AMBIENT pH SIGNALING INFLUENCES PHOSPHATE TRANSPORT IN *Neurospora crassa*

A Thesis

by

PATRICK WADE KENNEDY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Microbiology

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Approved by:

Chair of Committee, Wayne K. Versaw
Committee Members, Deborah Bell-Pedersen
Brian Shaw
Head of Department, Vincent Cassone

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ABSTRACT

Ambient pH Signaling Influences Phosphate Transport in

Neurospora crassa. (December 2005)

Patrick Wade Kennedy, B.S., Northeast Louisiana University

Chair of Advisory Committee: Dr. Wayne K. Versaw

In the course of our efforts to resolve Pi transport mechanisms in the model fungus *Neurospora crassa* we identified a null mutant, *par-1*, that displays enhanced Pi transport activity specifically under alkaline growth conditions. The PAR-1 protein is related to PalF of *Aspergillus nidulans*, which is one component of an ambient pH signaling pathway that is conserved among fungi. A deletion mutant for the PacC homolog, another component of the same pathway, phenocopies *par-1*, demonstrating that a defect in pH signaling is responsible for the altered Pi transport activity. Our results indicate that pH signaling in *N. crassa* plays an important role in coordinating high and low affinity Pi transport in response to ambient pH, but through different mechanisms. Sulfate acquisition also is influenced by pH signaling, suggesting that this regulatory system has a broad role in nutrient uptake and homeostasis.

DEDICATION

This thesis is dedicated to Andrion, Brianne, Herman, Carolyn and Thelma for all of their continued love, patience and support.

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I would like to especially thank Dr. Wayne Versaw for advice, encouragement, patience, guidance and support during my time as a graduate student at Texas A&M University. I will always have fond memories of our enlightening yet enjoyable discussions. Your energy and effort were definitely appreciated.

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CHAPTER I

INTRODUCTION

Phosphorous is an essential nutrient needed to make RNA, DNA, phospholipids and active metabolites such as ATP. Although phosphorus is plentiful in most environments it is often the limiting factor for growth of microorganisms mainly because orthophosphate (Pi), the assimilated form of phosphorus, exists primarily as insoluble salts or organic forms that cannot be used directly. Phosphate acquisition has been studied in many organisms (3, 4, 7, 24, 32, 37, 46, 55-57), and a shared characteristic is the use of two modes of phosphate uptake: a high affinity (low K_m) system, which is regulated in response to Pi availability, and a low affinity (high K_m) system, which is constitutively expressed. Despite this common theme, the roles of the two uptake modes appear to be uniquely divided in the filamentous fungus *Neurospora crassa*. The high affinity uptake system functions solely under Pi limiting conditions. This is evident from mutants devoid of high affinity transport, which are indistinguishable from wild type when grown on replete conditions (52, 54). Furthermore, the expression of other Pi regulated genes in such mutants is unaffected (52, 54). In contrast, the high affinity uptake systems in *Escherichia coli* and *Saccharomyces cerevisiae* play a significant role in Pi acquisition under replete as well as Pi limiting growth conditions. The high affinity system in both these model organisms is stimulated under Pi limiting conditions, but mutations in a high affinity transporter gene results in reduced growth rates under replete

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This thesis follows the style of Eukaryotic Cell.

conditions and leads to constitutive expression of at least some Pi regulated genes (6, 26). The basis for the difference in how *N. crassa* utilizes the two Pi uptake modes is unknown but may reflect a unique mechanism for sensing Pi, or alternatively, a significantly more robust low affinity system that is sufficient for growth under all but the most restricted conditions.

The high affinity system of *N. crassa* is composed of two structurally and mechanistically unrelated transporters, PHO-4 and PHO-5 (54). PHO-4, a Na^+/Pi symporter with a K_m of approximately 3 μM shares no sequence similarity with PHO-5, a H^+/Pi symporter with a K_m of approximately 37 μM (52, 53). These transporters are relatively insensitive to pH over a range of 4-8 although PHO-5 activity is limited at pH ≥ 7.6 (32, 54). The *pho-4*$^+$ and *pho-5*$^+$ genes, as well as other genes involved in scavenging Pi from the environment, are transcriptionally regulated via a phosphorus signaling cascade composed of the NUC-2, PREG, PGOV and NUC-1 proteins (24, 37) (Fig. 1). Briefly, the NUC-2, PREG and PGOV proteins are negative regulators that function to maintain NUC-1, a basic helix-loop-helix zipper protein, located predominantly in the cytosol when Pi concentrations are adequate. Under low Pi conditions, Pi repression is relieved and NUC-1 enters the nucleus where it activates transcription of Pi responsive genes (37). The components of this phosphorus signaling system and the respective functions are conserved in *S. cerevisiae* (26, 46).

The low affinity Pi uptake system of *N. crassa* is intriguing in that its affinity for Pi is strongly affected by extracellular pH. The apparent K_m increases nearly 400 fold as ambient pH increases from 4.0 to 7.3 (31). This effect cannot be explained solely based

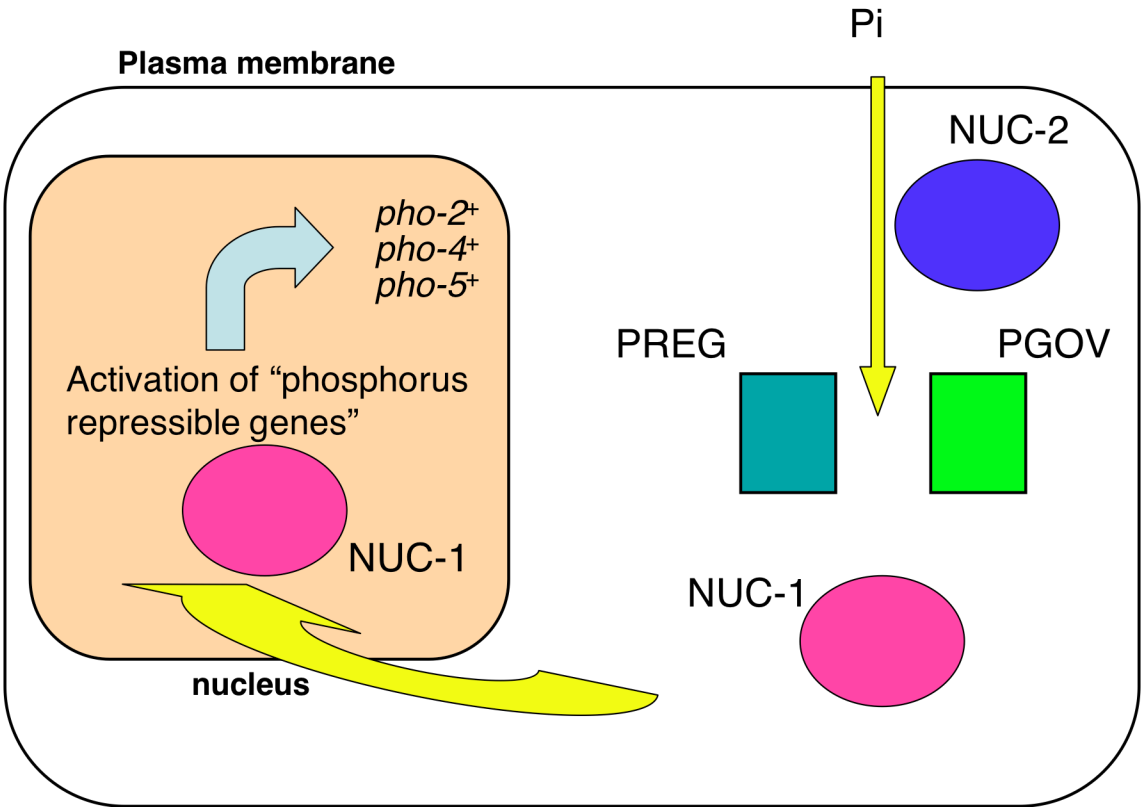

FIG. 1. Model of *N. crassa* phosphorus regulatory pathway (37).

upon the ionic form of Pi, suggesting that a complex mechanism exists for modulating activity under different ambient conditions. A consequence of increasing K_m with rising ambient pH is that the low-affinity system is unable to support growth under the combined conditions of low Pi concentration and pH above neutrality without the assistance of at least one of the high-affinity transporters. That is, a null *pho-4; pho-5* double mutant is unable to grow under selective conditions of low Pi (50 μM) and high pH (≥ pH 7.2) (52). A similar relationship between ambient pH and low affinity Pi transport has not been reported for other systems, but it may exist.

The transporter(s) responsible for low affinity Pi uptake in *N. crassa* is unknown. However, Metzenberg (21) isolated the *hpp* (housekeeping phosphate permease) mutant (38) as a suppressor of the *pho-4; pho-5* double mutant's growth defect on low Pi, high pH medium. Because this mutant lacks the high affinity transport system, growth under the selective conditions must reflect a change in the remaining low affinity mode of transport. This change could arise from a mutation either in the structural gene for a low affinity transporter or a gene involved in regulating transport. In an effort to distinguish these possibilities we cloned the affected gene and report here that it codes for a protein related to the ambient pH signaling regulator PalF of *A. nidulans* (41). Based on our findings we have renamed *hpp* as *par-1* (phosphate acquisition regulator).

