorganic minerals is influenced by a variety of factors, including pH (Brady, 1984; Tisdale et al., 1985); presence of soluble Fe, Al, and Mn (Brady, 1984); presence of anions or divalent cations (Sanchez and Uehara, 1980; Brady, 1984; Tisdale, et al., 1985); reaction kinetics;

saturation of sorption complexes (Tisdale et al., 1985); presence of amorphous colloids (Sanchez and Uehara, 1980; Tisdale et al., 1985); clay content and type (Wild, 1950; Sanchez and Uehara, 1980; Tisdale et al., 1985); and presence of $CaCO_3$ (Barrow, 1980; Tisdale et al., 1985); and hydrous Al- and Fe oxides (Barrow, 1980; Sample et al., 1980). A close positive linear correlation has been found to exist between the amounts of P sorbed by soils and their acetic acid-soluble Al and oxalate-soluble Fe contents (Ozanne, 1980).

Immobilization of P is generally greatest in acid soils which are high in P-fixing, finely divided sesquioxides; it is less frequently a problem in calcareous soils (Sanchez and Uehara, 1980). At pH values below 5.5, although P exists in the $H_2PO_4^-$ form considered most beneficial to plants, it is adsorbed by sesquioxides and bound in insoluble precipitates of Al-, Fe-, and Mn phosphates, which are more soluble at higher pH values (Brady, 1984). Availability of P has been found to be greatest at pH 5.5 to 7.0 (Englestad and Terman, 1980); above pH 7.0, adsorption by $CaCO_3$ (Barrow, 1980; Tisdale et al., 1985) or precipitation of Ca phosphates (Brady, 1984) may occur, contributing to the low P availability often found in alkaline soils (Marschner, 1986).

The presence of cations and anions may tend either to increase or decrease P sorption by soils. At pH 6.5 or less, sorption of P onto positively-charged edges of clays is enhanced by divalent cations, which make the edges more accessible to P than do monovalent cations (Tisdale et al., 1985). Sorption of P is reduced by competition for sorption sites by inorganic anions, particularly OH^-, SO_4^{2-}, MoO_4^{2-}, and SiO_4^{2-} (Tisdale et al., 1985), and by organic anions, such as citrate, capable of forming stable complexes with the Fe and Al of soil (Sample et al., 1980; Sanchez and Uehara, 1980; Brady, 1984; Tisdale et al., 1985). Additions of manure, for example, apparently release P already fixed as Al-, Ca-, or Fe phosphates due to the organic anions in the manure (Brady, 1984). Simple organic acids, however, were found by Appelt et al. (1975) to have no effect on P adsorption by volcanic ash-derived soils, which adsorbed P preferentially over organic acids. Availability of P also increases as the sorption

complexes become saturated and P is less tightly held by them (Tisdale et al., 1985).

The types and amounts of clays in the soil influence P sorption. Because of the large surface area:volume ratio of clay particles, soils with high clay content tend to adsorb more P than do those with less clay (Tisdale et al., 1985). Aluminosilicate clays and hydrous Fe- and Al- oxides appear to sorb P by the same mechanisms (Sample et al., 1980), although the aluminosilicates adsorb smaller amounts of P (Sanchez and Uehara, 1980). Amorphous colloids in soil adsorb high amounts of P due to their large surface areas (Tisdale et al., 1985). These colloids are prevalent in soils formed in volcanic ash (Sanchez and Uehara, 1980). Soils with high P-fixing potential are therefore frequently found in the suborder Andepts, which contain high levels of amorphous colloids, and in the orders Oxisols and Ultisols, which contain high percentages of Fe- and Al oxides (Sanchez and Uehara, 1980).

Immobilization of P by soil organic components

Although much of the P in soil is immobilized in inorganic forms, in many cases an even greater portion appears in the organic fraction. Soil organic P usually makes up 20-80% of total soil P (Brady, 1984). Of the P in soil solution, 20-70% may exist in organically bound forms (Speir and Ross, 1978; Marschner, 1986), further reducing P availability to plants, which appear to take up only P_i (Speir and Ross, 1978; Ozanne, 1980).

Organic P compounds appear in the soil as they are released from dead organisms (Anderson, 1980). The exact nature of most of these complex compounds is unknown (Speir and Ross, 1978); approximately 2/3 of them have yet to be identified (Brady, 1984; Tisdale et al., 1985). Most naturally occurring forms of soil P_o are esters of orthophosphoric acid, although phosphonic acid derivatives, containing C-P bonds and produced by rumen protozoa, have been found in some soils as well (Anderson, 1980). As with soil P_o in general, the nature of the P esters in soils is largely unknown, and nearly 40% of these

compounds remain to be identified (Anderson, 1980). Numerous mono- and di-esters of orthophosphoric acid have been identified, however (Cheshire and Anderson, 1975; Halstead and McKercher, 1975). The primary esters from low-molecular-weight soil extracts are phytin (myo-inositol hexaphosphate), nucleotides, phospholipids, and glucose-1-phosphate. By far, inositol phosphate makes up the largest portion of these esters. It has been estimated that 35% (Tisdale et al., 1985) to 60% (Halstead and McKercher, 1975) of the soil P_o, and approximately 25% of total soil P (Anderson, 1980) exist in this form. Inositol phosphate is released slowly into the soil during decomposition of plant materials, and quickly stabilizes by complexing with proteins or metal ions, or by forming insoluble salts. This renders it more resistant to hydrolysis than some of the more soluble ester salts (Tisdale et al., 1985). Nucleic acids are thought to be released quickly but break down rapidly in the soil, and extracellular RNA or DNA have not been isolated from soil. It has been estimated that 0.2-1.8% of total soil P_o originates as RNA and up to 2.4% as DNA (Anderson, 1980). Nucleotides, apparently released from humic-bound nucleic acids, may contain up to 2.4% of soil organic P (Halstead and McKercher, 1975). The phospholipids isolated from soil have been primarily phosphatidylcholine (lecithin) and phosphatidylethanolamine (Anderson, 1975). The phospholipids are released rapidly into soils (Tisdale et al., 1985), and may contain up to 1% (Halstead and McKercher, 1975; Tisdale et al., 1985) or 5% (Anderson, 1980) of soil P_o. A small amount of glucose-1-phosphate is present in soil (Anderson, 1975), and traces of other sugar phosphates and glycerophosphate have also been identified (Cheshire and Anderson, 1975). The remaining esters are thought to be mainly microbial in origin, with bacterial cell walls contributing heavily (Anderson, 1980).

Although some esters are quickly broken down in soil (Anderson, 1980), soil P_o is sometimes stabilized by formation of salts and by adsorption with soil colloids. Iron- and Al salts of inositol hexaphosphate, for example, are very stable in acid soils and resist hydrolysis by the enzyme phytase. In addition,

they are strongly adsorbed by clay minerals, which renders them even more resistant to enzymatic attack. Inositol phosphate is probably stabilized in calcareous soils by the formation of calcium salts (Anderson, 1975; Brady, 1984). Because they appear to be stabilized by sorption on soil colloids, P esters are found predominantly in the clay or silt fractions, and clay-rich soils tend to have higher P_o than do sandy soils. Nucleic acids, for example, are strongly adsorbed by clays, especially montmorillonite, causing P from this source to become unavailable, particularly in acid conditions. The nature of the clay minerals themselves is also important; allophane and amorphous Fe- and Al hydroxides tend to be associated with higher P_o levels than do the alumino-silicate clays (Anderson, 1980).

Soil P_o has been described as a reservoir of P that varies with changing conditions (Anderson, 1980; Brady, 1984). Some factors contributing to increases in P_o have already been discussed. Liming of acid soil decreases soil P_o; this decrease has been ascribed partly to increased solubility of P_o compounds (Anderson, 1975) and to mineralization of these substances by increased microbial action at a more favorable pH (Anderson, 1975; Tisdale et al., 1985). Decreases in P_o content of soils are related to increases in dilute acid-extractable inorganic P, supporting the idea that mineralization of P_o occurs (Tisdale et al., 1985). The mineralization of soil P_o is intimately associated with mineralization of organic matter as a whole (Speir and Ross, 1978). In order for mineralization of P to occur, however, soil organic materials must contain at least 0.2% P; at lower levels net immobilization of P occurs (Kaila, 1949; Anderson, 1975; Tisdale et al., 1985). Attempts have been made to determine mechanisms responsible for mineralization of soil P_o. In laboratory studies the growth of plant roots in soil causes reduction in P_o content, but addition of root phytase to soil without plants causes no reduction (Jackman and Black, 1952a). Thompson and Black (1970) demonstrated that the addition of plant phytase or phosphatase preparations to soil increases soil P_o. Incubation of soil in the absence of plants, however, results in mineralization of native P_o (Thompson and

Black, 1947). These findings indicate that the presence of plant roots and/or soil microflora results in mineralization of soil P_o, whereas phosphatase preparations alone serve as substrates for the microflora and result in immobilization of P as P_o.

SOIL PHOSPHATASE

Phosphatases are thought to play a major role in the mineralization of soil P_o through catalysis of hydrolytic cleavage of ester phosphate bonds (Speir and Ross, 1978; Tisdale et al., 1985). An understanding of their functions in this process is of major agricultural importance; however, many of the fundamental questions about the origins, locations, and persistence of these enzymes are still unanswered (Tabatabai, 1982).

Sources and types of soil phosphatases

It is generally accepted that soil enzymes have microbial, plant, or animal origins (Ladd, 1978). Phosphatases originate from plant roots (Speir and Ross, 1978), soil fungi (Casida, 1959; Bezborodova and Il'ina, 1970), bacteria (Mazilkin and Kuznetsova, 1964; Cosgrove et al., 1970), earthworms, yeasts (Hollander, 1971), and protozoa (Gould et al., 1979). Of the soil organisms that produce phosphatases, microorganisms are believed to contribute the largest amounts because of their high biomass in the soil and their short lifetimes (Ladd, 1978). Extracellular phosphatase activity in cultures of soil bacteria was reported by Mazilkin and Kuznetsova (1964) and by Cosgrove et al. (1970). A soil fungus, *Fusarium* sp., produces extracellular phosphatase activity (Bezborodova and Il'ina, 1970). Soil microorganisms in the rhizoplane and rhizosphere also stimulate root phosphatase (Estermann and McLaren, 1961; Gould et al., 1979; Tarafdar and Jungk, 1987).

In many cases, different types of organisms appear to produce only certain phosphatases. Plant roots produce phosphoric monoester hydrolases (EC

3.1.3), such as phytase, nucleotidases, sugar phosphatases, and glycerophosphatase; and phosphoric diester hydrolases (EC 3.1.4), including nucleases and phospholipases (Speir and Ross, 1978). All of these enzymes are acid phosphatases (Estermann and McLaren, 1961; Gould et al., 1979). With one exception (Bieleski, 1974), plant roots are not known to produce alkaline phosphatase (Estermann and McLaren, 1961). Both acid and alkaline phosphatases originate from earthworms, bacteria, fungi and yeasts (Casida, 1959; Hollander, 1971). Microbial respiration in soil is related significantly to soil alkaline phosphatase activity, but not with acid phosphatase activity (Frankenberger and Dick, 1983). Tabatabai (1982) concluded that the alkaline phosphatase activity in soils appears to be totally derived from microorganisms. Casida (1959) demonstrated that some *Aspergillus* species secrete particularly active phosphatases, including an acid phytase which catalyzes the partial dephosphorylation of ferric phytate, a compound previously considered inert in soil.

To date, only a few enzymes have been extracted from the soil (Ladd, 1978; Tabatabai, 1982). Because the soil environment differs radically from that of laboratory cultures, the enzyme activities of isolated soil microorganisms in culture may have little relationship to their relative activities in soil (Ladd, 1978). For these reasons, little is known about specific microorganisms' contributions to soil enzyme pools.

Assays of soil phosphatase activity

In 1927, Parker first suggested that enzymes of plant origin might catalyze decomposition of some organic P compounds in soil (Parker, 1927). Rogers (1942) first demonstrated phosphatase activity of soils by incubating calcium glycerophosphate and nucleic acid solutions separately with soil samples and toluene and noting that both substances were extensively hydrolyzed after 18 h incubation. Because the pH and temperature optima of the nuclease activity were similar to those of plant root nuclease, a plant origin was proposed for this activity.

The study of soil enzymes is difficult because soil is a heterogeneous organo-mineral complex which sorbs a variety of substances. In addition, soil is home to a diverse array of organisms (Speir and Ross, 1978). Because soil enzymes are difficult to extract and purify (Ladd, 1978), indirect procedures that measure enzymatic activity have been the primary means to evaluate enzyme status of soils. Progress in understanding the nature, origin, and role of soil phosphatase has been impeded by these constraints.

Initially, indirect phosphatase assays in soil involved measuring P_i released by the enzymes (Rogers, 1942; Jackman and Black, 1952a, b). With these methods, sorption of P_i by soils resulted in results of varying accuracy. In the mid-1950s, the use of artificial substrates for phosphatase assays was initiated. An artificial substrate is one which is "neither identified as nor even suspected of being a compound of soil organic phosphate" (Speir and Ross, 1978). These compounds have the advantage of being low molecular weight esters that are hydrolyzed rapidly compared with natural esters; in addition, they have an organic moiety which is easily and accurately quantifiable (Ladd, 1978; Speir and Ross, 1978). The degree to which these artificial substrates mimic the naturally occuring soil substrate is open to question, however (Speir and Ross, 1978). Commonly used artificial substrates include phenyl phosphate (Halstead, 1964), phenolphthalein phosphate (Speir and Ross, 1978); β-naphthyl phosphate (Ramirez-Martinez, 1966); and *p*-nitrophenyl phosphate, bis-*p*-nitrophenyl phosphate, and tris-*p*-nitrophenyl phosphate (Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977).

Soil sterilants

Because it has not been possible, in practice, to separate soil abiontic enzymes from those associated with living organisms (Ladd, 1978; Tabatabai, 1982), assays of abiontic enzyme activity are commonly performed using a soil sterilant to suppress or inhibit cellular enzyme activity. According to Tabatabai 0(1982),

> An ideal sterilization agent for extracellular enzyme detection in soil would be one that would completely inhibit all microbial activities but not lyse the cells and not affect the extracellular enzymes. Unfortunately, however, no such agent is yet available.

Toluene has become the most commonly used microbial inhibitor in soil phosphatase assays (Tabatabai, 1982). It halts synthesis of enzymes by living cells (Tabatabai, 1982) and prevents assimilation of reaction products (Drobnik, 1961). Toluene also increases the permeability of the cell membrane, allowing the protein-motive force, required for ATP generation, to dissipate (Burton, 1989); this increased permeability allows release of intracellular enzymes (Drobnik, 1961). *E. coli* cells are killed, but not lysed, by 5% toluene, and lose up to 25% of their intracellular proteins to the medium (Jackson and deMoss, 1965). Gram-positive bacteria and *Streptomyces* are more resistant than Gram-negative bacteria to the action of toluene (Beck and Poschenrieder, 1963).

Addition of toluene to soil incubation mixtures during assays does not significantly affect results for acid- or alkaline phosphatase or for phosphodiesterase when short incubation times are used (Eivazi and Tabatabai, 1977). In assay procedures requiring more than a few hours' incubation, however, toluene is not useful (Tabatabai, 1982). Kaplan and Hartenstein (1979) found that 6 of 14 tested bacteria were able to grow using toluene as the only C source, and Thompson and Black (1947) demonstrated that, at temperatures less than 50° C, toluene-treated soils appear to release more P_i than do non-treated soils after 7 d incubation.

The effect of toluene on soil bacteria depends on treatment of the soil during assay. In moist soils, at least 20% toluene is necessary to completely inhibit microbial activity, whereas 5 - 10% is sufficient in soil suspensions (Beck and Poschenrieder, 1963).

Alternatives to toluene sterilization have been proposed. At the minimum sterilizing dose, ionizing radiation decreases soil phosphatase activity by 10% (Speir and Ross, 1978). The dose required to kill or inactivate organisms varies, however, with the soil type and moisture content and the genus of organism

(Tabatabai, 1982), and irradiation is an expensive procedure (Speir and Ross, 1978). Ethanol has also been suggested as a more suitable sterilant for phosphatase assays because it has no reported plasmolytic characteristics (Frankenberger and Johanson, 1986). At this time, however, toluene remains the customary choice.

Effects of soil treatment on assay results

The treatment of soil samples prior to enzyme assays affects assay results (Ladd, 1978; Tabatabai, 1982). Although the effects of air-drying vary among soils, phosphatase activity generally decreases by as much as 50% upon drying, then remains fairly stable, even after several years of storage (Halstead, 1964). Eivazi and Tabatabai (1977) showed that air-drying increases the activity of acid phosphatase and phosphotriesterase, decreases that of alkaline phosphatase, and does not affect the activity of phosphodiesterase. Autoclaving of soil decreases or inactivates phosphatase activity (Kishk et al., 1976), with losses of 80 - 100% of activity after 1 h autoclaving (Speir and Ross, 1978). Eivazi and Tabatabai (1977) demonstrated total inactivation of alkaline phosphatase, phosphodiesterase, and phosphotriesterase when air dry soil was autoclaved 1 h; however, 1.7 - 6.7% of the original acid phosphatase activity remained.

Characteristics of soil phosphatase activity

Most of soil phosphatase studies have focused on phosphomonoesterases, primarily acid phosphatase (EC 3.1.3.2). Alkaline phosphatase (EC 3.1.3.1), phosphodiesterase (EC 3.1.4), and phosphotriesterase are less studied, but their activities have been evaluated in soils (Eivazi and Tabatabai, 1977).

Phosphatase assays of soils using artificial substrates have shown one or two pH optima, usually at pH 4 - 6 and 8 - 10 (Halstead, 1964; Hofmann and Hoffmann, 1966; Ramirez-Martinez, 1966; Eivazi and Tabatabai, 1977). In some cases, a "neutral" phosphatase with pH optimum near 7 is apparent (Halstead, 1964; Ramirez-Martinez, 1966). This optimal activity often spans

several pH units and possibly represents a mixture of acid and alkaline phosphatases (Speir and Ross, 1978). Phosphodiesterase and phosphotriesterase activities in soil have pH optima near 10 (Eivazi and Tabatabai, 1977). In the experiments of Eivazi and Tabatabai (1977), most soils had highest phosphatase activities near pH 7, although acid- and alkaline phosphatase activities predominated in acid and alkaline soils, respectively. Similar trends were noted by Juma and Tabatabai, 1977). Speir and Ross (1978), however, concluded that frequently the soil pH may be far removed from the pH optimum of the enzyme.

Fate of phosphatase in soils

Phosphatase activity in untreated soils is a combination of the activity of the soil biota and that of free enzymes which may be present (Tisdale et al., 1985; Ramirez-Martinez, 1966; Tabatabai, 1982). Free enzymes may originate as exoenzymes released from living cells or as endoenzymes from disintegrating cells, and may occur in soil solution or bound to disintegrating cell fragments or to dead or viable, nonproliferating cells (Ladd, 1978; Speir and Ross, 1978; Tabatabai, 1982). A high proportion of these enzymes is thought to be quickly denatured or degraded by soil protease (Ladd and Butler, 1975; Tabatabai, 1982).

Some of the phosphatases, however, appear to persist and become stabilized in the soil as abiontic enzymes (Burns, 1982), which are resistant to degradation but retain their catalytic activity (Ladd and Butler, 1975; Burns, 1982). This stabilization may occur by ionic- or hydrogen-bonding of the enzyme to existing humic materials in the soil or by covalent linkages between the enzyme and humic materials during formation of organic polymers (Ladd and Butler, 1975). Enzymes are also thought to become stabilized within the mineral component of soil. Ensminger and Gieseking (1942) explored this possibility in experiments in which they combined protein suspensions with either base-exchange clays or with kaolinite. Upon treating these mixtures with proteolytic enzymes, they found that the base-exchange clays interfered with enzymatic hydrolysis of the proteins, but that kaolinite had no such effect.

Smectite clays may protect proteins either by allowing them to move into the interlayer spaces within the clay crystal or by inhibiting the action of proteolytic enzymes (Brady, 1984). Although such protection of enzymes by clay minerals may occur, humus-free clays with enzyme activities have not been isolated from soil (McLaren, 1978). The stabilizing reactions of enzymes with soil colloids appear to decrease the activity of the enzymes (Ladd, 1978).

Factors influencing soil phosphatase activity

A variety of factors influence phosphatase activity of soils, including season of sampling (Ladd, 1978); cropping history and presence of decaying vegetation (Ladd, 1978; Tabatabai, 1982); soil amendments, agricultural chemicals, and pollutants (Tabatabai, 1982).