Cellular responses to ambient pH, and especially the regulation of these responses, have been studied in several fungal species (13, 14, 22, 29), and extensively in *A. nidulans* (2, 8, 19, 44, 45, 50) (Fig. 2). In short, when *A. nidulans* is exposed to alkaline pH, a signal is transduced via six Pal proteins, which results in the proteolytic processing

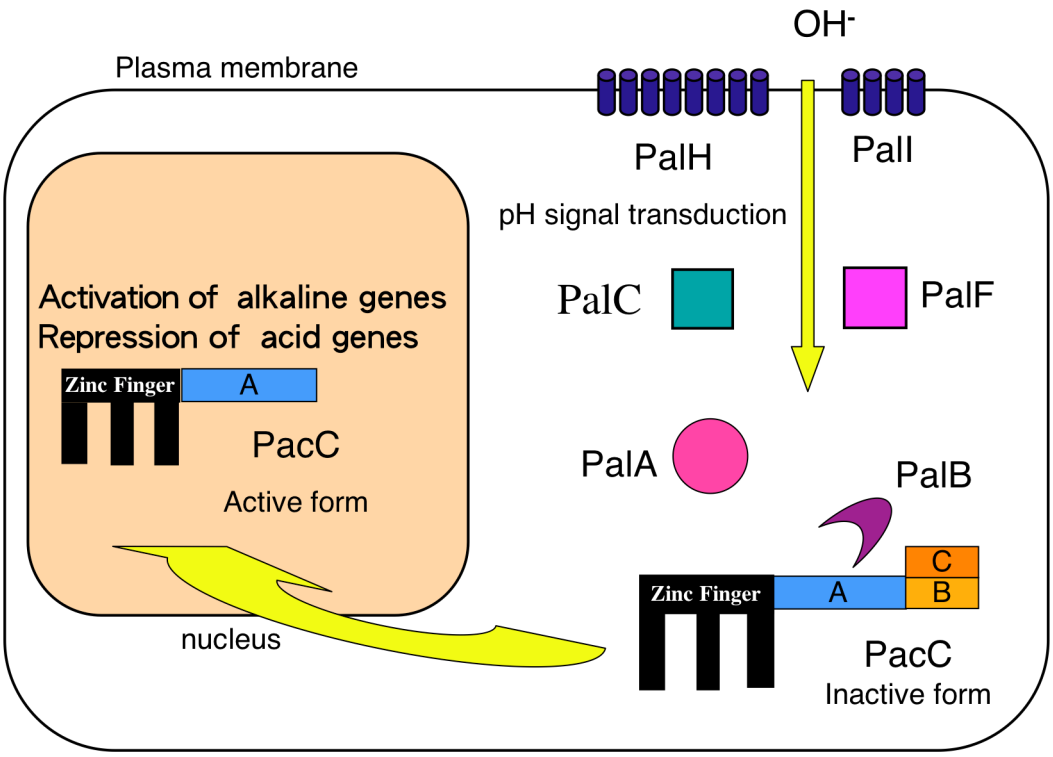

FIG. 2. Model of *A. nidulans* pH signal transduction pathway (44).

of the zinc-finger transcription factor PacC (15, 40). Once processed, PacC is able to enter the nucleus where it activates transcription of genes required for growth under alkaline conditions and also to repress transcription of a number of genes normally expressed solely under acidic conditions (43)**.** Thus, null mutations in *PacC* or any of the *Pal* genes, results in an "acid mimicking" phenotype.

The relationship between ambient pH and phosphate acquisition generally has been limited to the chemical properties of Pi (solubility, ionic form) and/or transport energetics. Here we report that *N. crassa* possesses an ambient pH signaling system analogous to those reported in other fungi (44), and that this signaling pathway influences both low-affinity and high-affinity Pi transport, as well as sulfate acquisition.

CHAPTER II

MATERIALS AND METHODS

Materials used

Strains *Neurospora crassa* strains used in this study are listed in Table 1. The Δ*par-1::Hph* and Δ*par-2::Hph* mutations are targeted deletions in which the respective coding regions were replaced with a hygromycin phosphotransferase cassette by homologous recombination (11). Homokaryotic isolates were obtained from backcrosses and the mutations were verified by Southern analysis. Null alleles of *pho-4* and *pho-5* were obtained by the repeat-induced point mutations (RIP) process (47) and have been described (54).

Growth Media Fries medium lacking KH_2PO_4, and the resulting deficit of K ions made up with KCl was prepared as described (39). Phosphate salts were added as indicated. Sucrose (1.5% w/v) was included in liquid growth media and a combination of sorbose, glucose and fructose (1, 0.05 and 0.05% w/v, respectively) was included in agar-solidified media. A single lot of agar that contributed less than 3 μM Pi to the medium when used at 1.5% (w/v) was used throughout this study. Unless specified otherwise, 100 mM Tris-Mes buffer was included to maintain pH at the indicated value.

Growth Measurements To assess vegetative growth quantitatively, conidia from 5 day old cultures were suspended in the appropriate liquid medium at $5x10^6$/ml in silanized flasks. Cultures were incubated at 30ºC with constant agitation (250 rpm), and aliquots were removed at intervals for determination of dry weight or total nucleic acids as

Table 1. *Neurospora crassa* strains

Strain	Genotype	Reference
FGSC #2489	74-OR23-IV *Mat* A "wild type"	
FGSC #8347	*pho-4; pho-5; Mat* A	(54)
FGSC #8348	*par-1 Mat* A	(38)
	Δ*par-1::Hph Mat* A	This study
	Δ*par-2::Hph Mat* A	This study
	Δ*par-1::Hph; pho-4; pho-5; Mat* A	This study
	Δ*par-2::Hph; pho-4; pho-5; Mat* A	This study

indicated. Nucleic acids were calculated from the absorbance of NaOH lysates at 260 nm as described (54).

Phosphate Uptake Conidia from 5 day old cultures were suspended at 10^5/ml in medium containing 10 mM Pi, and a final pH of 5.5 or 7.5 as indicated. Cultures were incubated at 30ºC with constant agitation (250 rpm) for 4 hr (pH 5.5 media) or 6 hr (pH 7.5 media). These conditions provided homogenous cultures at equivalent growth stages as determined by dry weight and total nucleic acids. Germlings were harvested by filtration then washed and suspended in ice-cold water at $2x10^6$/ml. An aliquot of the suspension was added to an equal volume of uptake solution (2x Fries medium lacking Pi and sucrose but containing 50 mM Tris-Mes at desired pH), and incubated at 30ºC for 10 min before the addition of ^{32}P-orthophosphate to the desired concentration. Samples were harvested at intervals by filtration, washed three times with 20 ml of 1 mM K-phosphate buffer (pH 6.5), and transferred to scintillation vials containing 4 ml of 0.1 N NaOH. Pi uptake was determined by Cerenkov counting. To normalize samples we harvested equivalent aliquots of germlings and determined total nucleic acids. Michaelis-Menten parameters were determined by nonlinear least squares regression analysis.

Sulfate Uptake Conidia from 5 day old cultures were suspended at 10^5/ml in Fries medium that lacked the usual sulfur and phosphorus sources but was supplemented with 10 mM Pi, 100 mM Tris-Mes at pH of 5.5 or 7.5 as indicated, and 0.25 mM methionine (36). Conidia were germinated, harvested and assayed as described for Pi uptake with the following modifications: uptake solution was 2x Fries medium containing 10 mM Pi

and 50 mM Tris-Mes but lacking sulfate and sucrose, ^{35}S-sulfate was substituted for ^{32}P-orthophosphate, and sulfate uptake was determined by liquid scintillation counting.

Nucleic Acid Manipulations Genomic DNA and total RNA were isolated from mycelia using TRI-reagent (Sigma Chemical CO., St. Louis, MO, USA). Blotting and hybridizations were performed as described previously (12). For RT-PCR, first strand cDNA was synthesized from 2 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) in a reaction volume of 20 μl that contained 1 μCi [α-^{32}P]dCTP. Unincorporated nucleotides were separated from first-strand cDNA by gel filtration and samples were normalized based on the incorporation of label. All samples were diluted to at least 0.1 ml with TE buffer. The numbers of PCR cycles were evaluated for each primer pair to ensure that signals detected on an agarose gel stained with ethidium bromide (0.5 μg/ml) were below saturation. Primers used for RT-PCR analysis are listed in Table 2.

Electrophysiology Double barrel micropipettes were fabricated as described by Lew (27). Hyphal trunks behind the growing edge were chosen for impalements. Upon impalement, the potential was measured for a period of about 4–5 minutes. To measure input resistance of the hypha, a 1 nA current was injected into the hypha through one of the barrels, while the voltage deflection was measured through the other barrel. Current injections through the two barrels were performed before and after the impalement to assure there was no crosstalk between the two barrels. The cable properties of the hyphal cells will result in current attenuation as current passes down the hyphal length and through the septal pores (21, 28). We did not correct for current attenuation because it

would require multiple impalements into the hypha, therefore resistivity (Ω cm^{-2}) was not calculated.