Fertilization effects on phosphatase activity vary, depending on the type and amount of fertilizer applied. In a study of 544 derno-podzolic soils, Chunderova and Zubets (1969) reported that phosphatase activity rose as soluble P increased up to 0.1 mg P_i g^{-1}. At higher P concentrations, activity declined, disappearing completely at 0.3 - 0.4 mg P_i g^{-1}. More recent studies have confirmed that both acid and alkaline phosphatases are competitively inhibited by P_i (Juma and Tabatabai, 1978; Speir and Ross, 1978). Low levels of P_i appear to stimulate phosphatase activity due to increased microbial numbers and plant growth, which result in the buildup of enzyme levels and soil organic matter over time. High P_i levels in soil solution, however, inhibit existing phosphatase activity and repress synthesis of bacterial phosphatases (Adebayo, 1973; Feder, 1973). Additions of organic P sources, such as composts and manures stimulate soil phosphatase activity (Halstead and Snowden, 1968). Fertilizers lacking P usually increase soil phosphatase activity (Levchenko and Lisoval, 1974), as do applications of fertilizers in general; however the increased activity is the indirect effect of plant and microbe growth and the production of new phosphatase, rather than activation of phosphatase originally in the soil (Speir and Ross, 1978).

In addition to P_i, a number of substances inhibit soil phosphatase activity, including fluoride in some soils (Halstead, 1964), vanadium (Tyler, 1976b), copper, zinc (Tyler, 1974, 1976a), and mercury in high concentrations (Speir and Ross, 1978). Juma and Tabatabai (1977) reported that all heavy metals and trace elements inhibit phsophomonoesterases in soils to at least some degree, and Halstead (1964) found that addition of lime to soil inhibits phosphatase activity.

Bacterial influences on P availability

The possible use of bacteria with the ability to release available P from soil organic matter to enhance P_i availability to plants has been explored for several decades. Before the Russian revolution, R. A. Menkina inoculated wheat seed with phosphobacterin, a culture of *Bacillus megaterium* var. *phosphaticum*, to enhance crop yields (Mishustin, 1963). Phosphobacterin, along with *B. adhaerens*, *B. cereus*, *B. mesentericus*, *B. subtilis*, and *Pseudomonas*, was shown to have high phosphatase activity (Mazilkin and Kuznetsova, 1964). Phosphobacterin was at first thought to increase crop growth and yield through mobilization of P (Mazilkin and Kuznetsova, 1964), but effects appeared inconsistent, and seemed to depend on soil, pH, amount of organic matter, and type of crop (Mishustin, 1963; Tisdale et al., 1985). Reports of results of phosphobacterin inoculation of seed within the Soviet Union varied greatly, possibly because some researchers omitted negative results (Samstevich, 1962). United States Department of Agriculture investigations concluded that, although phosphobacterin readily decomposed glycerophosphates, it did not influence favorably either P concentration or total P uptake by plants (Tisdale et al., 1985). Greenhouse studies of inoculated tomatoes showed yield increases of 7.5%, but wheat yield was not affected (Tisdale et al., 1985; Jay Hamilton Smith, personal communication).

Today, bacterial inoculants such as phosphobacterin are thought to promote crop yields by means of biologically active substances, including growth hormones, which they produce (Samtsevich, 1962; Berezova, 1963; Mishustin,

1963); they may also alter rhizosphere microflora, influencing plants indirectly (Berezova, 1963). Within the past ten years, studies of rhizosphere bacteria have concluded that some bacterial inoculants promote crop growth, either through disease suppression (Weller and Cook, 1986; Xu and Gross, 1986) or by supplying nutrients and growth factors to host plants (Davidson, 1988). Although soil microorganisms undoubtedly mineralize organic P, at this time there is no clear evidence that their activities directly benefit higher plants (Tinker, 1980).

PERIPLASMIC BACTERIAL PHOSPHATASES

The bacterial phosphatases considered in this study are those which have an impact on the surrounding medium and which can be detected when living, intact cells are presented with the synthetic substrate *p*-nitrophenyl phosphate (PNPP). As the following discussion will show, it is reasonable to assume that most of the phosphatases of *Escherichia coli* are periplasmic. The locations of phosphatases of other bacteria included in this study are largely unknown, but they may be in the periplasmic space or associated in some way with the outer membrane, because they act on the surrounding medium but are apparently not secreted into it in large quantities (Torriani, 1960).

Periplasmic enzymes have been defined by Beacham (1979) as enzymes which exist "between the inner and outer membranes, whose association with either of these membranes, if any, is so tenuous that they are released during spheroplasting." The periplasmic space in Gram-negative bacteria consists of the region between the cytoplasmic membrane and the outer envelope, and comprises 20-40% of the total cell volume (Beacham, 1979). The development of this space may have been an adaptation to dilute aquatic environments. Essential enzymes which otherwise may have been secreted into the growth medium, as occurs with Gram-positive bacteria (Costerton et al., 1974), are

localized in the periplasmic space. The periplasmic space also offers bacteria a means of isolating enzymes that would be toxic if retained in the cytoplasm.

In *E. coli*, 30 to 40 different periplasmic proteins have been identified, most of which are degradative enzymes, enabling bacteria to scavenge for nutrients found in low concentrations in the medium. The products of these enzymes can move across the cytoplasmic membrane, although their substrates cannot. The substrates are able to penetrate into the periplasmic space through hydrophilic channels in the outer membrane (Beacham, 1979).

Studies have indicated that many acid and alkaline phosphatases of *E. coli* are periplasmic in nature. Only 10-15% of the cells' phosphatase activity was demonstrated in cell-free supernatants, although the substrate in the surrounding medium appeared to be readily accessible to the enzymes. Intact and disrupted cells produced equivalent enzyme activities (Torriani, 1960), indicating that no significant contribution was made by intracellular phosphatases. Acid phosphatase and phosphodiesterase were obtained in solution by Neu and Heppel (1965) when they formed spheroplasts by treating *E. coli* cells with lysozyme and EDTA to remove the outer membrane. Most of the inducible alkaline phosphatase was released into solution as well, suggesting that these enzymes are found in the periplasmic space.

E. coli alkaline phosphatase

Bacterial alkaline phosphatase (BAP)(EC 3.1.3.1) was first purified and characterized by Garen and Levinthal (1960). Its mode of action and genetic control have been studied in greater depth than have those of other *E. coli* phosphatases. BAP is a nonspecific phosphomonoesterase that allows bacteria to utilize organic phosphates as sole sources of P. It is a stable, globular, dimeric metalloprotein, MW 86 000 daltons, composed of two chemically identical subunits, each made up of 449 amino acid residues. Each monomer contains an active center with three distinct metal binding sites and one phosphorylatable serine. A fourth metal site is found 25 Å from the active center, giving at least

six significant metal sites per dimer. Heterogeneous and nonsymmetrical distributions of metals among these sites appear to be the rule, resulting from differing solvent compositions and histories of specific samples. Under normal laboratory conditions of growth, however, BAP likely contains 4 Zn^{2+}, 2 Mg^{2+}, and 1 or 2 P per dimer. The release of P_i from the molecule is the rate-limiting step of the dephosphorylation reaction (Schlesinger et al., 1969; Wyckoff et al., 1983).

BAP has been shown to be inactive below pH 6.0 when acting on PNPP as a substrate (Garen and Levinthal, 1960; von Hofsten, 1961). Disagreement exists, however, regarding its optimum pH, which has been reported as 8.0 (Garen and Levinthal, 1960), 8.4 (Lazzaroni et al., 1985) and 8.8-9.8 (Torriani, 1960).

BAP is inhibited by P_i (Torriani, 1960; Schlesinger et al., 1969). Unlike some other bacterial phosphatases, its synthesis is dependent on the concentration of P_i in the environment (von Hofsten, 1961). Formation of BAP is repressed in exponentially growing cultures and is derepressed as the medium becomes depleted of P_i (Schlesinger et al., 1969). In 0.1 *M* P_i, as much as 70% of BAP activity is repressed. When the P_i concentration of the medium approaches 0.01 m*M*, the growth rate decreases to 0.2 times the original and BAP synthesis begins (Torriani, 1960; Lazzaroni et al., 1985). When cells are stressed by low P_i, BAP synthesis is increased more than 1000-fold; as much as 6% of the cellular protein produced may be BAP. Because BAP can hydrolyze a wide range of phosphomonoesters as well as pyrophosphate bonds, *E. coli* is able to grow on a number of substrates under alkaline conditions (Horiuchi et al., 1959; Kier et al., 1977; Torriani, 1960; Wanner, 1986).

Although P_i concentration has a dominating influence on BAP activity, the availability of other minerals and carbon (C) also has effects. Absence of Zn^{2+} in the medium results in complete inactivation of BAP (Schlesinger et al., 1969). Inhibition of all BAP activity has also been noted by Hafckenscheid (1968) when 0.6% glycerol was added to the inorganic salts + peptone medium

of Dvorak et al. (1967). These studies illustrate the complexity of phosphatase regulation in *E. coli* and possibly in other species.

The synthesis and transport of BAP into the periplasmic space have been studied and described. It has been shown that alkaline phosphatase subunits exist in the cytoplasm attached to polyribosomes. The folded subunits, consisting of polypeptide chains with amino-terminal peptide signal sequences, pass through the cell membrane into the periplasmic space (Schlesinger et al., 1969). The signal peptide, essential for export, is removed by an envelope protease (Yoda et al., 1980; Michaelis et al., 1986). In the periplasmic space, the subunits unfold and join to form inactive dimers, which are then activated by the addition of Zn^{+2} and Mg^{+2}. It is theorized that the structure of the polypeptide chain allows it to move into the periplasmic space and that no specific carriers are needed for transport (Ghosh et al., 1986).

BAP is but one of a series of gene products of 20 to 30 genes which comprise the *pho* regulon. This entire group of genes is induced by the product of the *phoB* gene and is repressed by P_i in the medium (Fig. 1.1) (Rao et al., 1986). The *phoS* and *pst* (phosphate-specific transport) genes have also been considered parts of the *pho* regulon (Amemura et al., 1982). The *phoR* product appears to have a negative function when it binds to a cofactor Y, which is synthesized under the control of *phoU* in the presence of P_i, and represses the transcription of *phoB*. If Y is not present, as in P_i starvation, the *phoR* product, PhoR-X, an alternative form of the *phoR* product which cannot repress *phoB*, induces transcription of the *pho* regulon (Garen and Echols, 1962; Tomassen et al., 1982; Wanner and Latterell, 1980; Rao et al., 1986). The *phoM* operon has been found to play both a positive and a negative regulatory role in the expression of *phoB* (Wanner and Latterell, 1980; Wanner, 1987; Wanner et al., 1988).

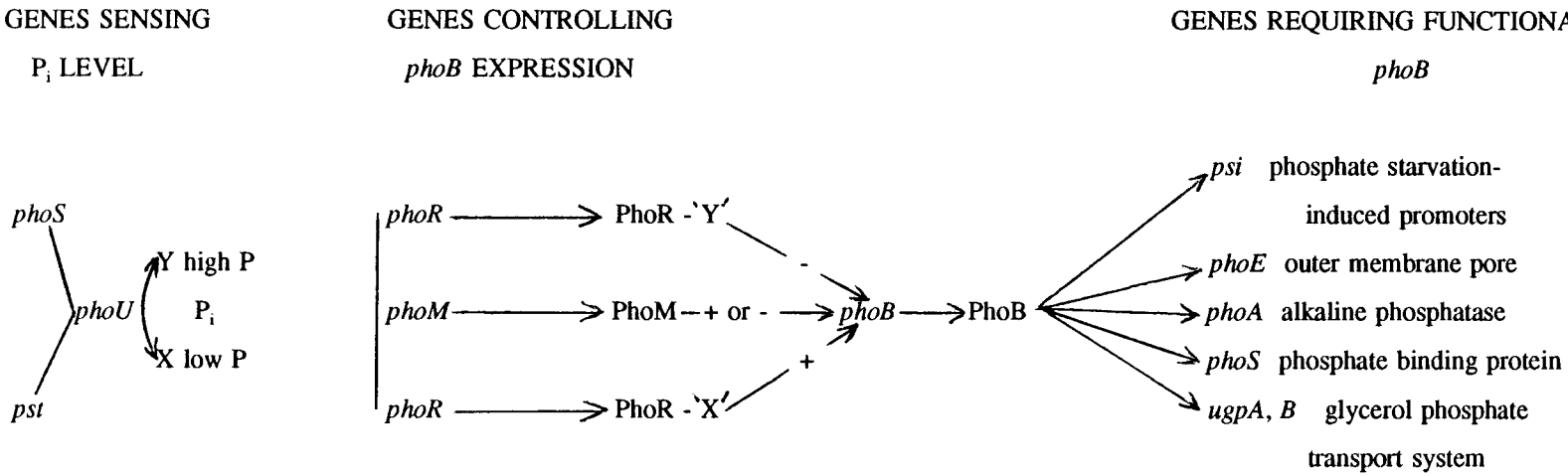

Fig. 1.1. Scheme of regulation of the phosphate (*pho*) regulon. Genes of the *pho* regulon (e.g. *phoA*) are derepressed by P_i limitation. Their expression is positively regulated by *phoB, phoM,* and *phoR,* and negatively by *phoR, phoS, pst,* and *phoU.* The *phoS* and *pst* genes are involved in the transport of P_i. They may regulate the *pho* regulon by influencing the level of a hypothetical effector(s) through the mediation of *phoU.* PhoR (the product of *phoR*) presumably binds to a nucleotide cofactor X to form the inducer PhoR-X or to Y to form the repressor PhoR-Y, both of which influence the expression of *phoB.* In the absence of PhoR, the *phoM* gene product activates the expression of *phoB*, independent of P_i levels. High levels of PhoB turn on the expression of genes like *phoE, phoA,* and *phoS.* The product of the *phoU* gene may change X to Y directly, or it may regulate the gene encoding this function (adapted from Rao et al., 1986).

The gene *phoA* is the structural gene for BAP. Constitutive ($PhoA^+$) mutants, which produce PhoA (BAP) in the presence of high P_i, include those of *phoR, phoS,* and *pst* genes. Uninducible mutants result from mutations in such genes as *phoB*, which cause blockage of the induction of the *phoA* gene product (Schlesinger et al, 1969; Brickman and Beckwith, 1975; Wanner and Latterell, 1980).

E. coli acid phosphatases

Unlike BAP, the acid phosphatases of *E. coli* have not been clearly characterized, and some remain poorly understood. A tentative list of *E. coli* phosphatases which are periplasmic in location and have been shown to hydrolyze PNPP appears in Table 1.1. Not all of these phosphatases are phosphomonoesterases. Acid phosphoanhydride phosphohydrolase hydrolyzes primarily inorganic phosphates with many phosphoanhydride bonds; PNPP appears to be the only phosphomonoester which it attacks readily (Dassa and Boquet, 1981; Dassa et al., 1982).

After cloning *E. coli* genes for different acid phosphatases with phasmid Mu dII4042, Pradel and Boquet (1988) isolated five different genes, including *agp*, distinguishable by pH optima, enzyme substrate preference and restriction enzyme pattern. They concluded that acid hexose phosphatase (AHP) and nonspecific acid phosphatase may correspond to several enzyme species. It is the overlap in pH optima and substrate preferences by this group of enzymes, as well as the variability in their activity as culture conditions change, that has resulted in conflicting classification schemes. Uridine 5'-diphosphate glucose hydrolase (EC 3.1.3.5) has demonstrated a broad optimum pH range (6.0 - 7.8), which is determined by the substrate present. In addition, it has been suggested that cyclic phosphodiesterase may have one site which cleaves phosphomonoesters and another which hydrolyzes 2',3'-cyclic phosphate linkages, and that this enzyme is responsible for most of the PNPP hydrolysis that occurs when BAP is suppressed (Dvorak et al., 1967).

Table 1.1. Periplasmic phosphatases of *E. coli* which use PNPP as substrate.

pH	Enzyme	Molecular Weight	Structural gene	*E. coli map site*	Major substrates	References
		Kdaltons				
2.5	Acid phosphoanhydride phosphohydrolase (EC 3.6.1.11)	45 (Monomer)	*appA*	22.5 min.	Inorganic polyphosphates; guanine nucleoside polyphosphates	Dassa et al., 1980; Dassa and Boquet, 1981; Dassa et al., 1982; Dassa and Boquet, 1985
2.5	Orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)				Fructose-1,6-diphosphate; 2,3-diphosphoglyceric acid	Hafckenscheid, 1968
4.0	Acid hexose phosphatase (AHP) (EC 3.1.3)	13 - 20 (Monomer)			Hexose- and phenyl monophosphate esters	von Hofsten, 1961; von Hofsten and Porath, 1962
4 - 5	Acid glucose-1-phosphatase (EC 3.1.3)	88 (Dimer)	*agp*		glucose-1-phosphate	Pradel and Boquet, 1988
6.0	2',3'-cyclic phosphodiesterase (EC 3.1.4.16)		*cpdB*	96 min.	Ribonucleoside-2'-3'cyclic phosphates	Rogers and Reithel, 1960; Anraku, 1964a, b; Dvorak et al., 1967; Hafckenscheid, 1968; Beacham and Garrett, 1980; Pradel and Boquet, 1988

(Continued)

Table 1.1., Continued.

pH	Enzyme	Molecular weight	Structural gene	*E. coli* map site	Major substrates	References
6.0-7.8	Uridine 5'-diphosphate glucose hydrolase (5'-nucleotidase) (EC 3.1.3.5)		*ush*	11 min.	Uridine-5'-diphosphate	Neu and Heppel, 1965; Beacham and Yagil, 1976; Kier et al., 1977
8.8	Orthophosphoric monoester phosphohydrolase (Bacterial alkaline phosphatase, BAP) (EC 3.1.3.1)	75 - 80 (Dimer)	*phoA*	8.5 min.	Phosphomonoesters, pyrophosphate bonds	Garen and Levinthal, 1960; Plocke et al., 1962; Wanner and Latterell, 1980; Wyckoff et al., 1983

Whereas BAP is normally produced in the stationary phase and only when P_i becomes limiting, an array of factors appears to affect synthesis of *E. coil* acid phosphatases. The following summary of observations for a few of these enzymes illustrates the variety of influences on their production and activities.

AHP (EC 3.1.3)(pH optimum 4.0). Addition of carbohydrates, especially glucose, slows synthesis of AHP, while simultaneously increasing the growth rate of the cells (von Hofsten, 1961). AHP activity was shown to be highest when the medium was supplemented with casamino acids (Dvorak et al., 1967), potassium succinate and 1 g Difco bacto-peptone L^{-1} (von Hofsten, 1961), or in medium where C is limiting, even in the presence of excess P_i (Torriani, 1960). Cells growing exponentially in glycerol or succinate medium had similar AHP activities, whereas those grown in lactate had activities only 50% as great as those in glycerol. Peptone concentrations greater than 1 g L^{-1}, or complex media such as Difco nutrient broth gave low AHP activities. In addition, cells grown in succinate medium gave higher AHP specific activities in the stationary than in the exponential phase (von Hofsten, 1961). A negative feedback mechanism has been proposed for AHP, whereby intracellular hexose phosphate levels suppress its activity, possibly as much as 100-fold (von Hofsten, 1961; Dvorak et al., 1967).

Acid phosphatase (EC 3.1.3)(pH optimum 4.0). An acid phosphatase with pH optimum 4.0 was found to be inhibited by 85% in the presence of 0.01 *M* NaF and inhibited by up to 60% by excess P_i (0.1 *M* K_2HPO_4) (Torriani, 1960). Relatively low activity was reported for cells grown under partial or complete anaerobiosis (von Hofsten, 1961).

Acid phosphoanhydride phosphohydrolase (EC 3.6.1.11)(pH optimum 2.5). The activity of this phosphatase contrasts in some respects with that of AHP. Like AHP, it appears to be synthesized only in the stationary phase; however, it is not induced by limited C, N or S, but but rather by P_i starvation and anoxia. It is negatively controlled by cyclic AMP, which requires *cya* and

crp gene function (Dassa et al., 1982).

Orthophosphoric monoester phosphohydrolase (EC 3.1.3.2)(pH optimum 2.5). Unlike that of other acid phosphatases, the activity of this enzyme increases in exponentially growing cells in glucose medium. As the culture grows, the pH of the glucose medium drops from 7.0 to 5.2. Orthophosphoric monoester phosphohydrolase synthesis during cell growth may be an adaptation to the increased level of organic acids in the medium (Hafckenscheid, 1968).

Structural genes for four of the acid phosphatases and their locations on the *E. coli* chromosome have been determined (Table 1.1). The control of these structural genes has not been elucidated.

Phosphatases of other bacteria

Relatively little is known about phosphatase production in bacteria other than *E. coli.* Kier et al. (1977) isolated and purified three phosphatases from *Salmonella typhimurium* LT2, a 2',3'-nucleotide phosphodiesterase (EC 3.1.4.d), an acid hexose phosphatase (EC 3.1.3.2), and a nonspecific acid phosphatase (EC 3.1.3.2), all of which are periplasmic. They found no evidence of the BAP or 5'-nucleotidase found in *E. coli.*

Pseudomonas aeruginosa has an alkaline phosphatase system which is induced after growth on proteose peptone as the sole source of P. Alkaline phosphatase activity is repressed by the addition of P_i to the medium, and increases more than 500-fold after P_i starvation. *P. aeruginosa* cells secrete this phosphatase into the surrounding medium during growth. The enzyme may be bound to extracytoplasmic cell components by electrostatic linkages, mediated by Mg^{2+}. The alkaline phosphatase is readily removed by washing cells in 0.1 or 0.2 *M* Mg^{2+} (Cheng et al., 1970; Beacham, 1979), indicating that it is extracellular. The production of extracellular phosphatases by other *Pseudomonas* species is unknown.