TABLE 2. Primers used for RT-PCR analysis

			Amplified size (bp)	
Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Genomic template	cDNA template
actin	TTCCATTCTCGCCTCGCTC	TACTTGACCGCGCCTATCC	438	438
ena-1	TGAAATTGTCCCCGGTGATCT	AAGACTTGAGGTTGCGCACAAT	727	655
par-1	CAGCATCACCATTCGACCACG C	CGTTCATACCGCGGCAGATACTC	807	648
par-2	CAGAGCTCAGGCTCCAGTTCA	CCACAGCGGTGTTCTCGTAGA	670	551
pho-2	CTCGGTCGTCAATGTCGCTT	AGGGAGGTCTTGTTGAGGCTGA	976	731
pho-4	TCAAAACGGTGACTCGGAAA	CAGTTGATGGTCCTCCACGT	836	752
pho-5	CGAAGGCAAGATGCCTACAA	TTGGAGTAATGGCGGAAGAAGT	781	717

CHAPTER III

RESULTS

Cloning of *par-1*$^{+}$ (*hpp*$^{+}$)

We hypothesized that enhanced Pi uptake and/or accumulation resulting from the *hpp* mutation (hereafter referred to as *par-1*) would be deleterious for growth if mutants were presented with high concentrations of Pi. To test this hypothesis we examined growth on media with varied Pi concentrations and pH. As shown in Figure 3, no significant growth phenotype was detected at any Pi concentration tested with media adjusted to pH 5.5. In contrast, growth on media adjusted to pH 7.2 revealed striking differences. The *par-1; pho-4; pho-5* triple mutant displayed the suppressed phenotype (poor but detectable growth) on medium containing 0.05 mM Pi, and markedly improved growth at 5 mM Pi suggesting that 0.05 mM Pi is still limiting. The parental *pho-4; pho-5* double mutant failed to grow at 0.05 mM Pi as expected, but grew at 5 mM Pi, confirming that the low-affinity transport system can support growth when provided with sufficient Pi. Strains carrying the *par-1* mutation, however, were unable to grow on medium containing 200 mM Pi (pH 7.2) regardless of genotypic background, whereas wild type and *pho-4; pho-5* strains grew relatively well. These results suggested that the *par-1* mutation causes hyper-accumulation of Pi, but only under alkaline growth conditions.

The conditional lethality of *par-1* on high Pi/high pH medium (200 mM, pH 7.2) was exploited first to map the *par-1* mutation, then to clone the wild-type allele by complementation. Standard genetic mapping indicated that the *par-1* mutation is located

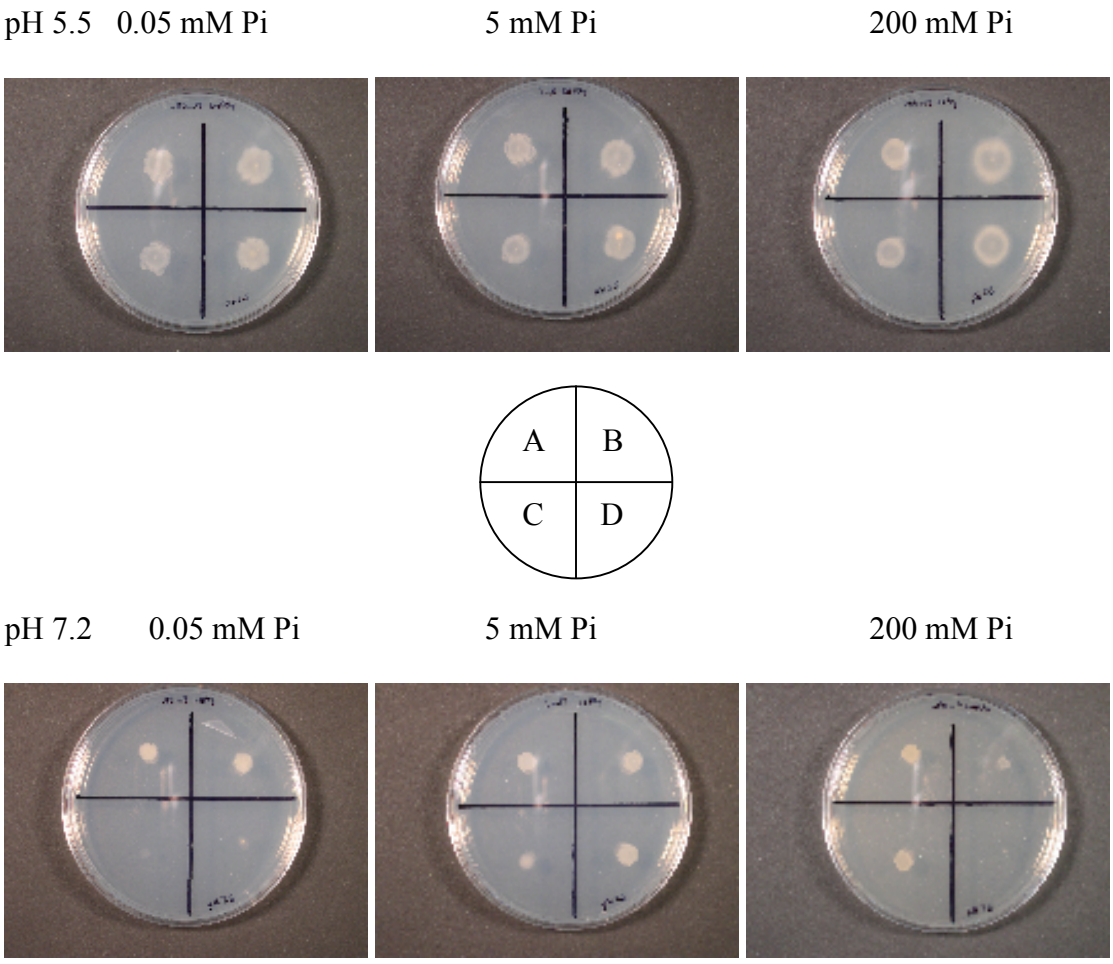

FIG. 3. Growth under varied Pi concentrations and pH.
Equivalent numbers of conidia from each strain were spotted on plates and incubated for 3 days. Quadrants (A) wild type, (B) *par-1*, (C) *pho-4; pho-5* and (D) *par-1; pho-4; pho-5*.

less than two units to the left of *nic-1* on linkage group I (data not shown). Wild type cosmid clones representing >150 kb of contiguous sequence encompassing the *par-1*$^{+}$ locus were tested individually for their ability to complement *par-1*. We found two complementing cosmids that share 12 kb of sequence, and a single candidate gene within this region corresponding to NCU03021.2 of *N. crassa* genome release 7 (20) was sufficient for complementation. Sequencing of the equivalent region from the *par-1* mutant revealed a single nonsense mutation within the predicted coding region. Based on those findings we designated this candidate gene *par-1*$^{+}$. This gene has a coding sequence of 2784 bp interrupted by a single 160 bp intron (confirmed by sequencing of RT-PCR products) and encodes a protein of 927 amino acids. The *par-1* mutation resulted in a premature stop at codon 500. We searched available sequence databases for proteins with similarity to PAR-1 and identified PalF of *A. nidulans* (33, 34) and other closely related fungal proteins (Fig. 4). The molecular function of these proteins is unknown and their sequences yield no compelling clues. However, genetic analyses indicated that all serve a role within an ambient pH signal transduction pathway that is conserved among fungi (41) (Fig. 2).

Because PAR-1 lacks hallmarks of a membrane transport protein, e.g. membrane spanning domains, it is a poor candidate for a low affinity Pi transporter. Instead, PAR-1 must have a role in the regulation of low affinity Pi transport. It was for these reasons that we re-named *hpp*$^{+}$ as *par-1*$^{+}$ (phosphate acquisition regulator-1).