A strain of *Erwinia herbicola* solubilizes mineral phosphate. A gene determining the mineral phosphate-solubilizing phenotype has been cloned and

its regulation studied (Goldstein and Liu, 1987). Hydrolysis of PNPP by *Erwinia* spp. has not been reported.

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CHAPTER 2

THE EFFECTS OF PHOSPHATE CONCENTRATION, pH, INCUBATION TEMPERATURE, AND PRESENCE OF THE *phoA* GENE ON PHOSPHATASE ACTIVITIES OF SOIL BACTERIA *IN VITRO*

ABSTRACT

Phosphatase activity in soils is thought to originate primarily from soil microorganisms, and such activity has been demonstrated *in vitro* for some soil bacteria. This enzymatic activity enables the cells to cleave orthophosphate (P_i) from organic P (P_o) compounds, but little is known about the mechanisms regulating expression of such activity, the types of phosphatases produced, or how this activity relates to P_i availability in soil.

This study was conducted to determine the effects of P_i concentration, pH, and temperature on acid- and alkaline phosphatase activities of various groups of soil bacteria and to determine if the observed activity could be related to the presence of *phoA*, the structural gene for alkaline phosphatase of *Escherichia coli*.

Seventeen soil bacteria were cultured in liquid medium containing either 0.02 m*M* P_i (low P_i) or 2.1 m*M* P_i (high P_i). Acid- and alkaline phosphatase activities were measured and compared with those of *E. coli* MPH43, which has a functional *phoA* gene ($PhoA^+$) and *E. coli* MPH69, which is $PhoA^-$. The effects of incubation temperature and pH on phosphatase activity were then examined for *Erwinia carotovora* subsp. *carotovora* W3C105 and *Pseudomonas fluorescens* Pf-5, as well as for the two *E. coli* strains. Southern blot hybridization studies were done using *E. coli phoA* gene to probe restriction enzyme digests of chromosomal DNA from the 17 soil bacteria.

Detectable acid (pH 6.5)- and alkaline (pH 8.0) phosphatase was expressed in low P_i medium by 14 and 12 strains, respectively, and in high P_i medium by 11 and 9 strains, respectively. Phosphatase activity was low for most strains. *E. carotovora* W3C105, however, expressed high alkaline phosphatase activity under low P_i conditions. Most of the pseudomonads and *Flavobacterium* sp. strain MtCa7 expressed higher acid- than alkaline phosphatase activity.

Chromosomal DNA fragments that hybridized with *phoA* were present in *E. carotovora* W3C105, *Enterobacter cloacae* E6, and *E. coli* strains MPH69 (PhoA$^-$) and C600(P1). Of these, only *E. carotovora* W3C105 exhibited more than low levels of alkaline phosphatase activity.

All of the four strains selected for tests of pH and temperature effects expressed lower phosphatase activity, if any, when grown at pH 6.5 than at pH 8.0. When grown at temperatures of 15, 20, 25, and 30° C, three of the strains expressed highest phosphatase activity near 25° C. The fourth strain, *E. coli* MPH69, gave low activities, or none, under all of the pH or temperature conditions.

The results of this study indicate that there is a wide range in phosphatase activity between even closely related soil bacteria, and that an individual strain may exhibit greatly varying activities with differences in incubation temperature, P_i availability, and pH.

The only strains that had genomic regions that hybridized to the *phoA* gene of *E. coli* were members of the family *Enterobacteriaceae.* Hybridization to *phoA*, however, did not insure alkaline phosphatase production under the conditions of this study. In addition, the fact that several strains expressed alkaline phosphatase activity but did not hybridize to the *phoA* gene of *E. coli* suggests that such strains possessed alkaline phosphatase genes distinct from *phoA*.

INTRODUCTION

Soil enzymes, which exist in viable microorganisms, in association with cell fragments, or as free enzymes complexed in clay or organic colloids (Burns, 1982), play important roles in cycling nutrients in soils (Speir and Ross, 1978). Because it is difficult to extract these enzymes from soil, they are usually studied indirectly using assay procedures that involve controlled artificial environmental conditions and artificial substrates (Ladd, 1978; Tabatabai, 1982). As a result, potential, rather than actual, enzyme activities are measured.

Recombinant DNA techniques now make it possible to identify genes that control enzyme activity in microorganisms and to introduce genes coding for production of specific enzymes into such organisms (Old and Primrose, 1989). Studying organisms that are identical except for the production of an enzyme offers potential for understanding the role of the enzyme in microbial ecology and nutrient cycling. In addition, use of DNA probes for a gene that codes for an enzyme might make it possible to quantify the enzyme associated with viable bacteria (Fredrickson et al., 1988; Holben et al., 1988). The *phoA* gene of *Escherichia coli*, which encodes bacterial alkaline phosphatase (BAP)(EC 3.1.3.1), has been well characterized (Garen and Levinthal, 1960; Garen and Echols, 1962; Wanner and Latterell, 1980; Tomassen et al., 1982; Rao et al., 1986); in addition, several acid phosphatases of *E. coli* have been described, and structural genes have been identified for some of them (von Hofsten and Porath, 1962; Hafckenscheid, 1968; Kier et al., 1977; Dassa and Boquet, 1985; Pradel and Boquet, 1988). Nearly all of these enzymes are of interest in soil enzyme studies because they catalyze the release of orthophosphate (P_i) from organic mono- and diesters of orthophosphoric acid. The majority of soil organic phosphorus (P_o) consists of these two types of compounds (Speir and Ross, 1978). Although soil bacteria exhibit phosphatase activity in culture (Mazilkin

and Kuznetsova, 1964; Cosgrove et al., 1970), much less is known about the phosphatases of bacteria other than *E. coli.* Nevertheless, because phosphorus (P) is frequently the limiting nutrient in agricultural systems and because large quantities of soil P are often immobilized in organic forms (Speir and Ross, 1978), characterization of the phosphatases of soil bacteria and understanding of their genetic controls are important in further studies of the roles these organisms may play in soil P cycling.

The objectives of this study were as follows: (1) to evaluate acid and alkaline phosphatase activities of a group of soil bacteria grown under low- and high P_i conditions in liquid culture. (2) to determine if genomic DNA of these bacteria hybridize to the *phoA* gene of *E. coli.* (3) to determine optimal temperature and pH for phosphatase activity of bacterial strains selected from this group for testing in soil inoculation experiments.

MATERIALS AND METHODS

Bacterial cultures

The bacteria used in this study were isolated previously from soil or the plant rhizosphere and characterized (Table 2.1). They included *Flavobacterium* sp., as well as several members of the family *Enterobacteriaceae* and the genus *Pseudomonas*, the two groups found to comprise 80-90% of bacteria in wheat rhizospheres (Freitas and Germida, 1990). Their sources, and the media in which they are normally cultured are shown in Table 2.1. *E. coli* MPH69 and MPH43 were included as negative and positive controls for alkaline phosphatase activity, respectively. MPH69 is a mutant which does not produce BAP; MPH43 contains the *phoA* gene on plasmid pHI-7, which confers ampicillin resistance (Ap^r), and a second functional *phoA* gene and streptomycin resistance (Sm^r) on the chromosome.

Chemicals and supplies

p-Nitrophenyl phosphate (PNPP, #104) and piperazine-N,N'-bis[2-ethanesulfonic acid]) (PIPES, #P-7643) were obtained from Sigma Chemical Co., St. Louis, MO; 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES, #7365-45-9) was purchased from Aldrich Chemical Co., Milwaukee, WI. Restriction enzymes were obtained from Bethesda Research Laboratories, Gaithersberg, MD, and were used as directed by the supplier. ^{32}P-labeled deoxyCTP (3 000 Ci $mmol^{-1}$) was obtained from New England Nuclear Corp., Boston, MA. A Qiagen DNA purification kit was supplied by Qiagen, Studio City, CA. Nytran Modified Nylon-66 membranes and NA-45 DEAE membranes were purchased from Schleicher and Schuell, Keene, NH.

Media

Bacterial cultures were grown on either Luria Bertani (LB) agar (Miller,

Table 2.1. Bacterial strains and media.

Strain		Medium	Source, reference
Enterobacter cloacae	E6	LB	E. Nelson (Cornell Univ.)
Enterobacteriaceae	Wasco 4	LB	M. N. Schroth (Univ. of Calif., Berkeley) (Suslow & Schroth, 1982)
Erwinia carotovora subsp. *atroseptica*	W3C37	LB	D. Gross (Washington State Univ.) (Xu and Gross, 1986)
Er. carotovora subsp. *Carotovora*	W3C105	LB	D. Gross (Xu and Gross, 1986)
Er. herbicola	952	LB	E. Nelson (Cornell Univ.)
Escherichia coli	C600(P1)	LB	C. I. Kado (Washington State Univ.)
E. coli (PhoA $^-$)†	MPH69	LB	J. Beckwith (Inouye et al., 1981)
E. coli (PhoA +)‡	MPH43	LB+Ap+Sm	J. Beckwith (Inouye et al., 1981)
Flavobacterium	MtCa7	NAGly	M. N. Schroth (U. C. Berlekey)(Suslow & Schroth, 1982)
Pseudomonas fluorescens	Pf-5	KB	D. Gross (Xu and Gross, 1986)
P. fluorescens biotype B	ATCC 17467	KB	American Type Culture Collection (Rockville, MD) (Stanier et al., 1966)
P. fluorescens bio. C	ATCC 17559	KB	ATCC (Stanier et al., 1966)
P. fluorescens bio. D	ATCC 9446	KB	ATCC (Stanier et al., 1966)
P. fluorescens bio. E	ATTC 13985	KB	ATCC (Stanier et al., 1966)
P. fluorescens bio. F	ATCC 17513	KB	ATCC (Stanier et al., 1966)
P. fluorescens bio. G	ATCC 17518	KB	ATCC (Stanier et al., 1966)
P. putida	ATCC 12633	KB	ATCC (Stanier et al., 1966)
P. putida biotype B	ATCC 17470	KB	ATCC (Stanier et al., 1966)
P. cepacia	POPS1-LG	NAGly	R. Lumsden (USDA, Beltsville, MD)

† F $^-$Δ*lac*, *galU*, *galK*, Δ(*leu-ara*), *phoA-E*-15, *proC*::Tn*5*.

‡ with pHI-7: alkaline phosphatase structural gene cloned into pBR322. Host: *araD*, Δ*lacU*, 196 Sm-resistance.

1972), Kings Medium B (KB)(King et al., 1974), or nutrient agar (Difco, Detroit, MI) amended with 1% glycerol (NAGly), as indicated in Table 2.1. *E. coli* MPH43 (pHI-7) was grown on media containing ampicillin (0.1 mg of the Na salt mL^{-1}) and streptomycin (0.02 mg mL^{-1}). Bacterial cultures were stored at -80° C in 2.0 mL polypropylene cryogenic vials containing Difco nutrient broth amended with 15% glycerol.

Bacterial cells to be assayed for phosphatase activity were grown in a modified CM9 liquid medium containing L^{-1}: 1.0 g NaCl; 1.0 g NH_4Cl; 3.0 mL 1% tryptophan; 15.0 mL 2% Difco casamino acids. Low P_i (LP) and high P_i (HP) media contained 0.028 g and 0.3 g KH_2PO_4, respectively. For buffering pH 7.2 and 8.0 media, 11.92 g HEPES was added L^{-1}; pH 6.5 medium was buffered with 18.93 g PIPES L^{-1}. After autoclaving, the following were added L^{-1}: 1.0 mL 0.2% thiamine and 10.0 mL 20% glucose, both filter sterilized; and 1.0 mL 1 *M* $MgSO_4$ and 2.0 mL 0.05 *M* $CaCl_2$, both autoclaved. This gave final concentrations as follows: NaCl, 17.0 m*M*; NH_4Cl, 19.0 m*M*; tryptophan, 0.15 m*M*; KH_2PO_4, 0.2 or 2.1 m*M*; HEPES or PIPES, 0.5 *M*; thiamine, 0.006 m*M*; glucose, 11 m*M*; $MgSO_4$, 1.0 m*M;* $CaCl_2$, 0.1 m*M*; and casamino acids, 0.03%. The LP and HP media contained 0.3 m*M* or 2.3 m*M* P as determined by sodium hypobromite digestion (Dick and Tabatabai, 1977).

Phosphatase assays of all strains

A modified universal buffer (MUB) adjusted to pH 6.5 was used for acid phosphatase assays because soil acid phosphatase activity is near optimum at this pH for most soils (Tabatabai, 1982). Alkaline phosphatase assays were buffered at pH 8.0 because *E. coli* alkaline phosphatase (BAP) has optimal activity at this pH (Garen and Levinthal, 1960).

The protocol for phosphatase assays is outlined in Fig. 2.1. Bacterial cultures were retrieved from cryogenic storage, streaked on appropriate agar media, and incubated at 30°C until colonies >1 mm had formed. Bacteria from single colonies were used to inoculate 15 mL LP, pH 7.2, in 125-mL Erlenmeyer

flasks with foam plugs. The flasks were incubated 48 h at 30°C and 200 rpm in a controlled environment shaker. Aliquots of 0.3 mL were transferred from each flask to six 250-mL Erlenmeyer flasks containing 30 mL LP or HP. These flasks were incubated an additional 48 h at 30°C and 200 rpm. All flasks were immediately chilled and all bacterial samples were kept on ice until the 37° incubation below.

Two 15-mL samples were removed from each flask to sterile screw-capped 50-mL polycarbonate centrifuge tubes and centrifuged 10 min in a refrigerated centrifuge at 2987 *g*. The cells in duplicate tubes were resuspended in 15 mL sterile, chilled MUB, pH 6.5 or 8.0. Two 6-mL aliquots of cell suspension were transferred from each tube to 150 x 20-mm sterile culture tubes with foam plugs. Cell suspensions remaining in the centrifuge tubes were diluted 1:10 with deionized water and the optical density at 600 nm (OD_{600}) of each was determined spectrophotometrically (Brickman and Beckwith, 1975). To one of each pair of culture tubes was added 0.2 mL of 0.036 *M* PNPP made in MUB of the same pH as the cell suspension; 0.2 mL of MUB, pH 6.5 or 8.0, was added to the second, control tube. After incubating tubes for 30 min in a 37°C water bath, 2.0 mL 0.5 *M* NaOH was added to stop the reaction. To each control tube was added 0.2 mL PNPP solution, and 0.2 mL MUB was added to each assay tube. The resulting suspensions were filtered through syringe filters (0.2 μm pore size) to remove cellular debris. The filtrate was diluted 1:10 with deionized water and OD_{410}

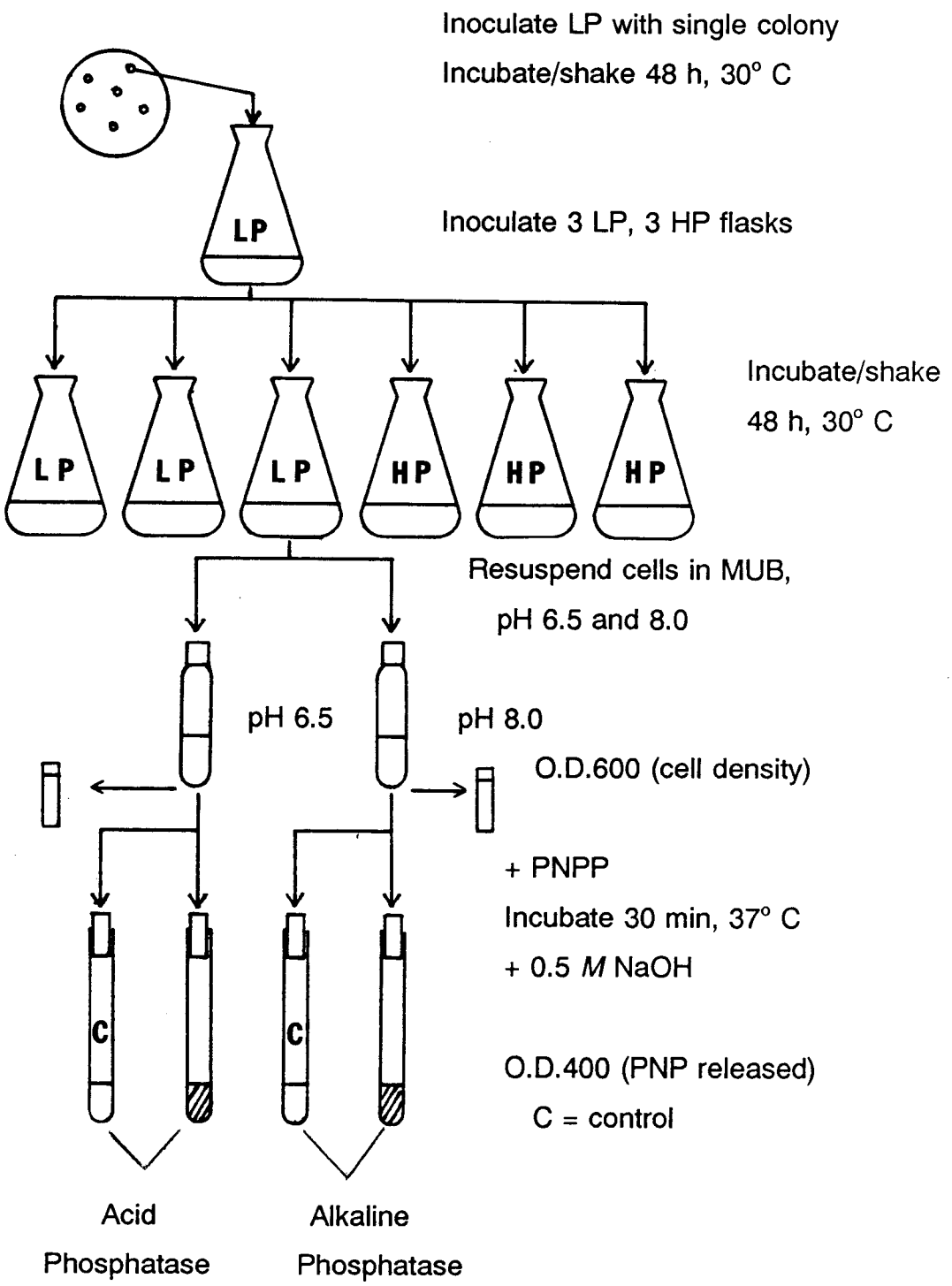


Fig. 2.1. Protocol for bacterial acid- and alkaline phosphatase assays.

determined spectrophotometrically (Tabatabai, 1982). When a reading exceeded 2.0, the sample was further diluted. OD_{410} was also measured for filtered reagent blanks containing buffer, PNPP, and NaOH.

The number of colony-forming units (cfu) OD_{600}^{-1} for the bacterial strains used in this study was determined by first incubating the bacteria in LP medium 48 h at 30°C and 200 rpm, then diluting each culture 1:2, 1:5, 1:10, and 1:25 and measuring OD_{600} of each dilution. Serial dilutions and triplicate plate counts were done for each dilution (Gerhardt et al., 1981). Linear regressions were performed on the resulting data to obtain the slope (OD_{600} [10^9 cfu mL^{-1}]$^{-1}$) for each strain. These values are shown in Table 2.2.

Phosphatase activity was expressed as nmol *p*-nitrophenol (PNP) released $(10^9 \text{ cfu})^{-1}$ min^{-1}, as calculated by the following formula:

$$\frac{OD_{410} \text{ x S x dilution factor}}{OD_{600} \text{ x 30 min x .011}}$$

where S = slope from Table 2.2,

.011 = slope of PNP standard curve.

pH and temperature effects on phosphatase activity

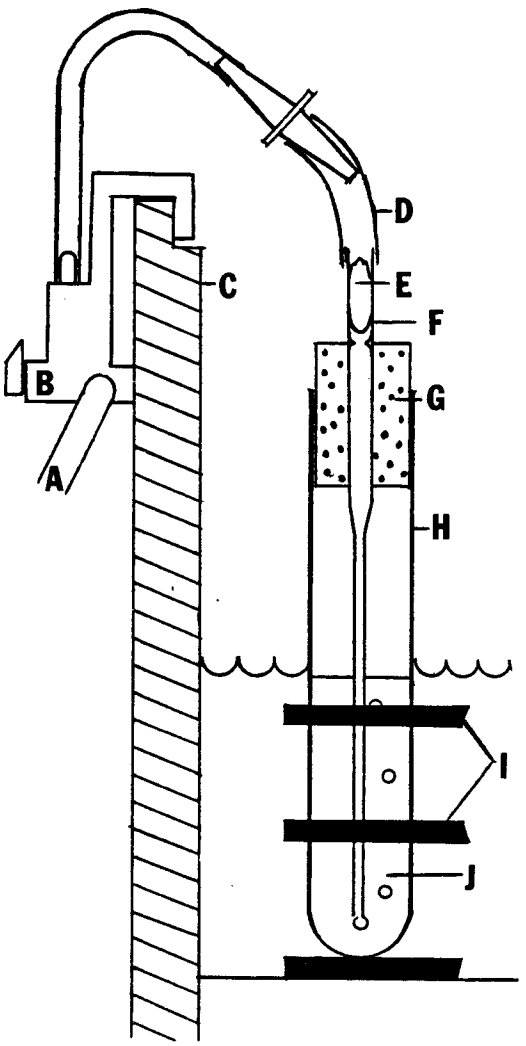
E. coli MPH43 and MPH69 (positive and negative controls for alkaline phosphatase, respectively), *E. carotovora* W3C105 (high alkaline phosphatase activity), and *Ps. fluorescens* Pf-5 (acid phosphatase activity) were grown at 30° C in LP, pH 6.5 and 8.0 for 48 h in a waterbath shaker (140 oscillations min^{-1}). Aliquots of 0.1 mL from each flask were used to inoculate duplicate 150 x 25-mm sterile culture tube assemblies (Fig. 2.2) containing 30 mL LP of the same pH as that in the flask. The tube assemblies were placed in racks and incubated in a constant temperature refrigerated water bath. Aeration was provided by two aquarium

pumps and was regulated by a valve for each tube to provide the maximum rate of aeration possible without causing large bubbles to rise up the tube and touch

Table 2.2. Bacterial culture densities (OD_{600}).