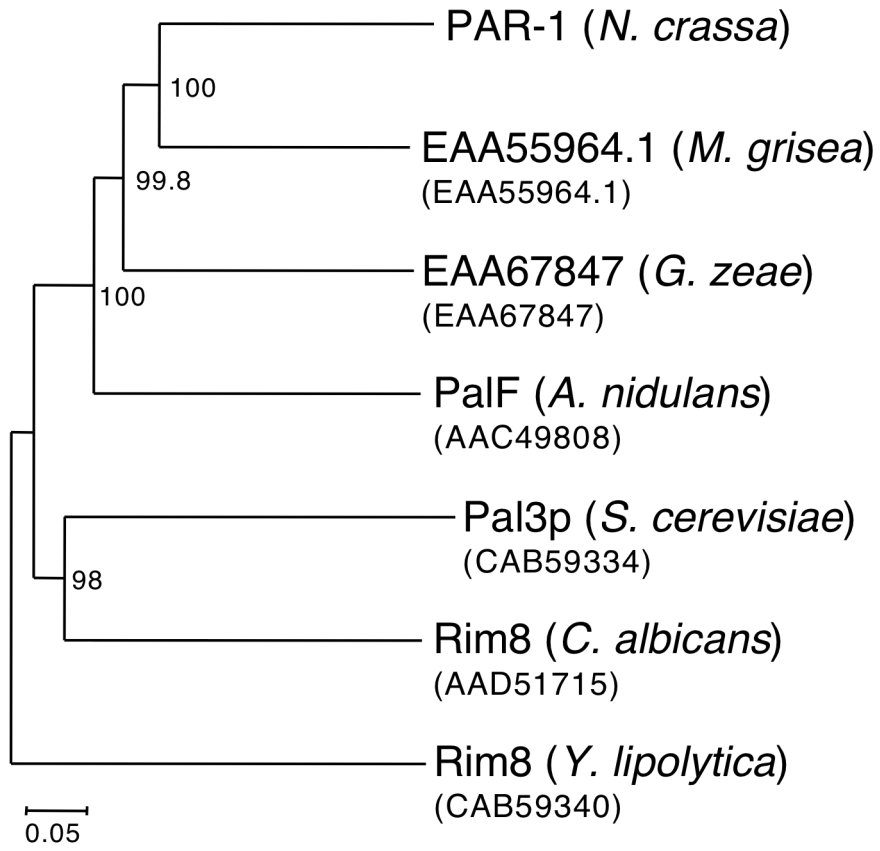

FIG. 4. Phylogenetic analysis of PAR-1. The unrooted, neighbor-joining tree was prepared from a Clustal X alignment using the NJ plot program. Bootstrap values (n=1000) are shown at the tree nodes. Accession numbers are listed below the protein name/designation. The scale bar indicates the number of amino acid substitutions per site.

Ambient pH signaling mutants phenocopy *par-1*

A homolog for each component of the pH signaling pathway in *A. nidulans* can be identified in the *N. crassa* genome, suggesting that pH signaling processes are likely to be analogous in these species. Therefore, it was possible that the *par-1* phenotypes were caused by a defect in pH signaling. Alternatively, PAR-1 may have had functions independent of this regulatory cascade. Also, because *par-1* transcript can be detected by RT-PCR in the *par-1* mutant at levels roughly equivalent to wild type it was possible that this mutant had neomorphic properties. To address these issues we used a gene-replacement strategy (11) to delete the *N. crassa* homolog of the *A. nidulans PacC* gene, which we have named *par-2*$^{+}$, and separately, to delete *par-1*$^{+}$. PacC is a transcription factor that represents the final step of the pH signaling pathway (Fig. 2) (41, 44). The *Δpar-2* mutation is a deletion of the 2041 bp predicted coding region corresponding to the 3nc420_070 locus (MIPS *N. crassa* database), and similarly, the *Δpar-1* mutation is a deletion of the entire *par-1*$^{+}$ coding region. Both deletion strains were crossed to a *pho-4; pho-5* strain to isolate the respective triple mutants, and all were tested for growth under the selective conditions. The *Δpar-1* and *Δpar-2* mutants phenocopy the original *par-1* mutant with respect to both the *pho-4; pho-5* suppressor and high Pi sensitivity phenotypes. These results confirmed that *par-1* is a null mutation and supports the idea that *par* phenotypes are the result of a defect in pH signaling.

Mutations in other fungi that are analogous to the null *par* mutations display “acid-mimicking” gene expression patterns and corresponding phenotypes (14, 41, 44, 45). That is, in such mutants the pH responsive genes that are normally expressed

predominantly under alkaline growth conditions are instead expressed at levels similar to those seen under acidic growth conditions. As shown in Fig. 5, the *par-1*$^+$ and *par-2*$^+$ genes are not pH responsive, but *ena-1*$^+$, which encodes a P-type Na^+-ATPase (9, 22, 23), is clearly pH responsive (1) with transcript levels that increase with rising ambient pH in a *par*$^+$ background. However, in a *par-1* background, *ena-1*$^+$ expression levels are acid-mimicking regardless of ambient pH. This result further supports our hypothesis that *par* mutations represent defects in pH signaling, and that in *N. crassa*, the signaling processes and eventual consequences of this pathway are similar to those reported for other fungi.

Salt sensitivity

Although we hypothesized that the *par-1* mutation causes hyper-accumulation of Pi, our growth media were routinely prepared using potassium phosphate salt as the sole source of Pi. Therefore it was possible that the growth phenotypes we observed were related to potassium concentration and/or ionic strength rather than Pi concentration. To test this possibility we prepared low Pi media (0.05 mM Pi) in which potassium phosphate was substituted with sodium phosphate, and variable amounts of KCl were added to make up the deficit in K^+ ions and to test the effect of increasing ionic strength. The suppressor phenotype of *par-1* under these conditions was indistinguishable from the original selective growth condition indicating no specificity or requirement for increased K^+ ions. However, when very high concentrations of KCl (>150 mM) were included, growth of Δ*par-1*; *pho-4; pho-5* was inhibited, whereas growth of the parental

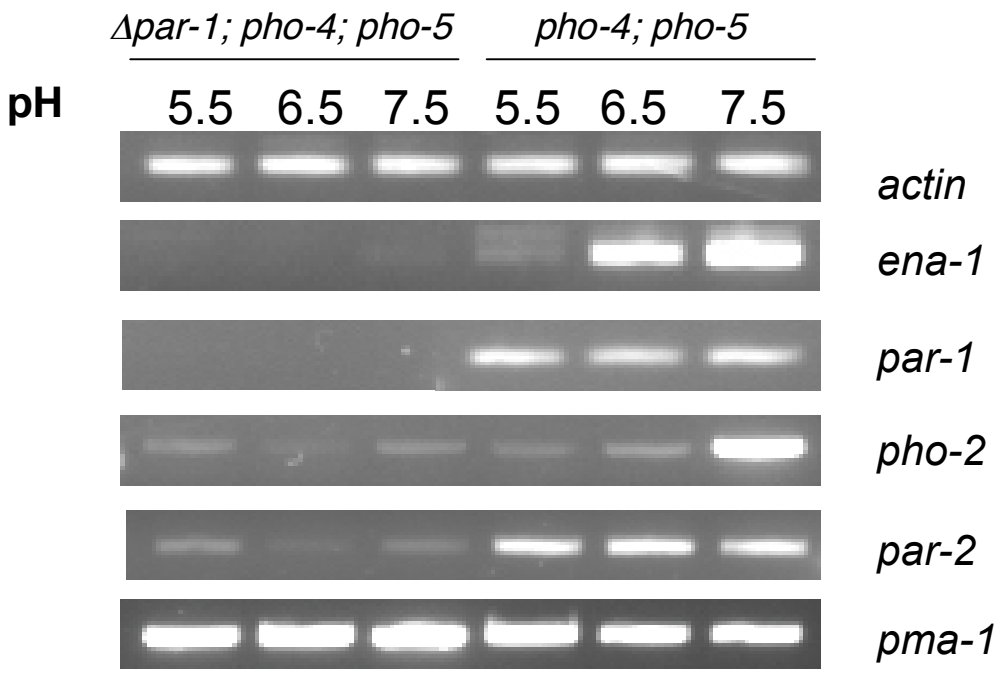

FIG. 5. RT-PCR analysis of *pho-4; pho-5* and *Δpar-1; pho-4; pho-5.* RNA was isolated from strains grown under different ambient pH conditions. Cycle numbers were 25 for *actin* and *ena-1*, 27 for *par-1*, *pho-2*, *par-2*, and *pma-1*.

pho-4; pho-5 strain was unaffected. These results suggested that unlike the suppressor phenotype, conditional lethality on high Pi medium was not specific for Pi, but rather a function of high ionic strength and/or osmotic stress. To explore these possibilities, we tested the effect of several different salts on growth. As shown in Table 3, all of the salts tested inhibited growth of *par* strains when present at high concentration (200 mM), whereas sorbitol, a non-ionic osmoticum, did not inhibit growth of any of the strains. It should be noted that LiCl inhibited growth at substantially lower concentrations (30 mM) than other salts, presumably due to the toxic effects of Li^+. These findings suggested that *par* mutations confer a general salt sensitivity and that osmotic stress is not a critical determinant for the conditional lethality.

All of our initial experiments were conducted using media containing Mops-NaOH buffer, and although the concentration of Na^+ contributed by the buffering system was relatively constant, we could not be certain of its influence on phenotypes, nor would the concentration be constant if we chose to vary the pH. To avoid this potential complication and to provide greater flexibility in experimental design we substituted the Mops-NaOH buffering system with Tris-Mes. Phenotypic analyses were repeated using the reformulated media, but no change was detected in any of the phenotypes listed in Table 3. Consequently, the Tris-Mes buffering system was adopted for all subsequent experiments.

TABLE 3. Phenotypes of pH signaling mutants

	Growth At pH 8.0[b]		Salt Sensitivity[c]	Osmotic Stress[d]	Li^+ Sensitivity[e]	Neomycin Resistance[f]
[Pi]	0.05	5.0 mM				
wt	+	+	-	-	-	-
par-1	-	+	+	-	+	+
Δpar-1	-	+	+	-	+	+
Δ*par-2*	-	+	+	-	+	+
pho-4; pho-5	-	-	-	-	-	-
Δ*par-1; pho-4; pho-5*	-	+	+	-	+	+

a. Unless otherwise specified, growth medium is Fries medium, 10mM Pi, 100 mM Tris-Mes pH 7.5.
b. 100 mM Tris-Mes pH 8.0
c. 200mM NaCl, KCl, NaH_2PO_4, KH_2PO_4, Na_2SO_4, or K_2SO_4.
d. 1M Sorbitol.
e. 30 mM LiCl.
f. 2 mg/ml neomycin sulfate

Mineral nutrient accumulation

Salt sensitivity has been reported as a phenotype associated with pH signaling defects, and has been attributed to diminished Na^+ efflux activity under alkaline conditions (9, 22, 44). This is consistent with our observations that *par* mutants were sensitive to high concentrations of sodium and lithium (a toxic analog of Na^+) salts (Table 3), and failed to express *ena-1*$^+$, which encodes a P-type Na^+-ATPase, at wild type levels (Fig 5). However, elemental analyses performed on *Δpar-1; pho-4; pho-5* and *pho-4; pho-5* strains grown under variable pH conditions revealed no detectable differences in Na content. In contrast, these strains did display pH-dependent differences in P, K and S contents (Table 4). Under pH 5.5 growth conditions the two strains had roughly equivalent mineral contents, but at pH 6.5 and 7.5, the *Δpar-1; pho-4; pho-5* strain accumulated 20-100% greater amounts of P, K and S than the parental *pho-4; pho-5* strain. Similar results were obtained from an independent experiment and the accumulation of none of the other elements examined (Ca, Cu, Fe, Mg, Mn, Na and Zn) differed between the two strains by greater than 5%.

Effects of neomycin and high ambient pH on growth

In addition to the high salt and lithium sensitivity phenotypes discussed above, pH signaling mutants in a number of fungi display resistance to neomycin and the inability to grow under high pH conditions (44, 45). As shown in Table 3, *par* mutations also confered resistance to neomycin, and this phenotype was observed in wild type as well as *pho-4; pho-5* backgrounds. Sensitivity to high pH, however, appears to be largely

TABLE 4. Mineral nutrient accumulation by ambient pH

Element	pH 5.5		pH 6.5		pH 7.5	
	pho-4; pho-5	Δ*par-1; pho-4; pho-5*	*pho-4; pho-5*	Δ*par-1; pho-4; pho-5*	*pho-4; pho-5*	Δ*par-1; pho-4; pho-5*
P	1.64	1.45	1.39	1.72	.81	1.22
K	1.44	1.43	.89	1.21	.15	.42
S	.66	.62	.53	.65	.43	.60

* Results reported % dry matter

related to Pi limitation (Table 3). At pH 8.0, wild type grows under limited (0.05 mM) and adequate (5.0 mM) Pi conditions whereas the null high affinity Pi uptake *pho-4; pho-5* double mutant is unable to grow under either condition. Surprisingly though, the *Δpar-1* and *Δpar-2* single mutants, which have functional high affinity Pi transporters, are unable to grow at pH 8.0 with 0.05 mM Pi, suggesting that the *par* mutations affect high affinity Pi transport. Indeed, RT-PCR experiments confirmed that both *pho-4*$^+$ and *pho-5*$^+$ genes were pH responsive with greatest expression at alkaline pH, and that expression was dramatically reduced in a *Δpar-1* background (Fig. 6).

The *Δpar-1; pho-4; pho-5* triple mutant failed to grow at pH 8.0 with 0.05 mM Pi but did grow when supplied with 5.0 mM Pi. In contrast, the parental *pho-4; pho-5* double mutant was unable to grow under either condition but did eventually grow at pH 8.0 if supplied with >10 mM Pi. These growth patterns indicated that the *par* suppressor phenotype defined at pH 7.5 persists at pH 8.0 but the higher pH value simply imposed a greater requirement for the amount of Pi needed to support growth, which presumably reflected specificity for the $H_2PO_4^{-1}$ ionic form of Pi.

Alkaline phosphatase

Some of the first pH signaling mutants identified in fungi were isolated based on their failure to produce alkaline phosphatase under limited Pi conditions (17). In *N. crassa,* the *pho-2*$^+$ gene encodes the predominant alkaline phosphatase that is produced under such conditions. Like *pho-4*$^+$ and *pho-5*$^+$, *pho-2*$^+$ is a Pi-repressible gene; its transcript and encoded enzyme is synthesized under both acidic and alkaline conditions provided that Pi is limiting (24, 25, 37). In contrast, alkaline phosphatase activity in wild-type *A.*

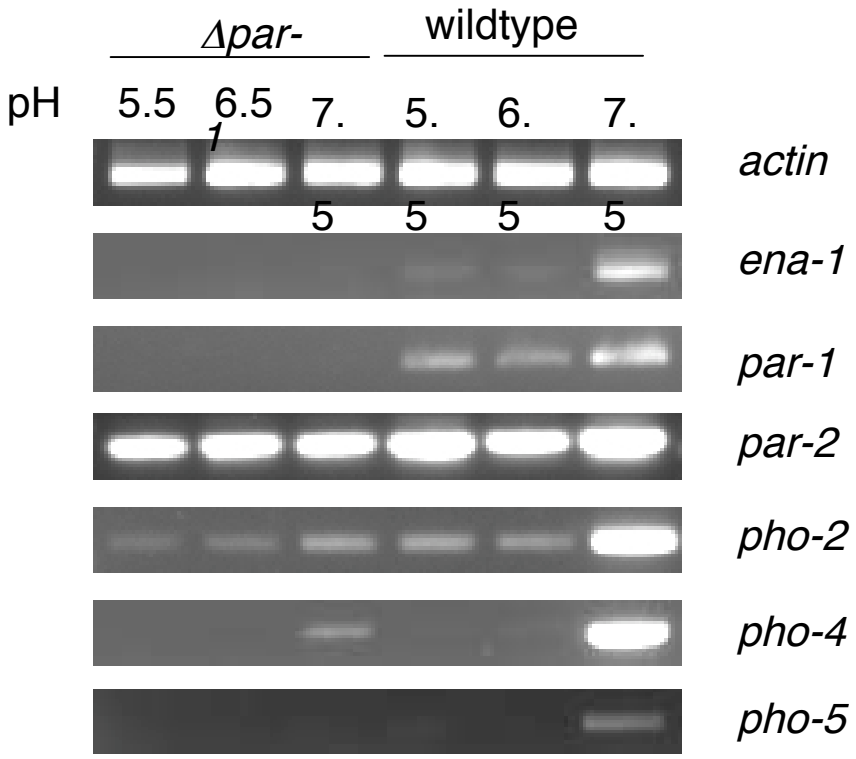

FIG. 6. RT-PCR analysis of Δ*par*-1and wild type. RNA was isolated from strains grown under different ambient pH conditions. Cycle numbers were 25 for *actin* and *ena-1*, 27 for *par-1*, *par-2*, *pho-2*, *pho-4* and *pho-5*.

nidulans is detected under Pi limiting growth conditions that are neutral or alkaline but not acidic (16, 17), suggested regulation by ambient pH that may not exist in *N. crassa*. However, we found that the *Δpar-1* mutation did affect PHO-2 activity, but the effects were only detected when Pi is present at relatively high concentrations (Table 5). At pH 7.5 with limited Pi (0.05 mM), all of the *pho-2*$^{+}$ strains had substantial alkaline phosphatase activity regardless of the *par-1* allele. As Pi concentration was increased, alkaline phosphatase activity diminished, as would be expected for a response solely regulated by Pi availability. This response was enhanced in strains carrying the *Δpar-1* mutation suggesting that those strains acquire Pi more efficiently, and as a result, were able to repress *pho-2*$^{+}$ expression at lower external Pi concentrations. This idea may be sufficient to explain the differences between the *pho-4; pho-5* and *Δpar-1; pho-4; pho-5* strains. However, expression of *pho-4*$^{+}$ and *pho-5*$^{+}$ was reduced in a *Δpar-1* single mutant (Fig. 6), which would limit the efficiency of Pi uptake. Yet, the wild type strain produced detectable alkaline phosphatase staining even at 20 mM Pi, whereas the *Δpar-1* single mutant failed to produce positive staining at 0.5 mM Pi. One possible explanation for these observations was that *pho-2*$^{+}$ expression, like that of *pho-4*$^{+}$ and *pho-5*$^{+}$, is affected by the *Δpar-1* mutation. RT-PCR experiments confirmed that *pho-2*$^{+}$ expression was pH responsive with greatest expression at alkaline pH, and that expression was dramatically reduced in a *Δpar-1* background (Fig. 6). These results suggested that a complex relationship exists between the phosphorus and ambient pH regulatory systems to coordinate Pi uptake and homeostasis.

TABLE 5. Alkaline phosphatase staining

Pi in medium (mM)	0.05	0.5	5	10	20
Wild type	++++	++	+	+	+ / -
pho-2	-	-	-	-	-
Δpar-1	++++	-	-	-	-
pho-4; pho-5	NG	++++	+++	++	+
Δpar-1; pho-4; pho-5	++++	++++	+	+	+ / -

NG, no growth.