Strain	Slope†	Strain	Slope
E6	0.41	ATCC 17467	1.10
Wasco 4	0.38	ATCC 17559	0.30
W3C105	2.63	ATCC 9446	0.42
W3C37	2.63	ATTC 13985	0.37
952	0.39	ATCC 17513	2.90
C600(P1)	0.89	ATCC 17518	0.33
MPH69	0.69	ATCC 12633	0.72
MPH43	1.53	ATCC 17470	1.08
MtCa7	2.13	POPS1-LG	0.76
Pf-5	0.44		

† OD_{600} $[10^9$ cfu$(mL)^{-1}]^{-1}$ from linear regressions ($p < .01$).



A Tubing to air pump

B Valve

C Wall of water bath

D Tygon tubing

E Cotton plug

F Pasteur pipet

G Polyurethane foam plug

H Culture tube

I Test tube rack

J Growth medium

Fig. 2.2. Sterile culture tube assembly for incubation in water bath. Components D through H were autoclaved as a unit.

the stopper. Sixteen tubes were used in each set (4 strains X 2 pH values X 2 replicates). Tubes were arranged so that each tube of a replicate pair was supplied by a different air pump. The original level of medium in the tube was marked with a Sharpie pen initially, and sterile deionized water was added every 24 h and at the end of the incubation to replace that lost to evaporation (approximately 10% d^{-1} at 25°C). Incubation proceeded for the following time intervals at the temperatures indicated: 15°C, 96 h; 20°C, 72 h; 25°C, 48 h; 30°C, 40 h. The tubes were removed from the aeration apparatus, chilled, and two 14-mL aliquots from each tube were centrifuged as already described and resuspended in 14 mL chilled MUB, pH 6.5 and 8.0. Acid and alkaline phosphatase assays were run on all samples.

Sources of variability

As a test for the source of variability in bacterial phosphatase assays, *E. coli* MPH43 was grown in 15 mL LP, pH 8.0 for 48 h at 30°C and 140 oscillations min^{-1}. Aliquots of 0.1 mL from this culture were used to inoculate each of four culture tubes containing 30 mL of the same medium, and the tubes were incubated at 30°C for 42 h with aeration as previously described. After 27 and 42 h, each tube was sampled by dipping a sterile applicator stick into the medium and streaking the bacteria on both NA and LB plates, which were incubated and examined for contaminants. After 42 h, serial dilutions of 1:2, 1:4, 1:8, and 1:16 were made for each culture tube. Two aliquots were taken from each of the 16 dilution tubes, and each of these duplicate subsamples was assayed for both acid- and alkaline phosphatase, as shown in Fig. 2.3.

Three types of variability were investigated. Subsampling error included pipetting errors and other errors inherent in the assay procedure. Dilution error was variability in phosphatase activity due to concentration of bacterial cells. Culture error was variability due to the differences in bacteria after 42 h growth in separate tubes. Computations of these types of variability were performed as indicated below. Values for acid- and alkaline phosphatase were calculated

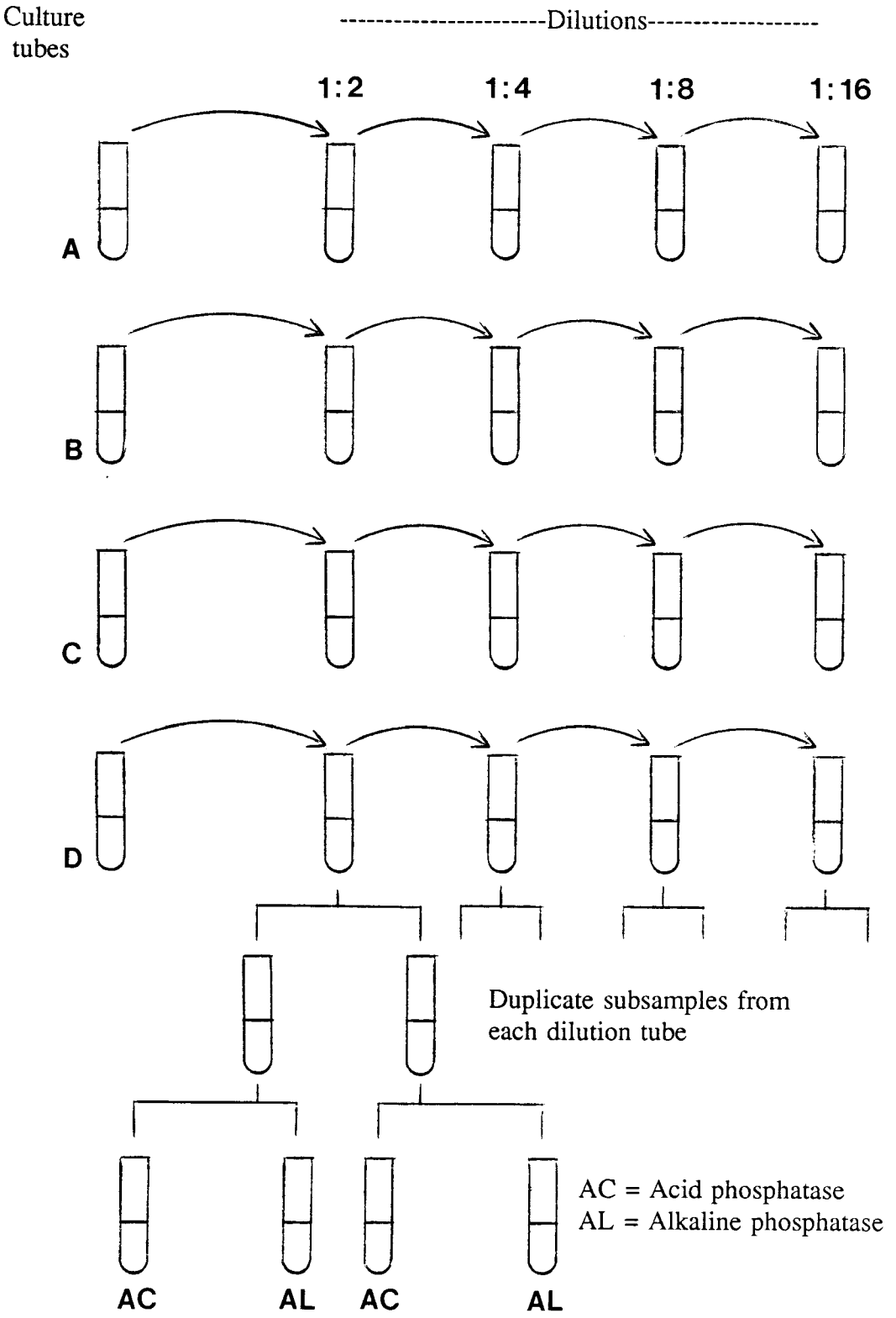


Fig. 2.3. Dilution scheme for variability test.

separately.

Subsampling error. The subsampling coefficient of variability (CV) were calculated for each subsample pair.

Dilution error. The mean of all eight subsamples of each dilution was obtained, and the CV of the resulting four means of all dilutions was calculated.

Culture error. The mean of all eight subsamples originating from each culture was computed, and the CV of the resulting four means of all cultures was then obtained.

DNA procedures

Overnight cultures of all bacterial strains grown in NA were centrifuged; pellets were resuspended in Tris-EDTA buffer (TE) (Ausbel et al., 1987) and lysed with 0.1 *M* Tris-HCl / 9% sodium dodecylsulfate, pH 8.5. After extraction with chloroform, DNA was precipitated with sodium acetate and ethanol, resuspended in TE, precipitated with sodium acetate and isopropanol, and suspended again in TE. Restriction enzyme digests were prepared by digesting these DNA samples with RNase and *Pvu*II. These digests were analyzed in 0.7% agarose gels in Tris-borate buffer (Ausbel et al., 1987); Plasmid digests were analyzed in 0.5% agarose gels with Tris-phosphate buffer (Ausbel et al., 1987). All gels contained 1 µg ethidium bromide (EtdBr) mL^{-1}. Samples were mixed with 10X stop buffer (Bethesda Research Laboratories, 1988) before loading in the gels.

Plasmid pHI-7 DNA was prepared from overnight culture of *E. coli* MPH43 by the method of Maniatis et al. (1982) and suspended in TE. It was purified by precipitation with 2.5 *M* ammonium acetate and ethanol, washed in ethanol and resuspended in TE to give approximately 1µg plasmid DNA mL^{-1}. The sample was digested with RNase and *Pvu*II, and the 0.9 kb *Pvu*II fragment of the *phoA* gene of pHI-7 was obtained by preparative gel electrophoresis (Maniatis et al., 1982) in a 1% gel in Tris-borate buffer. Band interception by a NA-45 DEAE membrane was used to recover the fragment from the gel. After

elution of the fragment from the membrane, EtdBr was removed by phenol extraction, and the DNA was precipitated with isopropanol and resuspended in TE.

^{32}P-labeled DNA probe was made by nick-translation of the *phoA* gene fragment using [^{32}P]dCTP (Dillon et al., 1985). The gene fragment was then purified with the Qiagen kit, resulting in a labeled probe containing 3.8474 cpm μL^{-1}. DNA-DNA hybridization was carried out by the method of Southern (1975), using Nytran-66 membranes.

Statistical analyses

Data were analyzed using standard analysis of variance procedures, with Waller-Duncan K-ratio T test. All probabilities were evaluated at the levels of significance indicated.

RESULTS

Phosphatase assays of all cultures

The majority of the bacteria in this study expressed detectable phosphatase activity (Table 2.3). Of the strains with phosphatase activity of greater than 0.5 nmol PNP $(10^9$ cfu$)^{-1}$ min^{-1}, *Pseudomonas* spp., *Flavobacterium*, and *E. herbicola* exhibited higher acid than alkaline phosphatase activity, whereas the reverse held for *E. carotovora* and *E. coli*. Closely related bacteria varied greatly in their phosphatase activities. For example, *E. carotovora* subsp. *carotovora* strain W3C105 expressed a mean alkaline phosphatase activity of 91.9 nmol PNP $(10^9$ cfu$)^{-1}$ min^{-1}, whereas *E. carotovora* subsp. *atroseptica* W3C37 expressed no activity. The *P. fluorescens* biotypes produced acid phosphatase activities ranging from 0 for biotypes C and E to 9.7 nmol PNP $(10^9$ cfu$)^{-1}$ min^{-1} for biotype F. Coefficients of variability ranged from <10% to >100%.

Hybridization study

The *phoA* gene of *E. coli* hybridized with a 14.5 kb fragment of genomic DMA of *E. carotovora* subsp. *carotovora*; a 6 kb fragment of *Enterobacter cloacae*; fragments of 6 and 2.1 kb from *E. coli* MPH69 (Fig. 2.4); and three fragments of 6, 2.1, and < 1 kb from *E. coli* C600 (Fig. 2.5). In addition, *phoA* hybridized with 4.4 kb and < 1 kb fragments of plasmid pHI-7. *The* phoA probe did not hybridize with the genomic DNA isolated from *E. herbicola*, *E. carotovora* subsp. *atroseptica*, *Enterobacteriaceae* Wasco 4, *Flavobacterium*, or any of the *Pseudomonas* spp.

pH and temperature effects on phosphatase activity

E. carotovora W3C105 grown in a culture medium at pH 6.5 did not express alkaline- or acid phosphatase activity, but it did express both activities in

Table 2.3. Acid- and alkaline phosphatase activities of the soil bacteria assayed.

Strain		Phosphatase activity			
		Low P_i†		High P_i‡	
		Acid pH 6.5	Alkaline pH 8.0	Acid pH 6.5	Alkaline pH 8.0
		nmol PNP (10^9 cfu)$^{-1}$ min^{-1}			
Enterobacter cloacae	E6	0.4	0.2	0.2	0.0
Enterobacteriaceae	Wasco 4	0.3	0.0	0.0	0.0
Erwinia carotovora subsp. *atroseptica*	W3C37	0.0	0.0	0.0	0.0
E. carotovora subsp. *carotovora*	W3C105	11.5	91.9	0.0	0.0
E. herbicola	952	5.6	0.9	7.9	1.0
Escherichia coli	C600(P1)	0.3	0.0	0.0	0.0
E. coli (PhoA $^-$)	MPH69	0.0	0.0	0.3	0.0
E. coli (PhoA $^+$)	MPH43	11.6	41.2	2.1	4.7
Flavobacterium	MtCa7	7.8	3.3	3.9	1.7
Pseudomonas fluorescens	Pf-5	1.8	0.6	0.6	0.4
P. fluorescens biotype B	ATCC 17467	2.7	0.8	1.4	0.5
P. fluorescens biotype C	ATCC 17559	0.0	0.0	0.0	0.0
P. fluorescens biotype D	ATCC 9446	1.8	0.2	1.9	0.2
P. fluorescens biotype E	ATTC 13985	0.0	0.0	0.0	0.0
P. fluorescens biotype F	ATCC 17513	9.7	3.1	5.4	2.4
P. fluorescens biotype G	ATCC 17518	0.3	0.3	0.0	0.2
P. putida	ATCC 12633	2.2	0.0	2.1	0.2
P. putida biotype B	ATCC 17470	2.1	1.0	1.5	0.0
P. cepacia	POPS1-LG	0.0	0.2	0.0	0.0

†Cells grown in LP medium (0.2 m*M* P_i).
‡Cells grown in HP medium (2.1 m*M* P_i).

Fig. 2.4. Southern blot autoradiogram (top) of chromosomal DNA digests of bacteria with ^{32}P-labeled *phoA* as the probe. Corresponding agarose gel stained with EtdBr is shown at bottom.

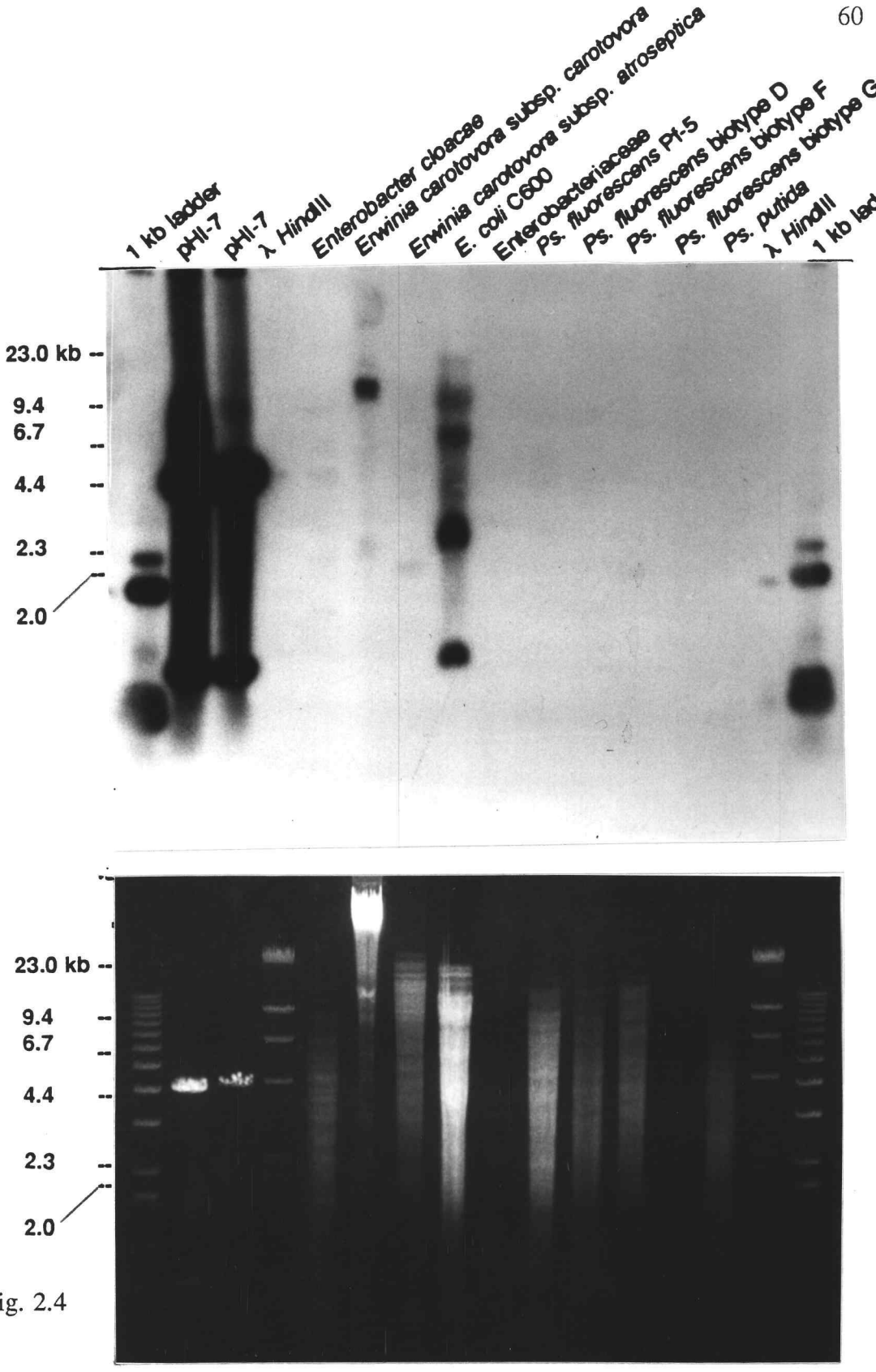

1 kb ladder
pHI-7
pHI-7
λ HindIII
Enterobacter cloacae
Erwinia carotovora subsp. carotovora
Erwinia carotovora subsp. atroseptica
E. coli C600
Enterobacteriaceae
Ps. fluorescens Pf-5
Ps. fluorescens biotype D
Ps. fluorescens biotype F
Ps. fluorescens biotype G
Ps. putida
λ HindIII
1 kb lad.
23.0 kb
9.4
6.7
4.4
2.3
2.0
23.0 kb
9.4
6.7
4.4
2.3
2.0

Fig. 2.4

Fig. 2.5. Southern blot autoradiogram (top) of chromosomal DNA digests of bacteria with ^{32}P-labeled *phoA* as the probe. Corresponding agarose gel stained with EtdBr is shown at bottom.