Pi transport

To evaluate the effects of pH signaling on Pi transport, we first identified growth regimes that would provide cultures at equivalent growth stages for each strain and growth treatment. Growth medium contained 10 mM Pi and was adjusted to either pH 5.5 or pH 7.5. The four strains examined (wild type, *Δpar-1* single mutant, *pho-4; pho-5* double mutant, and *Δpar-1; pho-4; pho-5* triple mutant) all grew at a comparable rate (doubling time of 2.29 ± 0.15 hr) in pH 5.5 medium. Similarly, all four strains grew at an equivalent rate (doubling time of 3.13 ± 0.19 hr) in pH 7.5 medium, but in this case growth was considerably slower than at pH 5.5. Therefore, to achieve equivalent growth under both pH conditions before analyzing Pi transport we incubated pH 5.5 cultures for 4 hr and pH 7.5 cultures for 6 hr.

We confirmed that Pi uptake was linear with respect to time under our growth and assay conditions for all four strains. As shown in Table 6, when strains were grown at pH 5.5, the *Δpar-1* mutation had no effect on Pi uptake. However, when cultures were grown at pH 7.5 noticeable differences in Pi uptake were detected. The *Δpar-1* single mutant showed reduced Pi uptake when compared to wild type, suggesting that the *Δpar-1* mutation has a negative effect on high affinity Pi transport. This was consistent with our finding that expression of the high affinity transporter genes, *pho-4*$^{+}$ and *pho-5*$^{+}$, was reduced in a *Δpar-1* background (Fig. 6). In contrast, the *Δpar-1* mutation had the opposite effect on low affinity Pi transport (Table 6). The *Δpar-1; pho-4; pho-5* triple mutant displayed increased Pi uptake relative to the parental *pho-4; pho-5* double mutant. The identity of the gene(s) responsible for low affinity Pi transport is unknown

TABLE 6. Pi uptake rates at acidic and alkaline growth conditions

	Uptake (pmol Pi min^{-1}N.A. mg^{-1})	
	Growth at pH 5.5	Growth at pH 7.5
wild type	0.38 ± 0.09	4.92 ± 0.39
Δpar-1	0.43 ± 0.07	0.26 ± 0.03
pho-4; pho-5	0.22 ± 0.05	0.20 ± 0.04
Δpar-1; pho-4; pho-5	0.21 ± 0.03	0.55 ± 0.05

* Assayed at pH 7.5 with 0.05 mM Pi

and it is possible that the enhancement of this activity is due to increased expression of the corresponding transporter gene(s). In this case, we would have anticipated that the amount of transporter protein per cell would be increased with a concomitant increase in transport Vmax. Alternatively, enhanced low affinity transport could have been a function of Km (increased affinity for Pi) or a combination of the two attributes. To distinguish between these possibilities we compared Pi transport kinetics for the *pho-4; pho-5* double mutant with those of the *Δpar-1; pho-4; pho-5* triple mutant.

Using the selective growth and assay conditions outlined above (growth at pH 7.5, assay at pH 7.5) we found that Pi uptake by the *Δpar-1; pho-4; pho-5* strain was greater than that of the *pho-4; pho-5* double mutant for all Pi concentrations tested (Fig. 7). However, we were unable to estimate values for Vmax and Km under these conditions because uptake rates were extremely variable when high concentrations of Pi were used. To approach saturation with lower total Pi concentrations we grew the strains as above (pH 7.5) but examined transport at pH 6.5 (Fig. 8A). Under these conditions the *Δpar-1* mutation has a pronounced effect on both Km and Vmax, resulting in a nearly 3-fold lower apparent Km and a 40% increase in Vmax. When we repeated this experiment using uptake medium adjusted to pH 5.5 (Fig. 8B), the apparent Km values decreased approx 8.5-fold compared to those at pH 6.5, but the relative difference between the two strains was the same. The Vmax values at pH 5.5; however, were essentially identical for both strains and only slightly lower than those measured at pH 6.5.

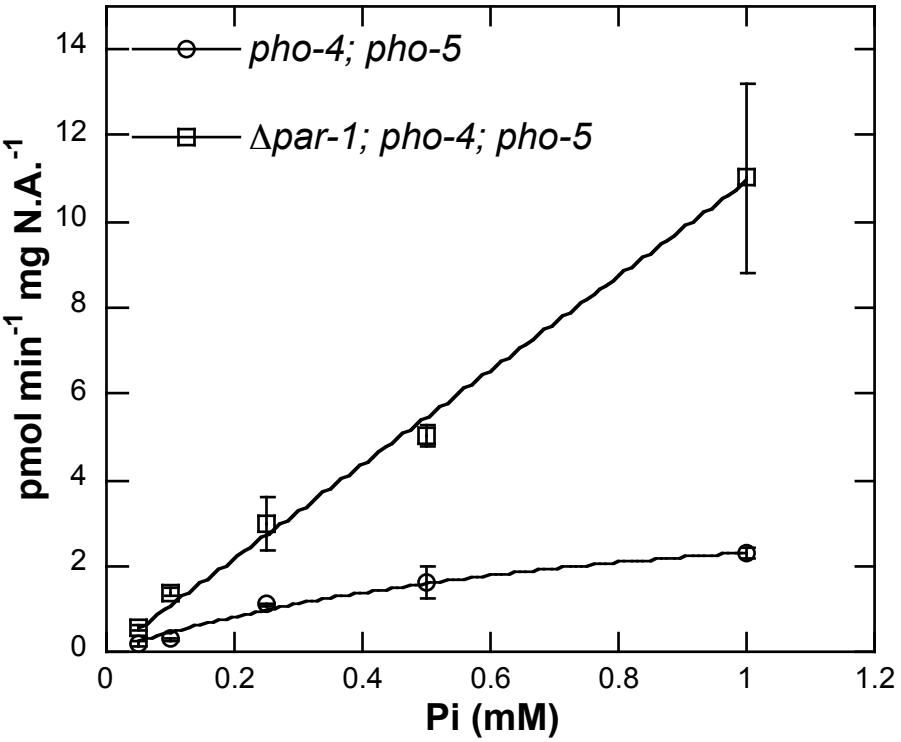

FIG. 7. Pi uptake for strains grown at pH 7.5 and assayed at pH 7.5. Values for one of three independent experiments are shown (means ± SE for 4 replicates).

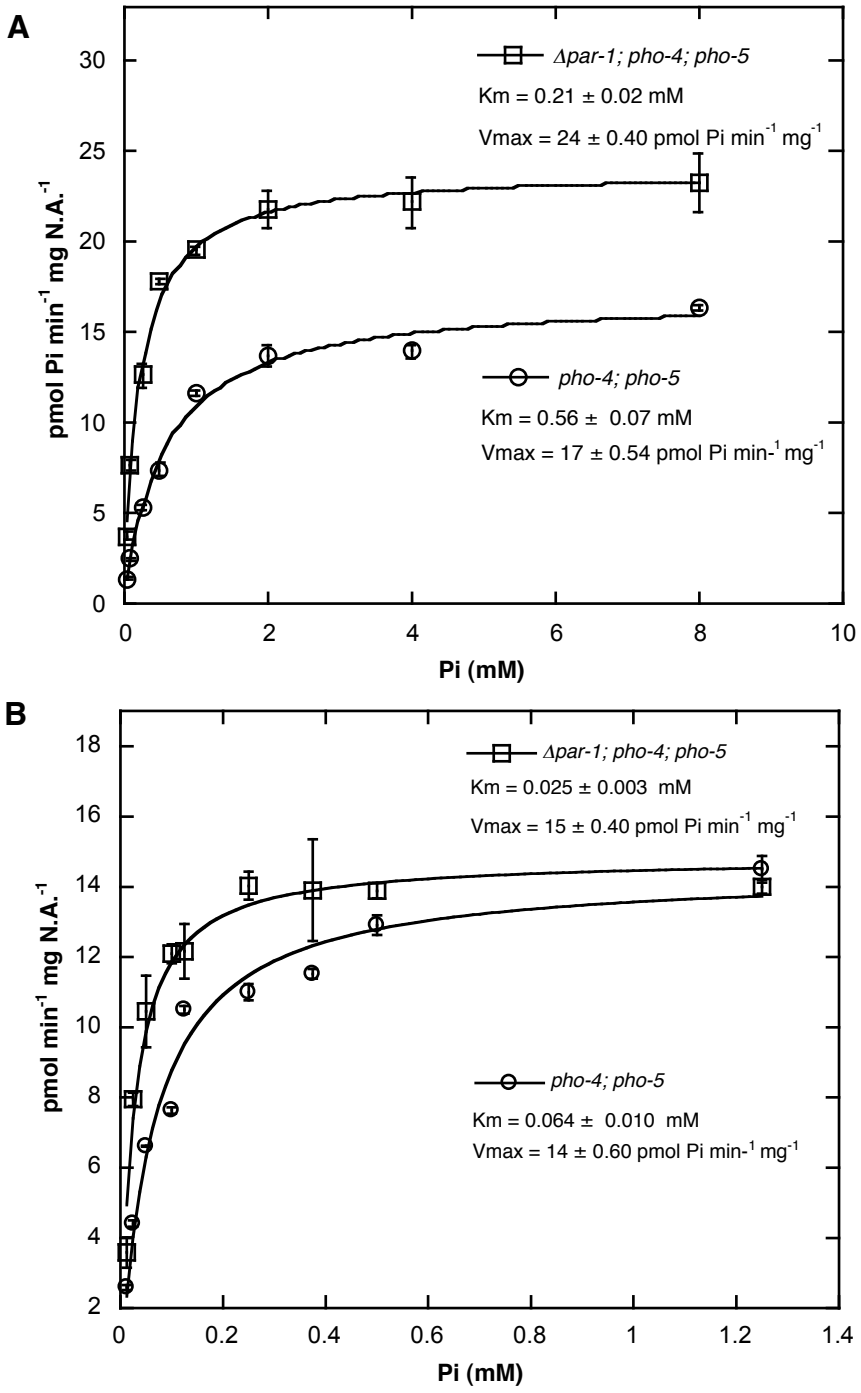

FIG. 8. Pi uptake kinetics for strains grown at pH 7.5 and assayed at varied pH values. (Panel A), assay pH 6.5; (panel B), assay pH 5.5. Values for one of three independent experiments are shown (means ± SE for 4 replicates).