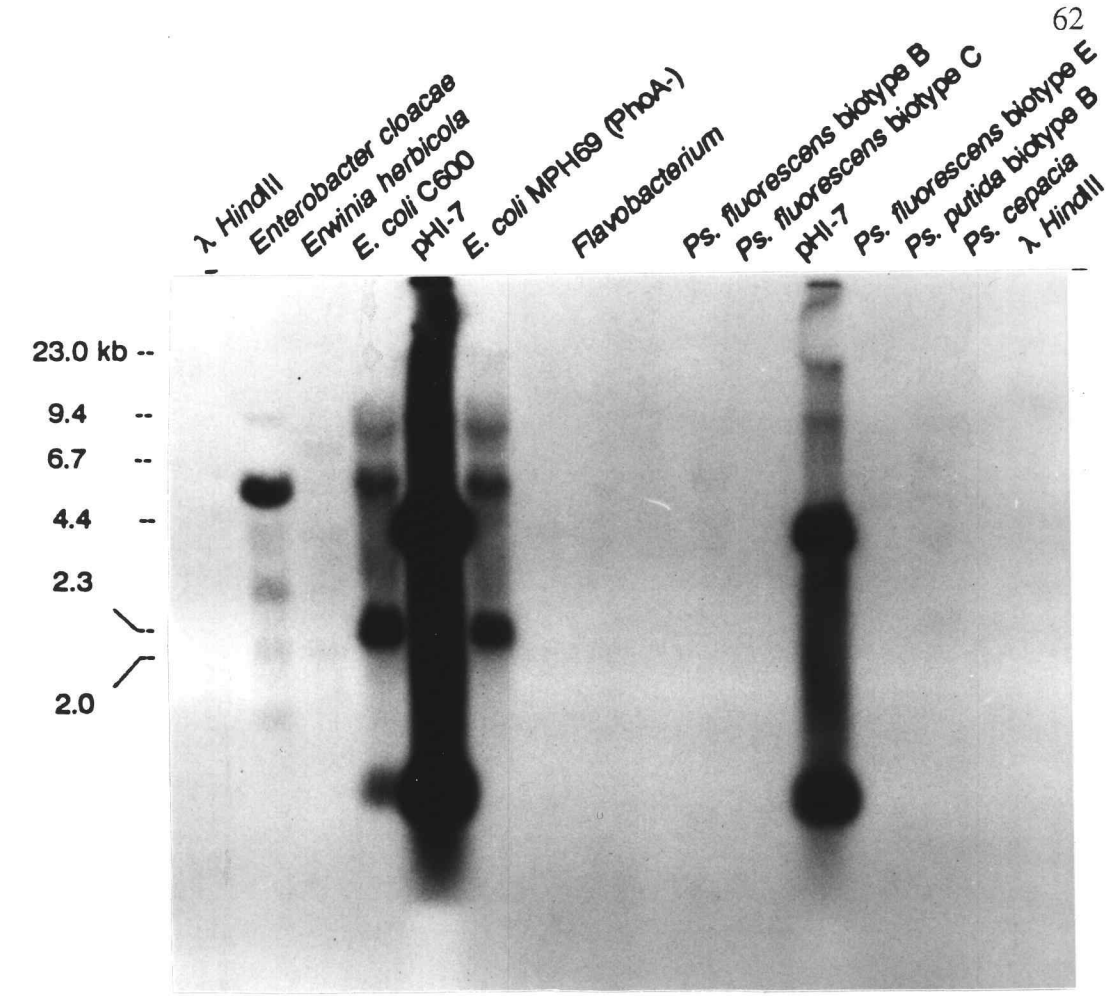

λ HindIII
Enterobacter cloacae
Erwinia herbicola
E. coli C600
pHI-7
E. coli MPH69 (PhoA-)
Flavobacterium
Ps. fluorescens biotype B
Ps. fluorescens biotype C
pHI-7
Ps. fluorescens biotype E
Ps. putida biotype B
Ps. cepacia
λ HindIII
23.0 kb
9.4
6.7
4.4
2.3
2.0
23.0 kb
9.4
6.7
4.4
2.3
2.0

Fig. 2.5

a medium buffered at pH 8.0. Greater than twice as much alkaline- as acid phosphatase activity was expressed by strain W3C105 grown in the pH 8.0 medium (Fig. 2.6). Like *E. carotovora* W3C105, the *E. coli* strains expressed greater phosphatase activities when grown at pH 8.0 than at pH 6.5. *P. fluorescens* strain Pf-5 had very little phosphatase activity at either pH value. Because of variability between values of individual samples, only the acid and alkaline phosphatase activity of *E. carotovora* W3C105 and *E. coli* MPH43, both grown at pH 8.0, were significantly different from 0 ($p < .05$).

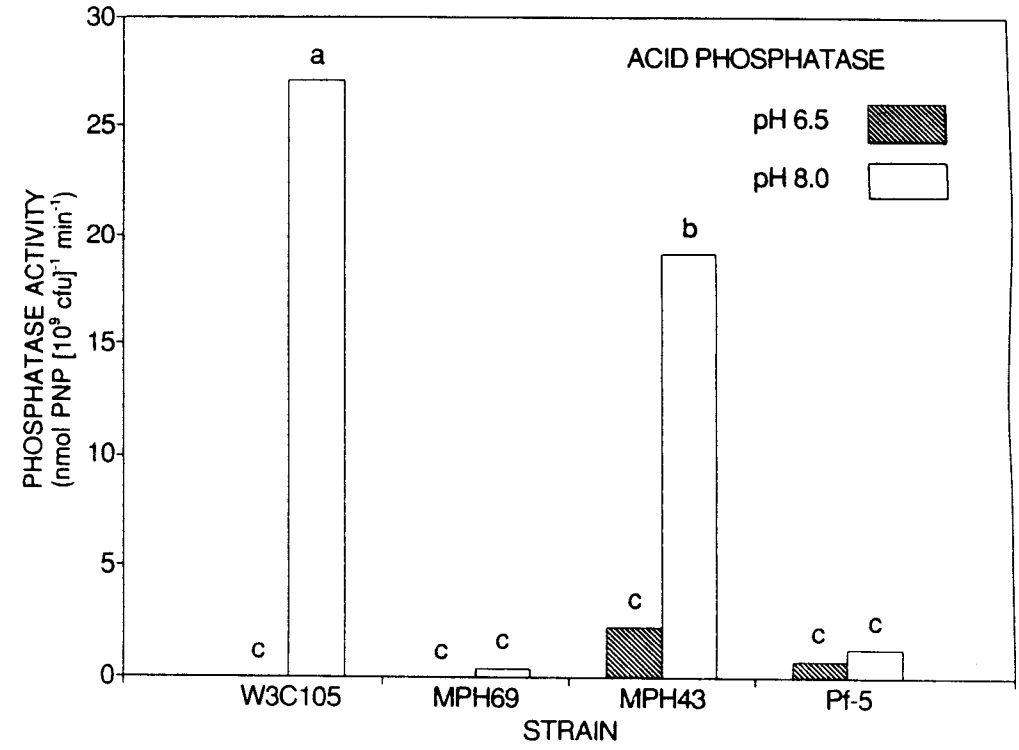
The temperature at which the cells were grown influenced greatly the expression of phosphatase activity (Fig. 2.7). Of all the organisms tested, *E. carotovora* W3C105 was most strongly affected by temperature. It had optimal acid and alkaline phosphatase activities at 25° C, which were more than five times greater than those at any other temperature. *E. coli* MPH43 had greatest phosphatase activity at 25° C, and none at 15° C. *P. fluorescens* Pf-5 appeared to produce more activity at lower temperatures, although its activity at all temperatures was very low and the differences between temperatures were not significant ($p = 0.05$). *E. coli* MPH69 expressed insignificant phosphatase activity at all temperatures.

Phosphatase activities of some of the bacteria in this study appeared to vary greatly with growth conditions. Table 2.4 summarizes phosphatase activities of *E. carotovora* W3C105 under different sets of conditions used throughout these studies. Phosphatase activities of other bacteria, such as *Ps. fluorescens* Pf-5, varied little with different growth conditions.

Sources of variability

Coefficients of variability (CV) for *E. coli* MPH43 subsamples ranged from 0.8 to 9.4% for acid phosphatase and from 0 to 11.2 % for alkaline phosphatase.

Fig. 2.6. Effects of pH of growth medium on acid- and alkaline phosphatase activities of *E. carotovora* W3C105, *E. coli* MPH69 and MPH43, and *P.fluorescens* Pf-5 grown at 25° C, pH 6.5 and 8.0. Values with the same letter are not significantly different ($p < 0.05$).


ACID PHOSPHATASE
pH 6.5
pH 8.0
PHOSPHATASE ACTIVITY
(nmol PNP [10⁹ cfu]⁻¹ min⁻¹)
30
25
20
15
10
5
0
a
b
c
c
c
c
c
c
W3C105
MPH69
MPH43
Pf-5
STRAIN

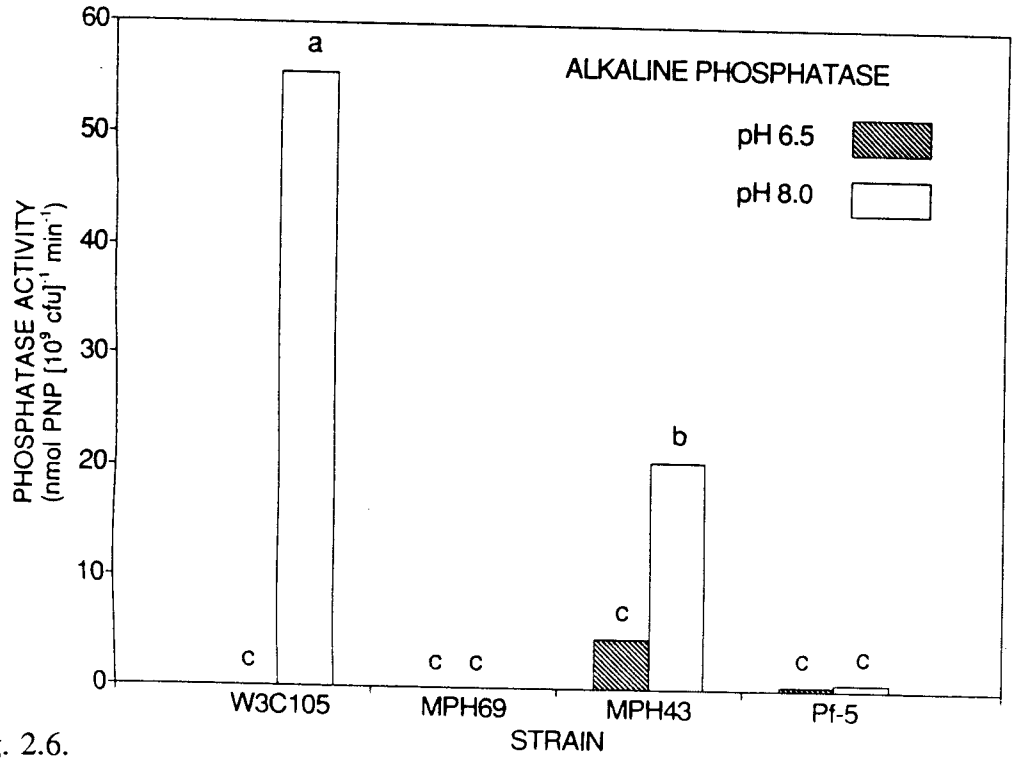

ALKALINE PHOSPHATASE
pH 6.5
pH 8.0
PHOSPHATASE ACTIVITY
(nmol PNP [10⁹ cfu]⁻¹ min⁻¹)
60
50
40
30
20
10
0
a
b
c
c
c
c
c
c
W3C105
MPH69
MPH43
Pf-5
STRAIN

Fig. 2.6.

Fig. 2.7. Effects of incubation temperature on acid- and alkaline phosphatase activities of *E. carotovora* W3C105, *E. coli* MPH69 and MPH43, and *P.fluorescens* Pf-5 grown at pH 8.0 and 15, 20, 25, and 30° C. Values with the same letter are not significantly different within strain ($p < 0.05$). Values without letters are not significantly different at this level.

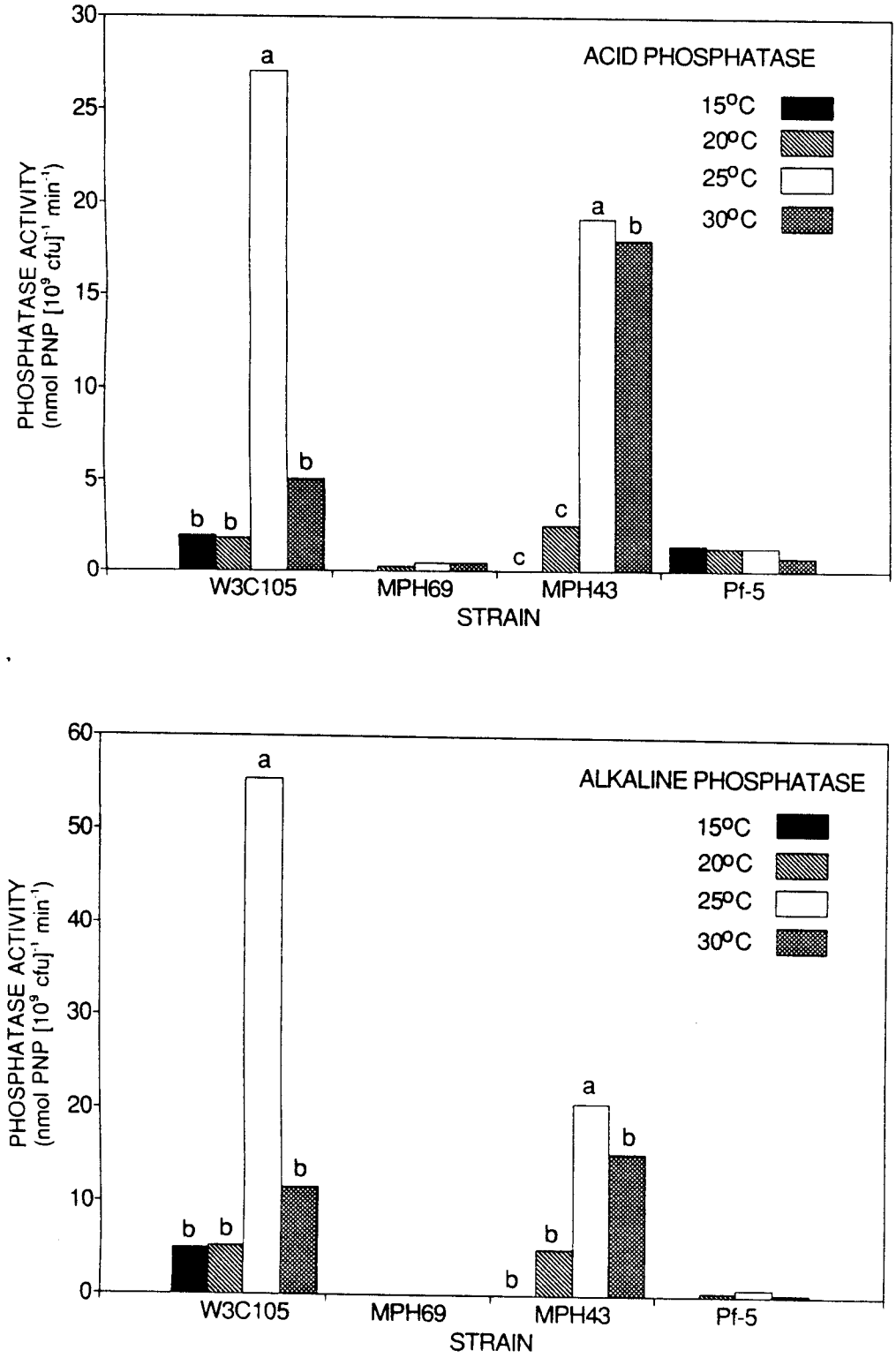

ACID PHOSPHATASE
15°C
20°C
25°C
30°C
PHOSPHATASE ACTIVITY
(nmol PNP [10⁹ cfu]⁻¹ min⁻¹)
30
25
20
15
10
5
0
a
b
b
b
c
c
a
b
W3C105
MPH69
MPH43
Pf-5
STRAIN
ALKALINE PHOSPHATASE
15°C
20°C
25°C
30°C
PHOSPHATASE ACTIVITY
(nmol PNP [10⁹ cfu]⁻¹ min⁻¹)
60
50
40
30
20
10
0
a
b
b
b
b
b
a
b
W3C105
MPH69
MPH43
Pf-5
STRAIN

Fig. 2.7.

Table 2.4. Phosphatase activities of *E. carotovora* W3C105 under different growth conditions.

Temperature	Time	pH	Medium	Acid phosphatase	Alkaline phosphatase
°C	h			nmol PNP $(10^9$ cfu$)^{-1}$ min^{-1}	
30	48	6.5	LP	0.0	0.0
30	42	7.2	LP	11.5	91.9
30	42	7.2	HP	0.0	0.0
30	48	8.0	LP	5.0	11.5
25	48	6.5	LP	0.0	0.0
25	48	8.0	LP	27.0	55.4
20	72	6.5	LP	0.0	0.0
20	72	8.0	LP	1.8	5.0
15	96	6.5	LP	0.0	0.0
15	96	8.0	LP	1.9	5.0

Dilution error CV values were 11.3 and 26.1% for acid and alkaline phosphatase, respectively, and culture error CV values were 27.9 and 41.9%. Most of the variation, therefore, was attributed to differences among replicate culture tubes. This variation may be caused by different aeration rates of replicate tubes or to other unknown differences in culture conditions. Variability appeared somewhat higher for alkaline- than for acid phosphatase for all groups. When mean acid- and alkaline phosphatase activities for all culture tubes were plotted against dilution (Fig. 2.8), alkaline phosphatase appeared to increase as the samples became more dilute, indicating that phosphatase values increased with decreasing cell density. A corresponding increase in acid phosphatase was not noted. No contaminant colonies were found on plates streaked from the original four culture tubes. Only a small amount of variation appeared to originate in the assay procedure itself.

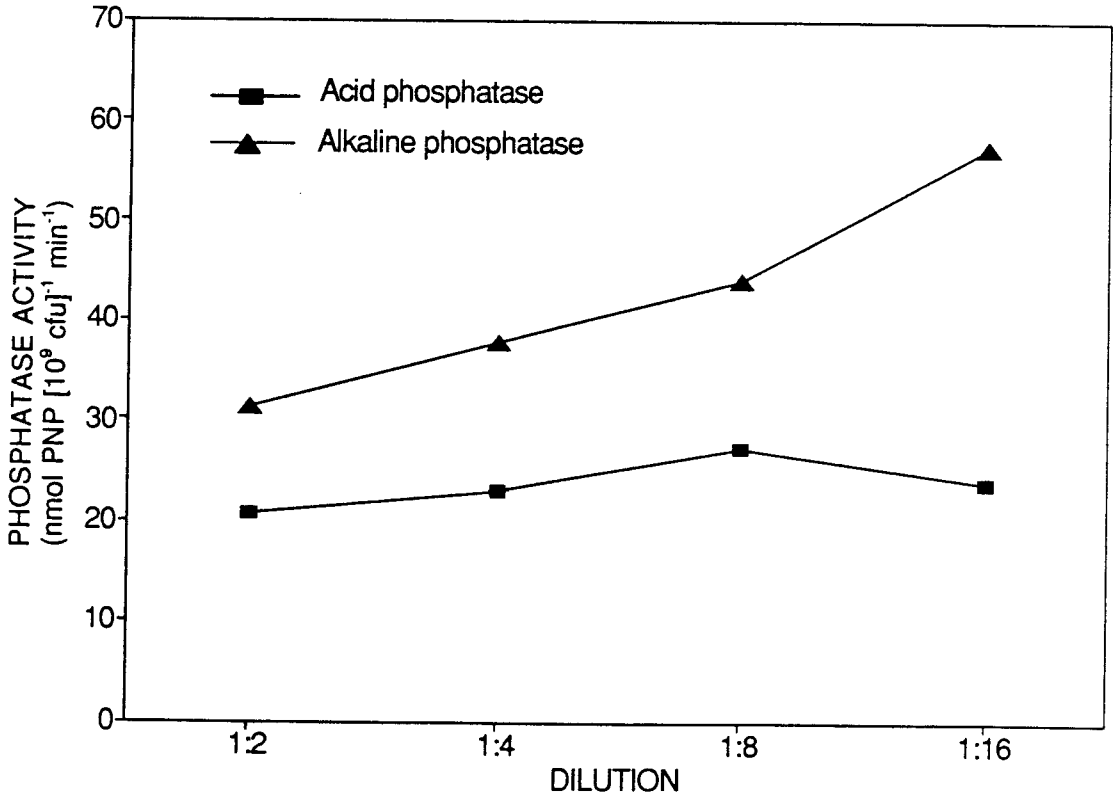


Fig. 2.8. Mean acid- and alkaline phosphatase activities of suspensions of *E. coli* MPH43 cells at four dilutions.

DISCUSSION

Phosphatase activities of all cultures

Results of the DNA hybridization study indicated that the genomic DNA of only those bacterial strains closely related to *E. coli* hybridized with *phoA*. Most of the strains that hybridized to the *phoA* gene did not express appreciable alkaline phosphatase activity under the conditions of this study. Nine strains which did not hybridize with *phoA* expressed alkaline phosphatase activity, indicating that these strains possess a *phoA* equivalent that does not have homology to *E. coli phoA*.

Of the 12 strains showing alkaline phosphatase activity, ten produced higher activities in LP than in HP. A similar trend was observed for acid phosphatase; 11 of the 14 positive strains expressed higher activities when in LP than in HP (Table 2.3). Phosphatase activity appeared to be at least somewhat repressed by P_i in the following strains: *Flavobacterium*; *P. fluorescens* Pf-5, biotypes B, F, G; and *P. putida* biotype B. Activities of *P. fluorescens* biotype D and *Ps. putida* appeared unaffected by P_i concentration, and *E. herbicola* showed higher acid phosphatase activity in HP than in LP.

With the exception of acid phosphoanhydride phosphohydrolase (pH optimum 2.5), *E. coli* acid phosphatases are known not to be repressed by P_i (von Hofsten and Porath, 1962). Therefore, any phosphatase activity expressed by *E. coli* in response to P_i limitation at pH values well above 2.5 may be attributed to *phoA*. Because BAP is active at pH values slightly above 6.0 (von Hofsten, 1961), it is likely that low levels of phosphatase activity at pH 6.5 that appear in LP but not in HP could have been due to *phoA* expression. *E. carotovora* W3C105, which appeared to be *phoA*$^+$, expressed activities of 91.9 (pH 8.0) and 11.5 (pH 6.5) nmol PNP $(10^9$ cfu$)^{-1}$ min^{-1} in LP,

but no activity in HP (Table 2.3). Thus all of the phosphatase activity of W3C105 may have been contributed by BAP. In contrast, *E. carotovora* W3C37, which did not hybridize to *phoA*, expressed no phosphatase activity under the conditions of this study. Unfortunately, information about other phosphatase systems for these two closely related strains is unavailable. Although the phosphatase activities of *Enterobacter cloacae* may be too low to be significant, it should be noted that it, too, hybridized to *phoA*, and that perhaps 0.2 units of the acid phosphatase reported in LP may have been contributed by BAP.

Alkaline phosphatase activity of *E. coli* MPH43 grown in HP was 11% of that in LP. The ratio of alkaline- to acid phosphatase activity declined from 3.6 in LP to 2.2 in HP, indicating a relative, as well as total, decrease in alkaline phosphatase activity with increased P concentration of the medium. Some of the alkaline phosphatase activity in HP could have been contributed by another enzyme such as 5'-nucleotidase, which is not repressible by P_i and has a pH optimum above 6.5, or by acid hexose phosphatase (pH optimum 4.0), which exhibits 75% of its maximum activity at pH 6.0 and as much as 35% at pH 8.0 (von Hofsten, 1961; Dvorak et al., 1967). If both enzymes were present, a higher activity at pH 8.0 than at pH 6.5 might be expected, unless other phosphatases with pH optima near 6.5 contributed greatly to the activity at that level.

In *E. coli* and other *Enterobacteriaceae*, the pH 6.5 activities could have been contributed by a variety of enzymes: 2'-3'cyclic phosphodiesterase, 5'-nucleotidase, acid hexose phosphatase, acid glucose-1-phosphatase, and nonspecific acid phosphatase. It is unlikely that orthophosphoric monoester phosphohydrolase (pH optimum 2.5) contributed to the activity at pH 6.5 (Hafckenscheid, 1968).

Brickman and Beckwith (1975) used 10 m*M* P_i to completely repress BAP synthesis, and 0.1 m*M* P_i has been shown to repress up to 70% of BAP

activity (Torriani, 1960). LP (0.2 m*M* P_i) would therefore repress a large portion of BAP activity, and much higher alkaline phosphatase activities would be expected for $PhoA^+$ strains if media containing 0.01 m*M* P_i were used. In this study, however, it was necessary to provide at least 0.2 m*M* P_i to maintain the pHI-7 plasmid in MPH43 and to allow adequate growth of *E. carotovora* strains.

Although the total P contents of LP and HP were found to be 0.3 and 2.3 m*M*, respectively, the exact form the of the 0.10 - 0.16 mmol P L^{-1} supplied by casamino acids was unknown. If this source of P was mainly P_i, it would have served to further repress BAP and any other phosphatases repressed by P_i. The presence of acid-hydrolyzed casein may have altered the acid phosphatase activity of some enzymes (von Hofsten, 1961) or increased synthesis of acid hexose phosphatase (Dvorak et al., 1967). The presence of glucose as a C source could have slowed synthesis of this enzyme. An attempt was made to use a defined minimal medium in this study, but some strains did not grow in it.

Temperature and pH effects on phosphatase activity

The four bacterial strains tested produced optimal phosphatase activities in culture if grown at 25°C and at a pH greater than 6.5 (Figs. 2.6 and 2.7). Activity of *P. fluorescens* Pf-5 appeared least sensitive to temperature and pH, whereas that of *E. carotovora* W3C105 was greatly affected by both factors. Based on these results, conditions of soil experiments were set at 25° C and pH near or greater than 6.5.

Variability

Sample variability within the same strain was frequently high throughout the experiments. As was shown for *E. coli* MPH43, a small amount of this variability was due to the assay itself, but more of it appeared to result from variations between replicate cultures and from differences in cell density of the

cultures evaluated.

CONCLUSIONS

As research with *E. coli, S. typhimurium, E. herbicola,* and *Ps. aeruginosa* has already shown, bacteria have developed a range of periplasmic/outer membrane enzymes which scavenge P from a variety of substrates under differing pH and nutritional conditions. The present studies indicate that this variability also exists among common soil bacteria.

Several strains studied express alkaline phosphatase activity, yet do not hybridize to the *phoA* gene of *E. coli.* Although the nonhybridizing strains clearly have genes determining alkaline phosphatase activity, these genes are distinct from those of *E. coli.*

As has already been shown for the *pho* regulon, the control of production of a single phosphatase can be mediated by a complex of genes, and the malfunction of any one of a number of them can prevent production of the enzyme. Such complex genetic controls for production of other phosphatases may have been involved in the current studies. If such controls exist, they would help to account for some of the variation in phosphatase activity observed between even closely related bacterial strains.

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CHAPTER 3

EFFECTS OF PHOSPHATASE-PRODUCING BACTERIA ON PHOSPHATASE ACTIVITY, ACID- AND BICARBONATE-EXTRACTABLE PHOSPHATE FRACTIONS, AND BIOMASS PHOSPHATE IN STERILE SOIL

ABSTRACT

Much of phosphorus (P) fertilizer added to soil is immobilized as organic P (P_o) by soil organisms and becomes unavailable to plants. Soil phosphatases, primarily of microbial origin, are known to catalyze the release of orthophosphate (P_i) from P esters and other P_o compounds in soil. Little is known about the contribution of individual types of soil bacteria to soil phosphatase activity or about the role these bacteria may play in releasing P_i from the soil P_o pool.

The purpose of this study was to determine the effects of phosphatase-producing bacteria on phosphatase activity and on some of the P pools of autoclaved soil samples.

Woodburn (Fine-silty, mixed, mesic, Aquultic Argixerolls) and Jory (Clayey, mixed, mesic, Xeric Haplohumults) soils were limed with CaO, and autoclaved 1-g samples were inoculated with one of the following three soil bacteria known to exhibit phosphatase activity *in vitro*: *Erwinia carotovora* subsp. *carotovora* W3C105, *Pseudomonas fluorescens* Pf-5, and *P. fluorescens* biotype F. In addition, samples were inoculated with *Escherichia coli* strains MPH43 ($PhoA^+$), known to express alkaline phosphatase activity, and MPH69 ($PhoA^-$), which does not exhibit such activity. Samples were incubated at 25° C for 7 d. At the beginning and end of this period the following determinations were made: bacterial plate counts; acid- and alkaline phosphatase activity; 0.5 *M* $NaHCO_3$-extractable P; 0.5 *M* H_2SO_4-extractable P; and biomass P.

E. carotovora W3C105 and *E. coli* MPH43 did not grow well in Woodburn soil. In Jory soil, however, numbers of recovered bacteria on Day 7 were approximately 10^8 colony-forming units (cfu) g^{-1} for all strains.

Samples containing *P. fluorescens* Pf-5 had acid phosphatase activities 8

to 17 times greater than did uninoculated control samples on Day 7 for both soil types. Jory soil samples containing *E. carotovora* W3C105 and *E. coli* MPH43 ($PhoA^+$) had alkaline phosphatase activities significantly higher than those of control samples on Day 7 ($p = 0.05$). Samples containing *E. coli* MPH69 ($PhoA^-$) and *P. fluorescens* biotype F did not have significantly elevated phosphatase activities.

Levels of both $NaHCO_3$-extractable P and H_2SO_4-extractable P were lower on Day 7 than on Day 0 for all samples. Biomass P increased between Day 0 and Day 7 in inoculated samples, but remained the same in controls. The increases in biomass P were greater than the decreases in $NaHCO_3$-extractable P for all inoculated samples, indicating that the bacteria were mobilizing P from a less available source, possibly P_o. The uninoculated control samples, however, showed a net loss in available P. This loss was presumably due to fixation.

The results of this study led to the following conclusions: (1) the soil phosphatase activity contributed by a bacterial strain was not predicted by its activity *in vitro*. (2) some bacterial strains may contribute more to soil phosphatase activity than do others. (3) bacteria may be able to use soil P_o that is not readily available to them, thus releasing, if only temporarily, some of the formerly unavailable P in soil.

INTRODUCTION

Phosphorus (P), an important plant nutrient, is often the limiting macronutrient in soils because of its low mobility and high rate of fixation by both clay minerals and incorporation into organic P (P_o) compounds in soil (Speir and Ross, 1978). Naturally occurring soil P originates from the parent minerals (Brady, 1984) and exists in a variety of organic and inorganic forms, which are in dynamic chemical equilibrium with one another (Tisdale et al., 1985).

Measurable components of the P cycle include stable P_o which is either chemically resistant to attack or protected in aggregates; moderately labile P_o; labile P_o; labile inorganic P (orthophosphate) (P_i); solution P; and stable inorganic P consisting of primary and secondary minerals and occluded P (Chauhan et al., 1981). Extraction of soil with 0.5 *M* $NaHCO_3$ recovers the most labile, biologically available P_i, labile P_i and P_o adsorbed on the soil surfaces, and a small amount of microbial P (Bowman and Cole, 1978). Fumigation of soil with chloroform before $NaHCO_3$ extraction releases additional P_i and P_o from lysed microbial cells, which can constitute as much as 40% of the microbial P present in the soil (Hedley and Stewart, 1982). Extraction of soil with 0.5 *M* H_2SO_4 recovers labile P_i and P_o as well as recently fixed P_i (Dick and Tabatabai, 1978).

Soil phosphatases are thought to play important roles in mineralization of organic P (Speir and Ross, 1978; Tisdale et al., 1985). Plants, soil fauna, and microorganisms have been shown to produce phosphatases, but most soil phosphatases are thought to originate from soil microorganisms (Ladd, 1978).

Soil bacteria have been shown to exhibit phosphatase activity in culture (Mazilkin and Kuznetsova, 1964; Cosgrove et al., 1970; Nelson, Ch. 2), but the

effects of these bacteria alone on phosphatase levels and available P in soil have received little study.

The purposes of this study were to determine the effects of five soil bacteria on soil phosphatase activity, $NaHCO_3$-extractable P, H_2SO_4-extractable P, and chloroform fumigation-released P (biomass P) of autoclaved soil samples after 7 d incubation, and to relate net increases in biomass P to *in vitro* phosphatase activity of these bacteria.

MATERIALS AND METHODS

Bacterial cultures

Three soil bacteria were used to inoculate soils based on their phosphatase activities in liquid culture (Nelson, Ch. 2). *Erwinia carotovora* subsp. *carotovora* W3C105 (Xu and Gross, 1986) demonstrated high alkaline phosphatase activity, whereas *Pseudomonas fluorescens* Pf-5 and biotype F (ATCC 17513) (Stanier et al., 1966) produced acid phosphatase. *Escherichia coli* MPH43 and MPH69 (Inouye et al., 1981) were used as the positive and negative control, respectively, for alkaline phosphatase activity. Genotypes of these two strains are as follows: *E. coli* MPH43 contains pHI-7: alkaline phosphatase structural gene cloned into pBR322. The host genotype is *araD*, Δ*lacU* 196 Sm-resistance. *E. coli* MPH69 genotype is F'Δ*lac, galU, galK,* Δ(*leu-ara*), *phoA-E*-15, *proC*::Tn*5*.

Soil Samples

The soils used in this study were Woodburn (Fine-silty, mixed, mesic Aquultic Argixerolls), a Willamette Valley floor soil with low P fixation rates, and Jory (Clayey, mixed, mesic, Xeric Haplohumults), an upland soil with high P fixation rates. Characteristics of the two soils pertinent to this study are summarized in Table 3.1. Surface (0 - 15 cm) samples were passed through a 2-mm sieve and air dried at room temperature for 48 h by spreading a thin (1 - 2-mm) layer on clean paper in the laboratory. One gram samples of the dry soil were added to 50 mL polycarbonate centrifuge tubes with screw caps and moistened to field capacity by adding 0.28 mL deionized water for Woodburn samples (Shiping Deng, personal communication) and 0.37 mL for Jory samples

Table 3.1. Some physical and chemical properties of Jory and Woodburn soils.

Characteristic	Jory soil	Woodburn soil
Clay (%)	38	25
Sand (%)	19	8
pH (1:2, soil to m*M* $CaCl_2$ ratio)	5.5	4.6
Total C (g kg^{-1})	32.7	20.9
Total P (g kg^{-1})	1.87	1.85
Inorganic P (mg kg^{-1})	1560	1525
Organic P (mg kg^{-1})	312	324

(Faustin Iyamuremye, personal communication). Five glass beads, approximately 2 mm in diameter, were added before autoclaving to all soil tubes to be used for extraction and enumeration of bacteria. The samples were weighed, allowed to sit overnight at room temperature, and autoclaved (125° C) for 30 min on three consecutive days with moisture maintained at field capacity. Fredrickson et al. (1988) reported that this procedure minimized changes in soil chemical properties compared to longer autoclaving periods.

Soil pH was measured with a glass electrode (2:1 water:soil ratio). The autoclaving procedure was found to cause a pH decrease from 5.3 to 4.9 in Woodburn soil, and from 4.9 to 4.7 in Jory soil. The bacteria in these studies would not grow in the autoclaved soil, so the samples were limed before autoclaving by adding 3 and 4 g CaO kg^{-1} soil for Woodburn and Jory soil, respectively (Peter Bottomly, personal communication). The pH of the limed autoclaved soil was 6.4 for Woodburn and 6.2 for Jory soil.

Inoculation and incubation of soil samples

Bacteria were grown in a low P_i (LP) modified CM9 liquid medium, pH 7.2, was used (Nelson, Ch. 2). Ampicillin (0.1 mg of the Na salt mL^{-1}) and streptomycin (0.02 mg mL^{-1}) were routinely added to all growth media for *E. coli* MPH43 to select for pHI-7. Bacteria from a single colony of each strain were inoculated into 15 mL LP medium in a 25 mL flask and incubated with shaking in LP medium at 30° C and 120 rpm. After 48 h, 10 mL of each bacterial culture was centrifuged at 2987 *g* for 10 min. The cells were washed with and resuspended in 0.1 *M* $MgSO_4$ (Dupler and Baker, 1984). Sterile soil samples were then inoculated with 0.1 mL of bacterial suspension, and sterile deionized water was added as needed to return the soil to field capacity. Only sterile deionized water was added to control samples.

Soil samples were incubated at 25° C for 7 d. Soil moisture was maintained at field capacity gravimetrically, adding sterile deionized water as needed on days 3, 5, and 7.

Experimental Design and Statistics

Woodburn soil experiments were run with four replicates. Bacterial plate counts and analyses for soil acid- and alkaline phosphatase were done on day 0 and day 7 for samples inoculated with each bacterial strain and for uninoculated control samples. The same procedures were followed for Jory soil except that three replicates were used and analyses for 0.5 *M* $NaHCO_3$-extractable P, 0.5 *M* H_2SO_4-extractable P, and biomass P were included on both day 0 and day 7.

Data were analyzed using analysis of variance, with Waller-Duncan K-ratio T tests and Dunnett's T tests for all treatments against a control. All probabilities were evaluated at the level of significance indicated in Results.

Soil analysis procedures

Viable bacterial counts were obtained by extracting cells from the soil samples and making serial dilutions in sterile deionized water following the method of Wollum (1982). The dilutions were then plated on Kings Medium B agar (King et al., 1974)(*P. fluorescens*) or LB agar (Miller, 1972) (other strains), as described by Gerhardt et al. (1981), and incubated at 30° C until colonies were large enough to count.

Acid- and alkaline phosphatase assays were performed by the method of Tabatabai (1982). In addition to the inoculated and uninoculated samples, three autoclaved soil samples were included in assays. These were treated in the same way as the experimental samples except that the substrate, *p*-nitrophenyl phosphate, was added to the reaction mixture after the 1-h incubation period. Values obtained for these samples were then subtracted from those for both inoculated and uninoculated soil samples. Phosphatase assays were performed on group of both inoculated and uninoculated soil samples without added toluene to determine if addition of toluene altered assay results. For comparison with *in vitro* phosphatase activity, Day 7 phosphatase activity in inoculated Jory soil samples was corrected for the background activity of control samples and expressed as nmol *p*-nitrophenol (PNP) released (10^9 colony-forming units

[cfu])$^{-1}$ min^{-1}, based on the numbers of viable bacteria recovered from the soil samples on Day 7.

Extractable P was determined by extracting soil samples with 0.5 *M* $NaHCO_3$ (Olsen and Sommers, 1982) and then developing color by the ammonium molybdate-ascorbic acid method of Murphy and Riley (1962). Biomass P was determined by chloroform fumigation of soil samples (Brookes et al., 1982), followed by extraction with 0.5 *M* $NaHCO_3$. A 2-mL aliquot of each extract was digested by acidified ammonium persulfate oxidation (EPA, 1971) and color was developed as for extractable P samples. Biomass P was then calculated as the difference between fumigated and nonfumigated samples. Recently fixed P was determined by 0.5 *M* H_2SO_4 extraction, according to the method of Dick and Tabatabai (1978).

The gain in bacterial biomass P in excess of that derived from the $NaHCO_3$-extractable P pool between Day 0 and Day 7 was calculated as follows.

$$\text{Net mobilized P} = (N_7 - N_0) + (B_7 - B_0),$$

where N_0 and N_7 = $NaHCO_3$-extractable P on Day 0 and Day 7, respectively, and B_0 and B_7 = biomass P on Day 0 and Day 7, respectively.
Net mobilized P, therefore, represents bacterial biomass P which originated either from recently fixed inorganic P or from soil P_o.

Assuming that $NaHCO_3$-extractable P is a part of the H_2SO_4-extractable P pool, the change between Day 0 and Day 7 in recently fixed P was calculated as follows:

$$\Delta \text{ Recently fixed P} = (A_7 - A_0) - (N_7 - N_0),$$

where A_0 and A_7 = H_2SO_4-extractable P on Day 0 and Day 7, respectively.

RESULTS

Bacterial numbers in soil samples

Approximately 100% of inoculated bacteria were recovered from Jory soil on the same day of inoculation (Table 3.2). The number of viable bacteria was estimated from plate counts of soil samples on Day 7 (Fig. 3.1). *E. coli* MPH43 and *E. carotovora* W3C105 either did not increase in numbers or could not be recovered from Woodburn soil on Day 7, but increased in Jory soil. *P. fluorescens* strains increased in both soils. The greatest increase, from 1.8 X 10^4 to 2.3 X 10^8 cfu g^{-1} was shown by *E. carotovora* W3C105 in Jory soil. No viable bacteria were counted in control samples on Day 0 or Day 7, indicating no detectable contaminants.

Soil acid- and alkaline phosphatase

In both Jory and Woodburn soils, samples inoculated with *P. fluorescens* Pf-5 produced greatly elevated acid phosphatase activity on Day 7 (Figs. 3.2 and 3.3). Other inoculated strains had no effect on acid phosphatase activity (Fig. 3.3). Alkaline phosphatase activities in Jory soil containing *E. coli* MPH43 and *E. carotovora* W3C105 were significantly higher than control samples on Day 7 (Fig. 3.4). Samples inoculated with *P. fluorescens* biotype F and *E. coli* MPH69 showed no significant alteration of acid- or alkaline phosphatase activity in Jory soil.

The addition of toluene to soil samples during assay procedures did not significantly alter phosphatase values ($p < 0.05$).

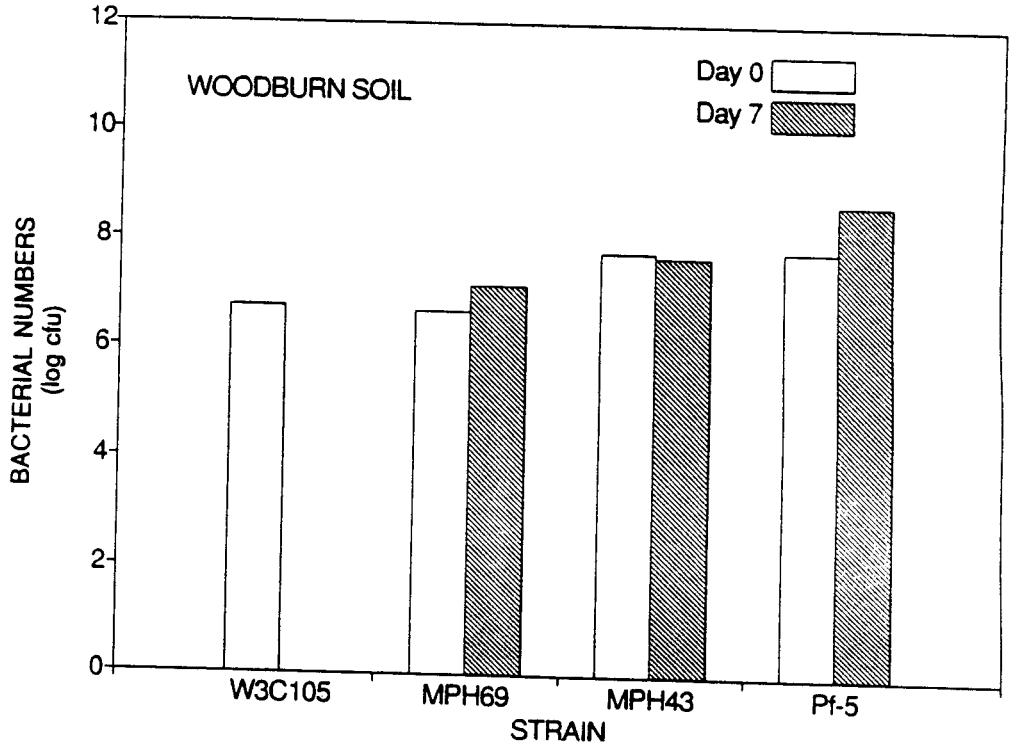
Acid-extractable P

Results of extraction of soil samples with 0.5 *M* H_2SO_4 on Day 0 were

Table 3.2. Numbers of bacteria recovered from inoculated Jory soil samples on Day 0, as determined by plate counts.