Sulfate transport

Elemental analyses indicated that the *Δpar-1; pho-4; pho-5* triple mutant hyper-accumulated S relative to the *pho-4; pho-5* parental strain, and that hyper-accumulation was pH dependent (Table 4). To determine if this hyper-accumulation was the result of an increased rate of sulfate uptake, we grew these strains at pH 5.5 and 7.5 and compared sulfate uptake rates using the same basic procedures as for Pi. Similar to our results with Pi transport, no significant differences in the rate of sulfate uptake was detected when strains were grown at pH 5.5, but the relative rate of sulfate uptake was enhanced in the *Δpar-1; pho-4; pho-5* triple mutant when strains were grown at pH 7.5 (Fig. 9). These results suggested that pH signaling influences sulfate transport and low affinity Pi transport via the same general mechanism.

Inhibition of Pi and sulfate transport by CCCP

It seemed possible that the similar effect of the *Δpar-1* mutation on low affinity Pi uptake and sulfate uptake may be due to a common aspect of their respective transport mechanisms. The low-affinity Pi transport system is reported to function by H^+/Pi symport (28). Similarly, sulfate is suggested to occur via H^+/sulfate symport that is catalyzed by two transport proteins, CYS-13 and CYS-14 (19). In each case protons serve as an essential co-substrate, and thus it is conceivable that the *Δpar-1* phenotypes are a direct or indirect function of protons. To obtain evidence in support of a proton-symport mechanism for Pi and sulfate uptake we tested the effect of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) on the respective transport activities. CCCP is a lipid-soluble weak acid that increases membrane permeability for

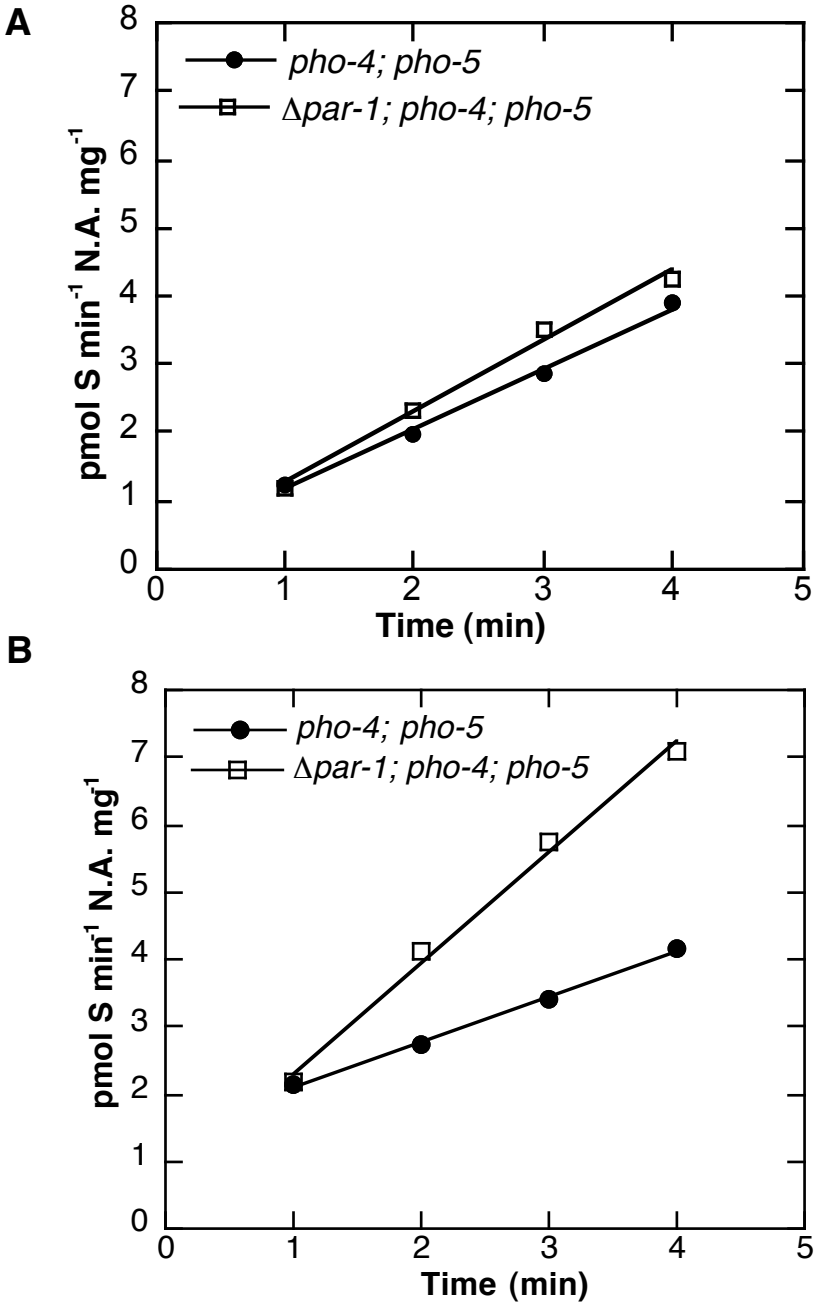

FIG. 9. Alkaline growth condition increases the rate of sulfate uptake in Δ*par-1*. Uptake of 0.05 mM [^{35}S] grown in (panel A) acidic conditions (pH 5.5) and (panel B) alkaline condition (pH 7.5).

protons, which discharges the pH gradient. If transport of Pi and sulfate are dependent on the pH electrochemical gradient, as would be the case for proton-coupled transport, then CCCP should have been an effective inhibitor of uptake. As shown in Fig. 10, CCCP abolishes low affinity Pi transport in the *pho-4; pho-5* strain at pH 5.5 as well as at pH 7.5. Sulfate uptake was similarly abolished by treatment with CCCP (data not shown). These results were consistent with the idea that transport of Pi and sulfate is coupled to that of protons.

Extracellular acidification

We hypothesized that the effects of the *Δpar-1* mutation on low affinity Pi transport and sulfate transport may be related by the common role served by protons as a co-substrate. One possibility was that the *Δpar-1* mutation promotes proton efflux to effectively increase the concentration of this essential co-substrate. We first examined transcript levels for *pma-1*$^{+}$, which encodes the plasma membrane H^{+}-ATPase that is responsible for the vast majority of proton efflux from the cell. As shown in Fig 5, the *Δpar-1* mutation had no effect on *pma-1*$^{+}$ transcript levels nor was *pma-1*$^{+}$ expression responsive to ambient pH. We then tested directly for an effect of *Δpar-1* on acidification of growth medium. The *Δpar-1; pho-4; pho-5* triple mutant and the *pho-4; pho-5* double mutant were each grown in medium containing 10 mM Pi and adjusted to pH 7.5 but no additional buffer was included. Fig. 11 illustrates that the *Δpar-1* mutation had no effect on growth or acidification of the medium under these conditions.