Strain	Bacterial numbers	
	Inoculated	Extracted
	----------cfu----------	
E. carotovora W3C105	1.0×10^4	1.8×10^4
E. coli MPH69	4.4×10^7	3.6×10^7
E. coli MPH43	5.5×10^7	5.0×10^7
P. fluorescens Pf-5	1.1×10^8	8.8×10^7
P. fluorescens Biotype B	2.9×10^7	2.6×10^7

Fig. 3.1. Plate counts of bacteria recovered from Woodburn and Jory soil samples after 0 and 7 d incubation.


WOODBURN SOIL
Day 0
Day 7
BACTERIAL NUMBERS (log cfu)
0
2
4
6
8
10
12
W3C105
MPH69
MPH43
Pf-5
STRAIN

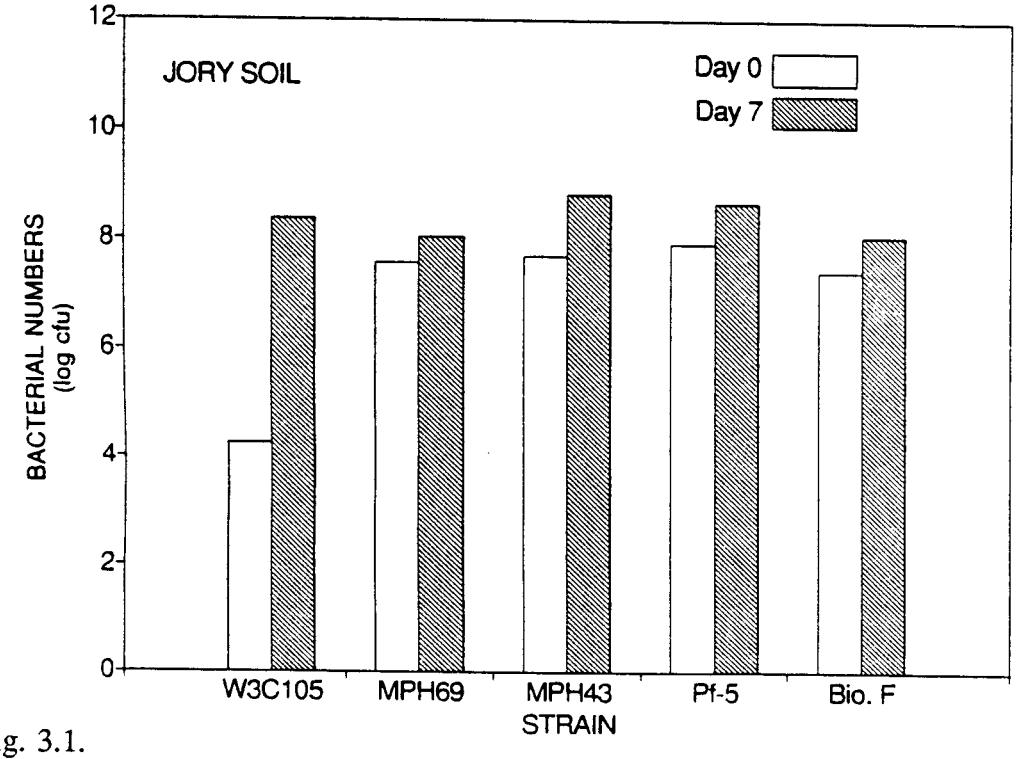

JORY SOIL
Day 0
Day 7
BACTERIAL NUMBERS (log cfu)
0
2
4
6
8
10
12
W3C105
MPH69
MPH43
Pf-5
Bio. F
STRAIN

Fig. 3.1.

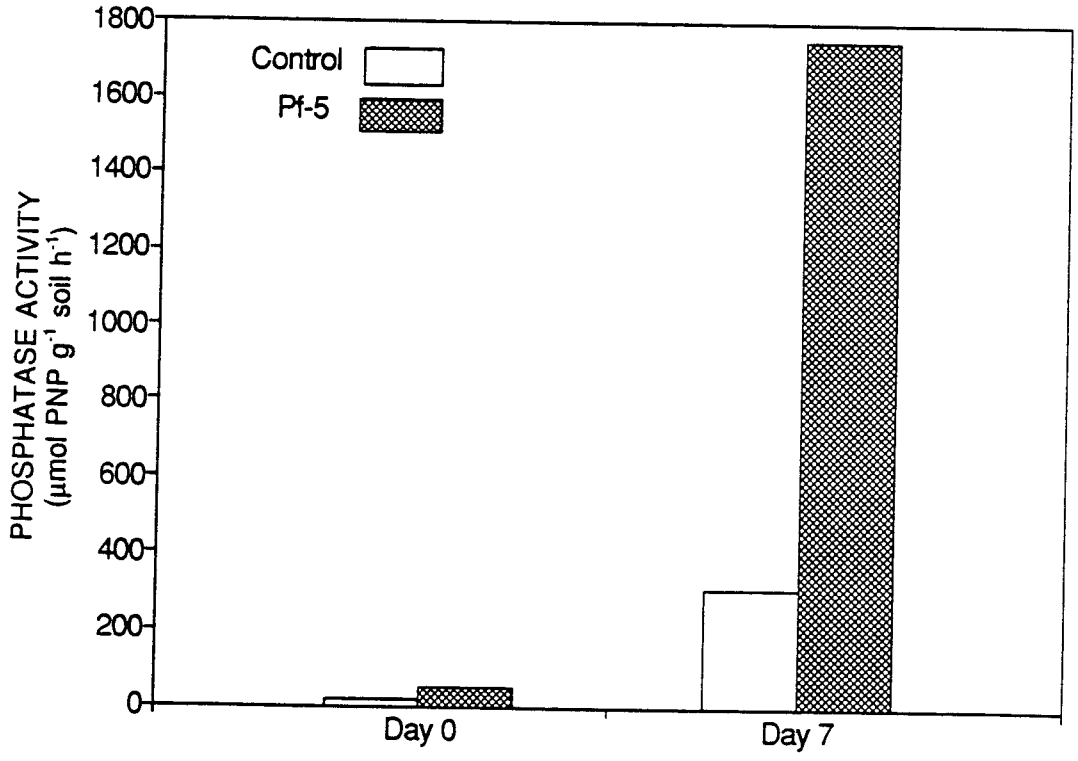
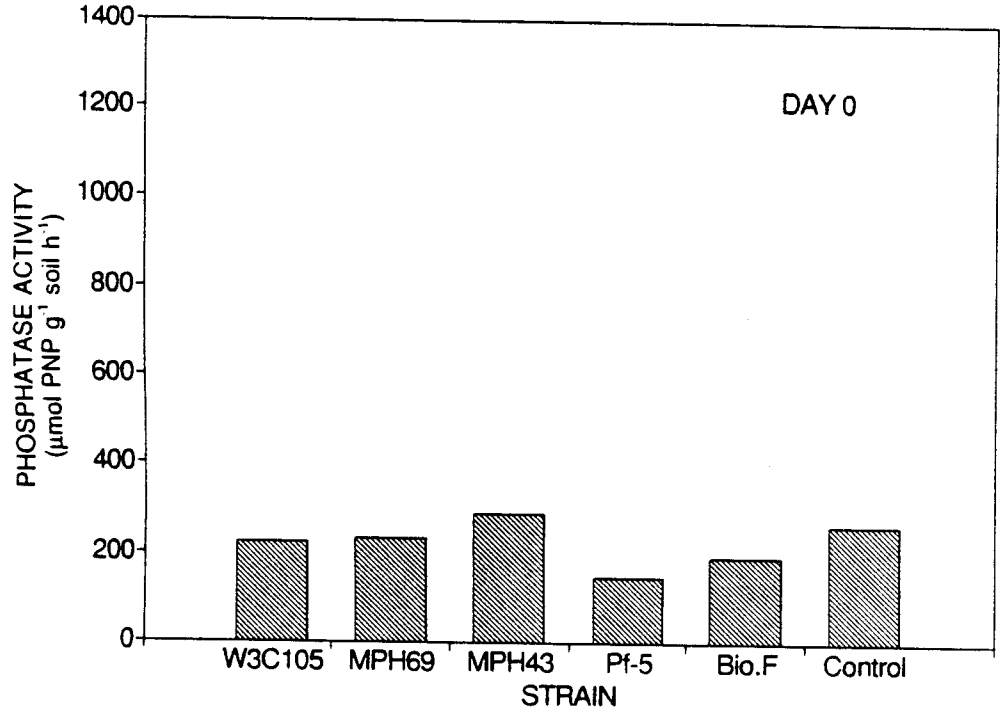


Fig. 3.2. Acid phosphatase activity in *P. fluorescens* Pf-5-inoculated and uninoculated (control) Woodburn soil samples after 0 and 7 d incubation. Values with the same letter are not significantly different ($p = 0.0001$).

Fig. 3.3. Acid phosphatase activity in inoculated and control samples of Jory soil after 0 and 7 d incubation. Values with the same letter are not significantly different. Values with * are significantly different from the control ($p = 0.05$).


1400
1200
1000
800
600
400
200
0
PHOSPHATASE ACTIVITY
(µmol PNP g-1 soil h-1)
DAY 0
W3C105
MPH69
MPH43
Pf-5
Bio.F
Control
STRAIN

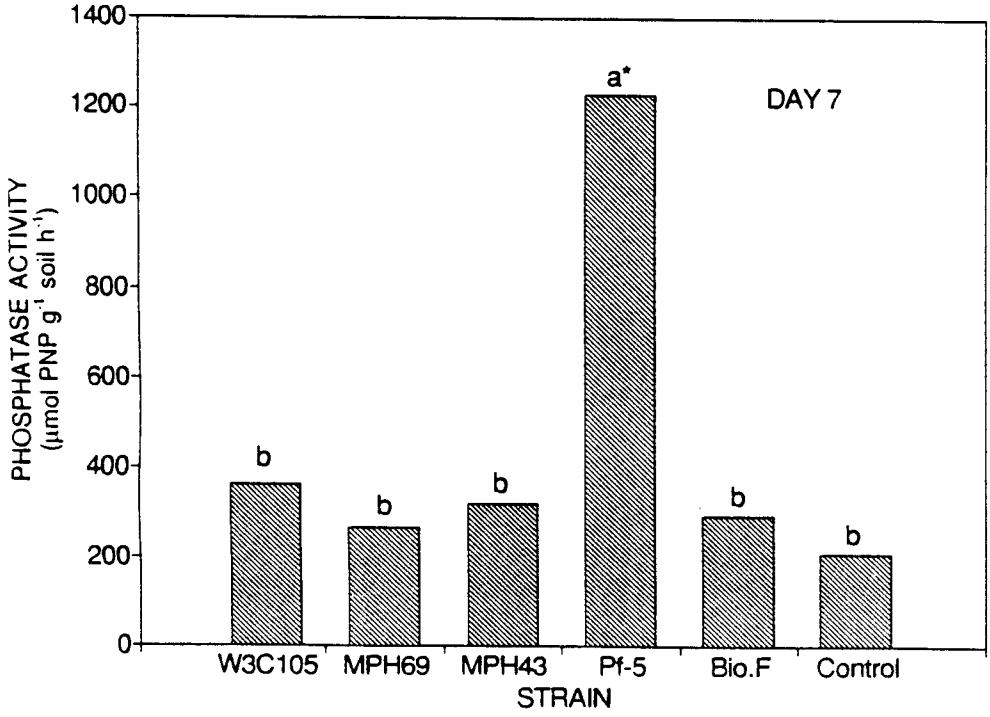
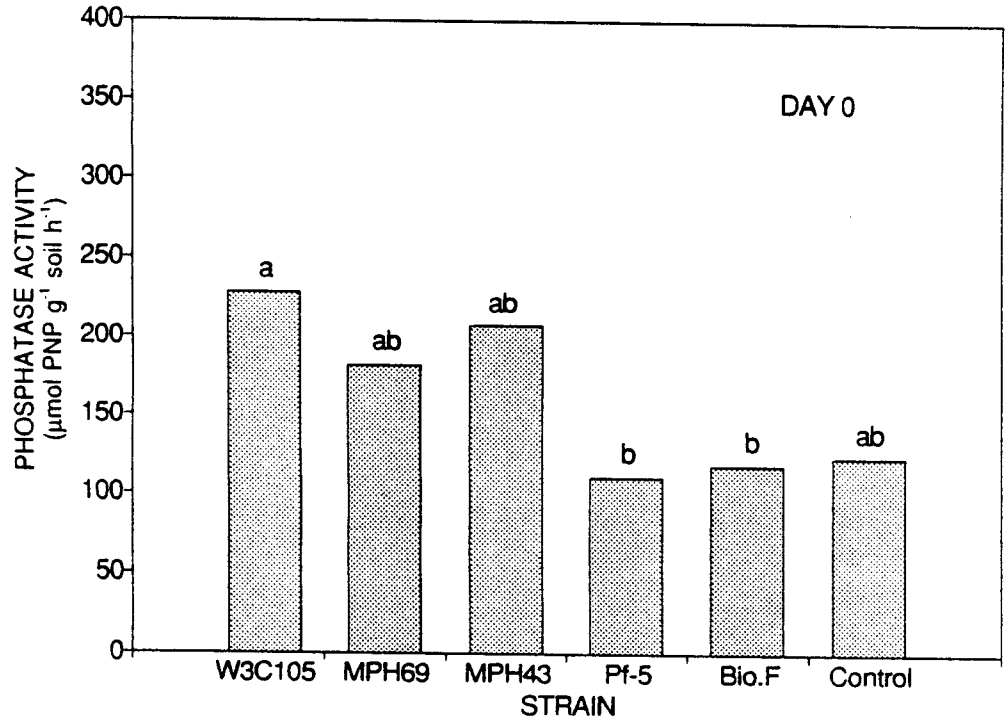

1400
1200
1000
800
600
400
200
0
PHOSPHATASE ACTIVITY
(µmol PNP g-1 soil h-1)
DAY 7
a*
b
b
b
b
b
W3C105
MPH69
MPH43
Pf-5
Bio.F
Control
STRAIN

Fig. 3.3.

Fig. 3.4. Alkaline phosphatase activity of inoculated and control samples of Jory soil after 0 and 7 d incubation. Values with the same letter are not significantly different. Values with * are significantly different from the control ($p = 0.05$).


DAY 0
PHOSPHATASE ACTIVITY
(µmol PNP g-1 soil h-1)
400
350
300
250
200
150
100
50
0
a
ab
ab
b
b
ab
W3C105
MPH69
MPH43
Pf-5
Bio.F
Control
STRAIN

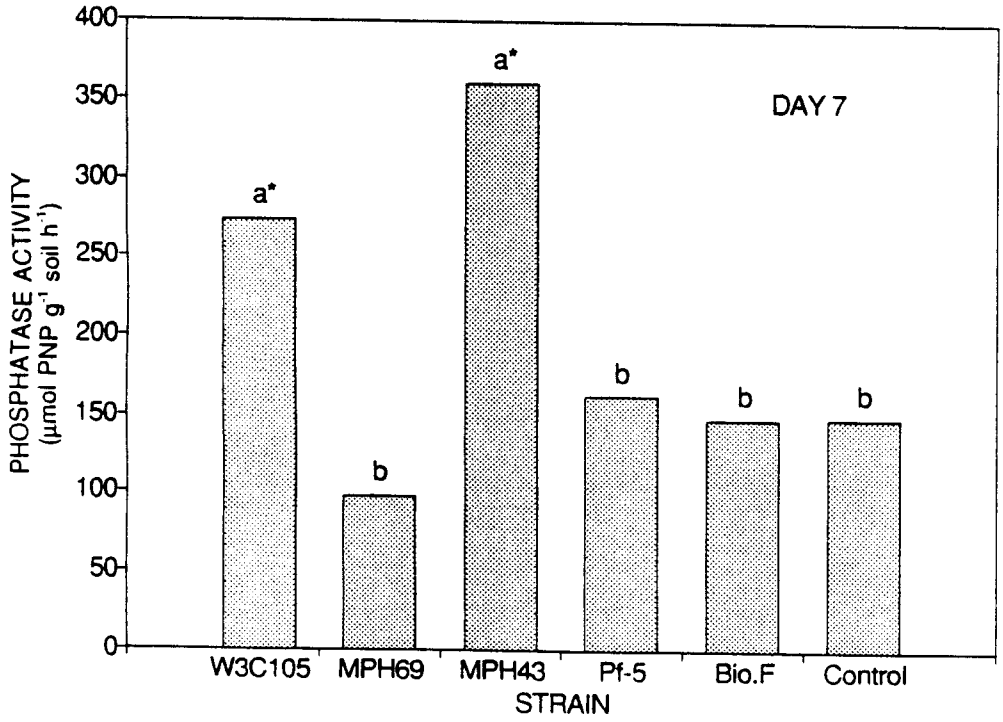

DAY 7
PHOSPHATASE ACTIVITY
(µmol PNP g-1 soil h-1)
400
350
300
250
200
150
100
50
0
a*
b
a*
b
b
b
W3C105
MPH69
MPH43
Pf-5
Bio.F
Control
STRAIN

Fig. 3.4.

not significantly different from one another (Fig. 3.5). The same was true on Day 7. Significant ($p = 0.05$) decreases between Day 0 and Day 7 were observed for *E. coli* MPH69 and MPH43, *P. fluorescens* Pf-5, and control samples; although not significant at that level, the same trend was noted for *E. carotovora* W3C105 and *P. fluorescens* biotype F.

Bicarbonate-extractable P

The amount of P extracted by 0.5 *M* $NaHCO_3$ from control samples was significantly higher than that extracted from the inoculated samples on Day 0; on Day 7, P extracted from control samples exceeded that in *E. coli* MPH43 samples, but did not differ significantly from that from other samples (Fig. 3.6). For all samples except those containing *E. carotovora* W3C105, significant P decreases occurred between Day O and Day 7 ($p = 0.05$), and a similar trend was observed for samples containing W3C105.

Biomass P

No significant differences in biomass P were noted between strains on Day 0 (Fig. 3.7). On Day 7, however, the control samples contained significantly less biomass P than did the inoculated samples. A significant increase in biomass P between Day 0 and Day 7 was noted for *E. carotovora* W3C105, *E. coli* MPH43, and *P. fluorescens* Pf-5 ($p = 0.05$). Although not significant at this level, the same trend was observed for *E. coli* MPH69 and *P. fluorescens* biotype F.

Changes between Day 0 and Day 7 in biomass P, H_2SO_4-extractable P, $NaHCO_3$-extractable P and recently fixed P are shown in Table 3.3. Inoculated soil samples gave increases in biomass P more than ten times that for control samples. No clear trend was observed within the other P fractions, although the control samples appeared to lose more P from the recently fixed fraction than did inoculated samples.

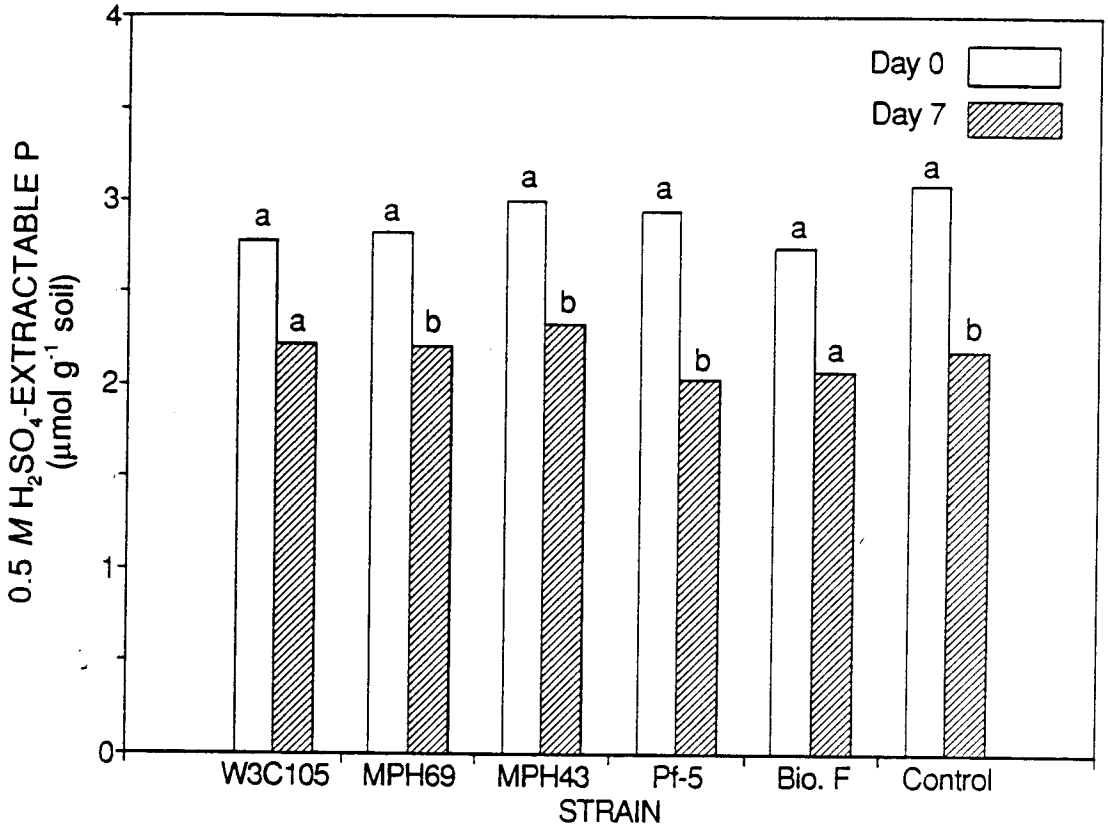


Fig. 3.5. Phosphorus extracted by 0.5 *M* H_2SO_4 from inoculated and control samples of Jory soil after 0 and 7 d incubation. Values were not significantly different from controls at either time. Values with the same letter are not significantly different within each strain (p = 0.05).

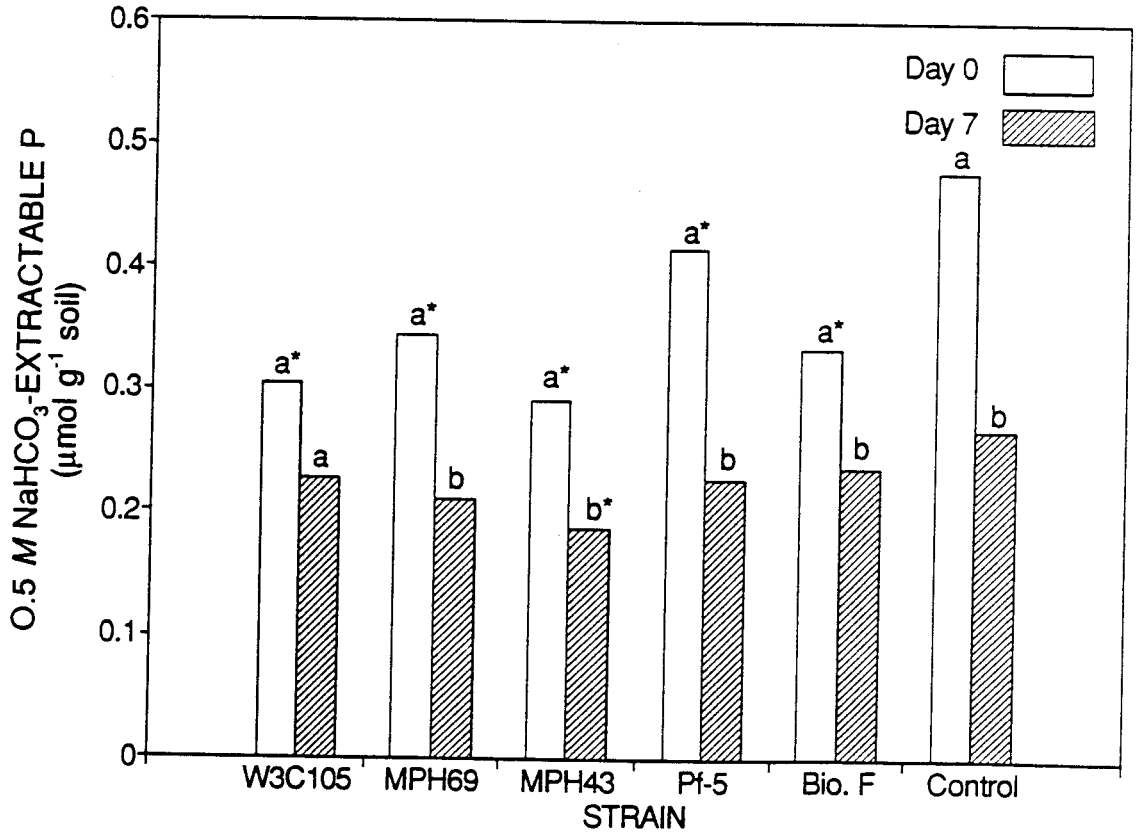


Fig. 3.6. Phosphorus extracted by 0.5 *M* $NaHCO_3$ from inoculated and control samples of Jory soil after 0 and 7 d incubation. Values with the same letter are not significantly different within each strain. Values with * are significantly different from the control on that day ($p = .05$).

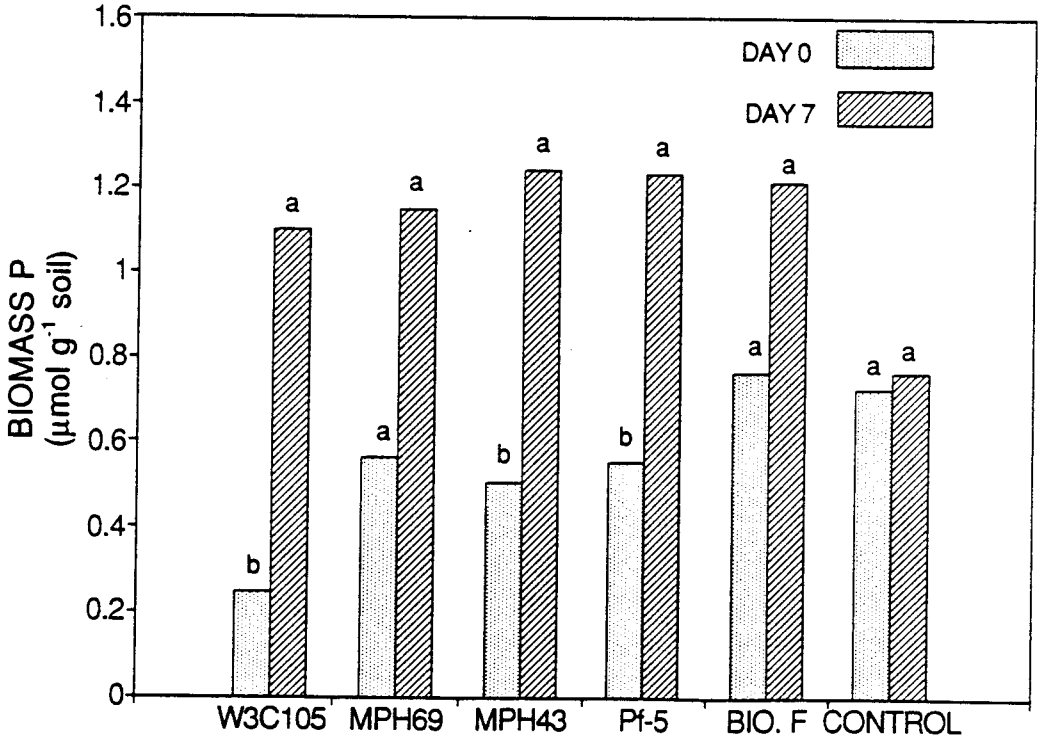


Fig. 3.7. Biomass P content of inoculated and control samples of Jory soil after 0 and 7 d incubation. Values with the same letter are not significantly different within each strain. Values with * were significantly different from the control on that day (p = 0.05).

Table 3.3. Changes in soil P pools of inoculated and control samples of Jory soil during 7 d incubation.

Strain	Δ Biomass P	Δ H_2SO_4-extractable P	Δ $NaHCO_3$-extractable P	Δ Recently fixed P†
	---------- μmol P g^{-1} soil----------			
E. carotovora W3C105	0.85	-0.57	-0.08	-0.49
E. coli MPH69	0.58	-0.62	-0.13	-0.49
E. coli MPH43	0.74	-0.67	-0.10	-0.57
P. fluorescens Pf-5	0.68	-0.91	-0.19	-0.72
P. fluorescens biotype F	0.45	-0.67	-0.10	-0.57
Control	0.04	-0.90	-0.21	-0.69

†(Δ H_2SO_4-extractable P) - (Δ $NaHCO_3$-extractable P)

Net mobilized P

All of the inoculated soil samples showed an increase in biomass beyond that lost from the $NaHCO_3$-extractable fraction (Fig. 3.8). The control samples, however, exhibited a net loss of biomass P.

Phosphatase activities of inoculated Jory soil samples, corrected for activity of controls and converted to nmol PNP released $(10^9 \text{ cfu})^{-1}$ min^{-1}, are compared with phosphatase activities of the same strains *in vitro* (Table 3.4). There appears to be a relationship between *in vitro* alkaline phosphatase activity and that in soil for *E. carotovora* and *E. coli* strains, but none for acid phosphatase activity of all strains or for alkaline phosphatase activity of the two *Pseudomonas* strains.

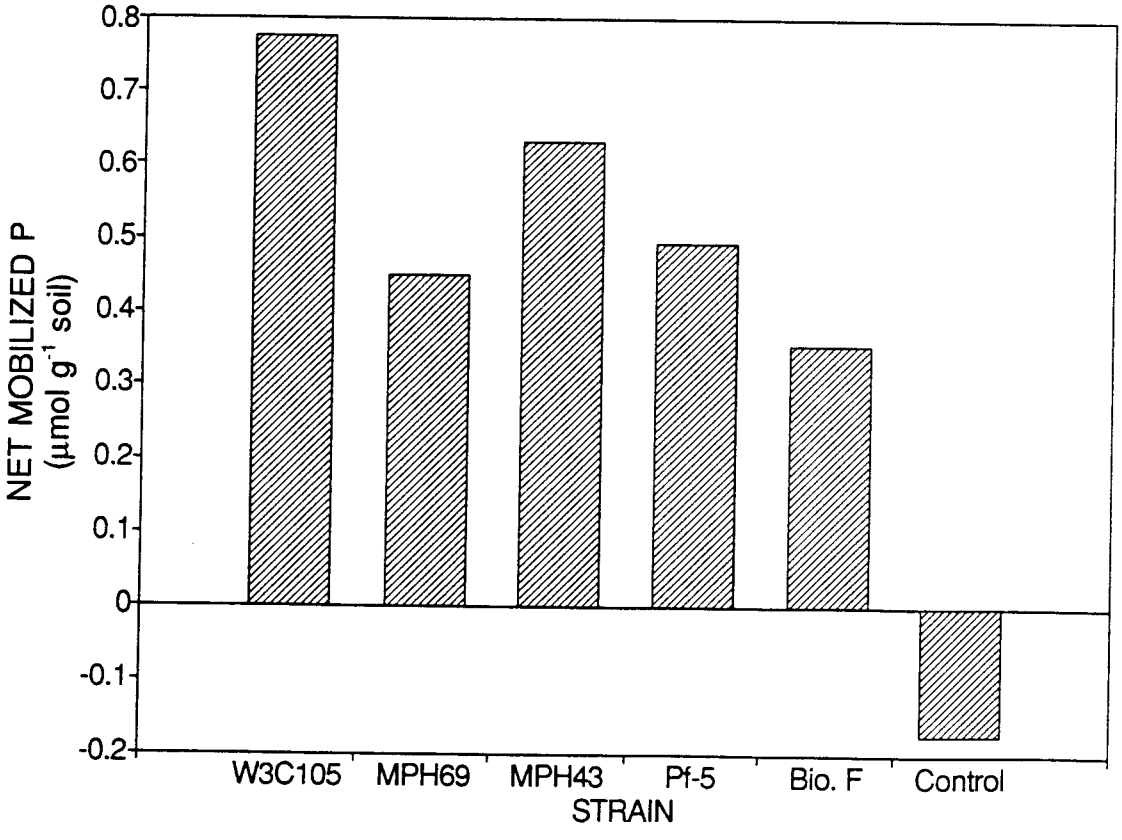


Fig. 3.8. Net increase in mobilized P of inoculated and control samples of Jory soil during the 7 d incubation period.

Table 3.4. Comparison of phosphatase activity of five soil bacteria strains *in vitro* and after 7 d incubation in Jory soil.

Strain	Acid phosphatase		Alkaline phosphatase	
	In vitro†	In soil, Day 7	*In vitro*	In soil, Day 7
	----------nmol PNP released (10^9 cfu)$^{-1}$ min^{-1}----------			
E. carotovora W3C105	11.5	81.2	91.9	65.9
E. coli MPH69 (Pho^-)	0.0	60.4	0.0	0.0
E. coli MPH43 (Pho^+)	11.6	18.4	41.2	35.3
P. fluorescens Pf-5	1.8	256.4	0.6	3.5
P. fluorescens biotype F	9.7	83.5	3.1	0.0

†Culture medium contained 0.2 m*M* P_i. Cultures were incubated 48 h.

DISCUSSION

Phosphatase activity in soil

Inoculation of soil samples with *P. fluorescens* Pf-5 increased soil acid phosphatase activity more than 8-fold and 17-fold in Jory and Woodburn soils, respectively. *P. fluorescens* biotype F, which gave somewhat higher acid phosphatase activity than Pf-5 *in vitro*, showed no significant acid- or alkaline phosphatase activity in soil. Jory soil samples inoculated with both *E. carotovora* W3C105 and *E. coli* MPH43 (Pho^+) showed significant increases in alkaline phosphatase activity on Day 7, whereas samples inoculated with *E. coli* MPH43 (Pho^-) gave no significant increase in either acid- or alkaline phosphatase. These results are consistent with those of *in vitro* studies of these three organisms. It is interesting to note that alkaline phosphatase levels of Day 0 samples inoculated with W3C105 were significantly higher than those inoculated with Pf-5 and biotype F, despite the fact that W3C105 inoculum levels were only 10^{-4} those of Pf-5 and biotype F. This is consistent with results of *in vitro* studies, in which W3C105 exhibited alkaline phosphatase activity over 30 times that of Pf-5 and biotype F.

Autoclaving did not completely inhibit phosphatase activity in uninoculated samples (Figs. 3.2 - 3.4) which runs contrary to work done by Eivazi and Tabatabai (1977). Air-dry Woodburn soil autoclaved for 1 h gave acid- and alkaline phosphatase activities of 0.88 and 1.33 μmol PNP g^{-1} h^{-1}, respectively, considerably higher than the 0.0 to 0.03 μmol PNP g^{-1} h^{-1} on a range of Midwest soils reported by Eivazi and Tabatabai (1977). Liming Woodburn soil with CaO did not change these values appreciably. Wetting the soils to field capacity and autoclaving 30 min on 3 consecutive days, as was done in this study, resulted in acid phosphatase activities of 0.2 and 1.9 μmol PNP g^{-1} h^{-1} for Woodburn and Jory soil, respectively, and alkaline phosphatase activities of 1.1 and 0.9 μmol PNP g^{-1} h^{-1}. Perhaps some of the soil phosphatase

was protected by other soil components from denaturation during the short autoclaving periods. It may be, also, that PNP is released from *p*-nitrophenyl phosphate not only by phosphatase, but also by inorganic chemical processes occurring in the soil samples. It should be noted, too, that the soils in this study formed under conditions somewhat different than those that influenced the Midwest soils.

The similarity in phosphatase assay results between toluene-treated and untreated soil samples is consistent with the conclusion of Eivazi and Tabatabai (1977) that phosphomonoesterase and phosphodiesterase activity of soil is not affected by addition of toluene during short assays.

Numbers of bacteria in soil

Little is known about the changes that occur in soil that is repeatedly autoclaved. Inorganic chemical reactions and denaturation of organic soil components probably occur, resulting in the lowering of soil pH observed in this study as well as other unobserved effects which may be deleterious to microorganisms. It was not surprising, therefore, to find that *E. carotovora* and *E. coli* MPH69 did not flourish in autoclaved Woodburn soil, despite the addition of CaO to raise the pH. Why these same organisms appeared to thrive in limed, autoclaved Jory soil is unknown.

Plate counts of bacteria in Jory soil samples on Day 7 showed 10^8 - 10^9 cfu g^{-1}, which compares favorably with 10^8 cfu g^{-1} found in the top 5 cm of native grassland soil (Paul and Clark, 1989) by the same method. Thus, the numbers of bacteria recovered in this study were typical of total numbers of rhizosphere bacteria recovered in the field.

Although *E. carotovora* W3C105 numbers were relatively small (10^4 cfu g^{-1}) on Day 0, they had surpassed 10^8 cfu g^{-1} by Day 7. Informal counts indicated that this increase in numbers occurred early in the incubation period, probably within the first three days, and that bacterial numbers remained fairly constant during the fourth through seventh day.

Fate of soil P components

It is clear that biomass P increased in inoculated soil samples during incubation (Fig. 3.7). That some of this P came from a source other than readily available P_i or P_o is shown by the positive net immobilized P values for soil inoculated with all strains (Fig. 3.8). It could be argued that as the readily available P_i is depleted more of the recently fixed P_i may become available; however, net immobilized P of *E. coli* MPH43 and *E. carotovora* W3C105 exceeded losses of recently fixed P by .066 and .283 μmol P g^{-1}, respectively. Thus, at least this excess net immobilized P may have originated from organic P fractions of the soil.

It appears that a relationship exists between increases in numbers and net immobilized P, at least within closely related groups such as *Enterobacteriaceae* or the pseudomonads (Table 3.5). Net immobilized P also appears to be related to soil alkaline phosphatase activity on Day 7, but both net immobilized P and alkaline phosphatase activity may be reflections of increases in bacterial numbers. No relationship appears to exist between acid phosphatase activity and either net immobilized P or increases in numbers.

Comparison of in vitro- and soil phosphatase activity

Information available about *E. coli* phosphatases indicates that alkaline phosphatase induction and repression are under the control of elaborate mechanisms which respond to levels of P_i in the environment, and that acid phosphatases appear to be influenced by a variety of substrates and conditions. Although little is known about control of phosphatase production in the other organisms studied, they are probably as complex and varied as those of *E. coli*. It is not surprising to find that in most cases acid- and alkaline phosphatase activities of a bacterial strain in soil do not correlate with the activities of the same organism grown in liquid culture (Table 3.4), given the differences that must exist between the environment in autoclaved soil and that of a liquid medium.

Table 3.5. Comparison of increases in bacterial counts in Jory soil with net biomass P for the five strains studied.

Strain	Magnitude of increase in numbers	Net biomass P	Soil phosphatase activity, Day 7	
			Acid phospha-tase	Alkaline phospha-tase
		μmol P g soil^{-1}	nmol PNP released (10^9 cfu)$^{-1}$ min $^{-1}$	
Enterobacteriaceae				
W3C105	10 000 X	0.78	81.2	65.9
MPH69	3 X	0.45	60.4	0.0
MPH43	12 X	0.63	18.4	35.3
Pseudomonads				
Pf-5	52 X	0.49	256.4	3.5
Biotype F	40 X	0.36	83.5	0.0

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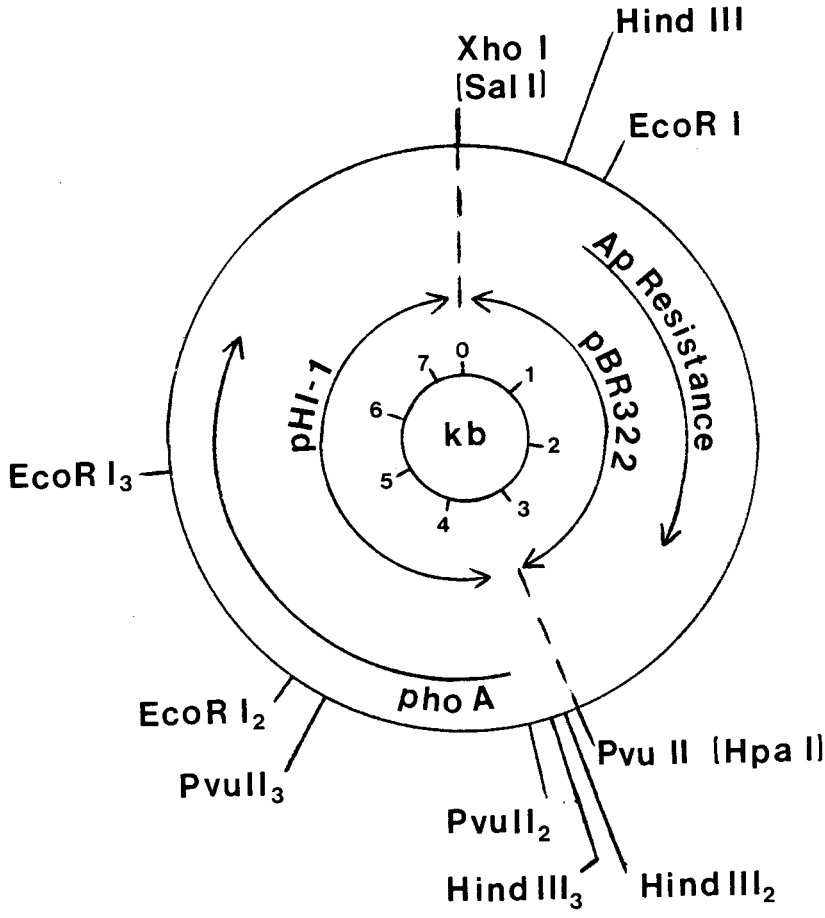
APPENDIX



Map of *E. coli* plasmid pHI-7, formed by joining fragments of pBR322 and pHI-1. The resulting plasmid bears a gene for ampicillin (Ap) resistance and the *phoA* gene, which has two *Pvu*II cleavage sites.