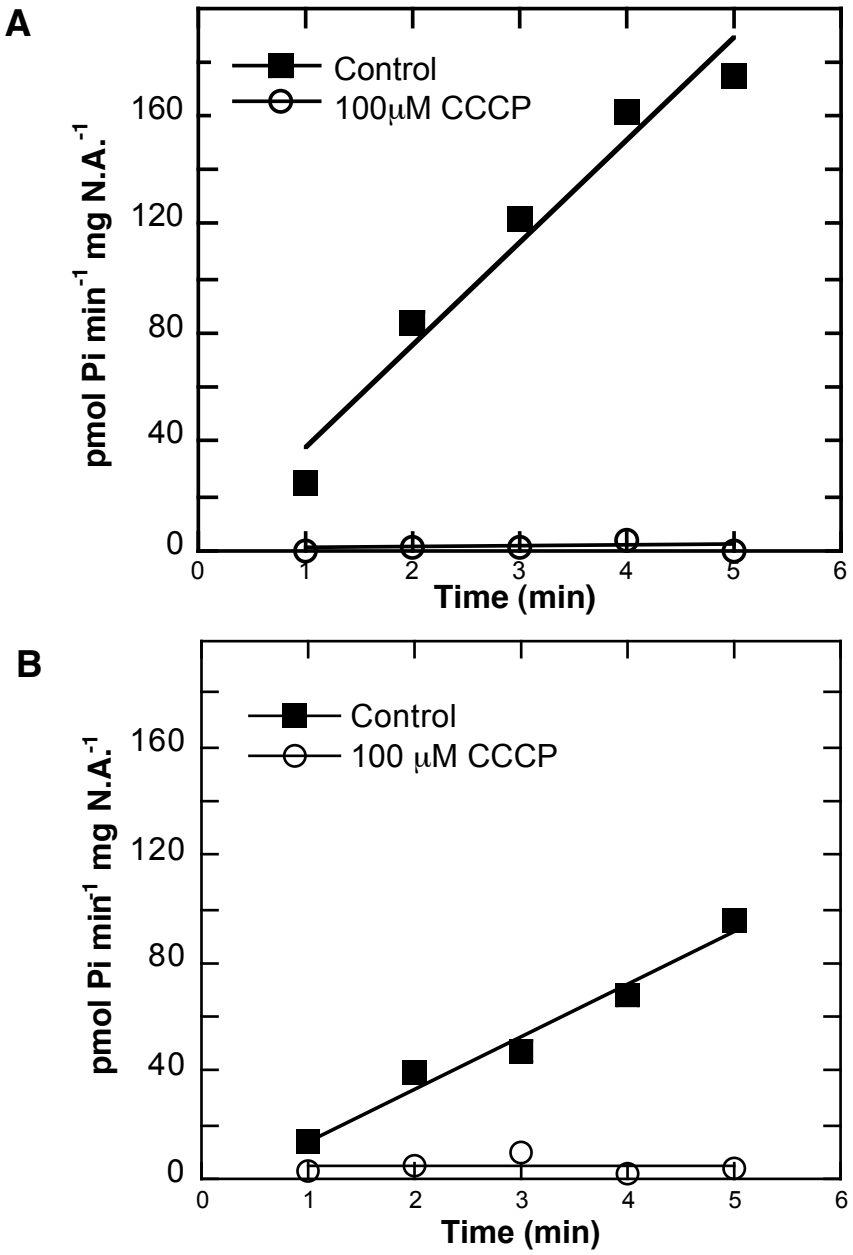

FIG. 10. CCCP disrupts phosphate uptake. The *pho-4; pho-5* strain was grown in Fries medium at pH 7.5. Uptake of [^{32}P] was assayed at (panel A) acidic conditions and (panel B) alkaline conditions.

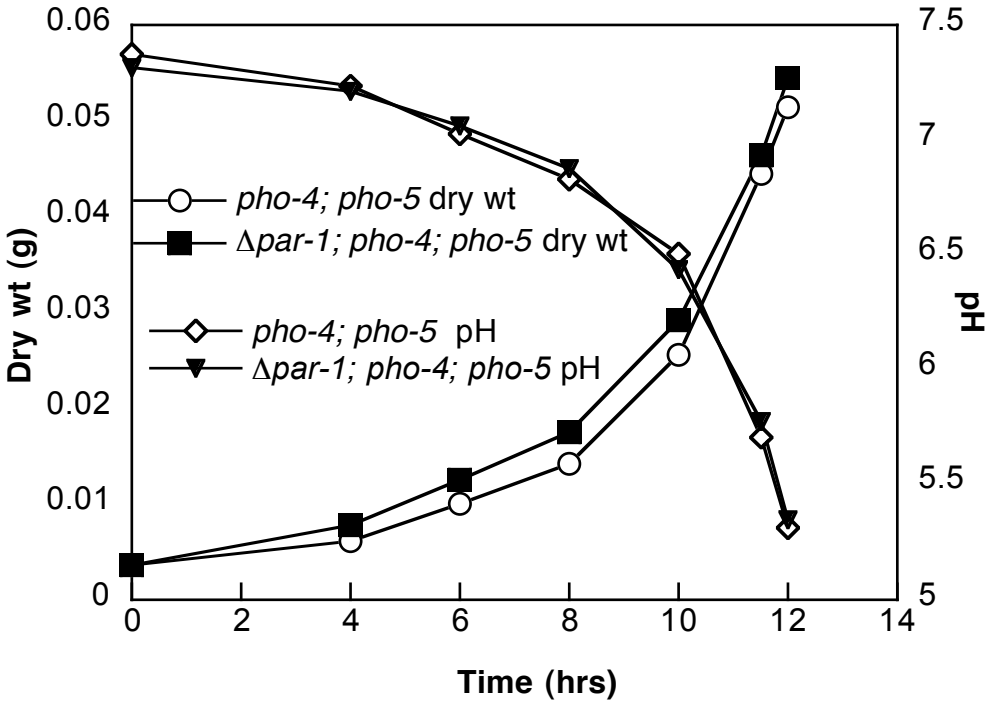

FIG. 11. Medium acidification and biomass accumulation during growth of *N. crassa.* The mutant *pho-4*; *pho-5* and Δ*par-1*; *pho-4*; *pho-5* strains were grown in Fries medium with 10 mM Pi as sole buffering source.

It should be noted that only gross changes in pH could have been detected with this experimental approach, and that differences restricted to the periplasmic space also would not have been detected.

Plasma membrane potential

We hypothesized that the *par* phenotypes of salt sensitivity and enhanced low affinity Pi transport could be the result of a steady-state depolarization of the plasma membrane (inside more positive) relative to wild type. The *pho-4; pho-5* double mutant and *Δpar-1; pho-4; pho-5* triple mutant were grown under the same selective conditions used for Pi transport studies (10 mM Pi, pH 7.5) and potentials were measured for six individual hypha as described in materials and methods. As shown in Table 7, no statistically significant differences in membrane potentials or in membrane resistance (a measure of total ionic flux) were detected. Membrane potentials were, however, depolarized relative to the approximately -200 mV value commonly reported for *N. crassa* (48, 49). The most likely cause of the depolarized potentials is the relatively high concentrations of potassium and/or ammonium in the medium. This is because when measurements were repeated using hyphae equilibrated in a basal salts (BS) solution (48), the membranes were highly polarized (Table 7).

TABLE 7. Plasma membrane potential measurement

Fries, 100 mM Tris-Mes (pH 7.5)	Em (mV)	R (MΩ)
pho-4; pho-5	- 48 ± 9.5	8.1 ± 4.2
Δpar-1; pho-4; pho-5	- 44 ± 13.5	7.8 ± 1.5
BS (pH 7.2)[a]		
pho-4; pho-5	- 174.5 ± 7.6	8.3 ± 3.7
Δpar-1; pho-4; pho-5	- 186.2 ± 23.4	17.0 ± 9.7

a. 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 4.6% sucrose, 10 mM Mops (pH 7.2)

CHAPTER IV

CONCLUSION

A bloom of *N. crassa* is often the first sign of life after a fire. The charred and partially desiccated vegetation that supports this growth presents a harsh environment very different from standard laboratory media. The remaining moisture is rendered alkaline and will contain some inorganic salts at concentrations that may approach limits of solubility. Based on our findings presented here, we suggest that the ambient pH regulatory system is essential for growth under such conditions and that its unexpected role in Pi acquisition is paramount.

Although it was predicted from sequence homologies that an ambient pH regulatory system similar to that in *A. nidulans* would exist in *N. crassa,* and perhaps all fungi (44), until now evidence confirming that the function of this signaling pathway is conserved in *N. crassa* has been indirect (42). Direct evidence for function is provided by the *Δpar-1* and *Δpar-2* mutations, which result in phenotypes that closely mirror those of analogous mutations in *A. nidulans* and other fungi (9, 22, 45). These phenotypes include salt sensitivity, resistance to neomycin, the inability to grow at alkaline pH, and importantly, the expected (acid-mimicking) effect on expression of pH responsive genes.

Our research efforts have focused primarily on the elucidation of Pi transport mechanisms, and the *par-1* mutation provided our first clue that pH regulation can affect this process. The low affinity and high affinity Pi transport systems both are affected by pH regulation, but in opposite directions and appear to be modulated by different primary mechanisms.

The high-affinity Pi transporter genes, *pho-4*$^+$ and *pho-5*$^+$, both display pH dependent expression patterns in wild type but a constant, acid-mimicking pattern in the *Δpar-1* background regardless of ambient pH (Fig. 6). Thus these genes appear to fulfill the criteria specified by Arst and Penalva (1) for regulation by ambient pH. It is important to note that these expression analyses are complicated by the fact that *pho-4*$^+$ and *pho-5*$^+$ are both Pi-repressible genes (35, 52). Repression by high Pi concentration (10 mM) at pH 6.5 and 5.5 may be responsible for why *pho-4*$^+$ and *pho-5*$^+$ show a sudden onset of detectable expression at pH 7.5 rather than a progressive increase in transcript levels with increasing ambient pH as observed for *ena-1*$^+$ (Fig. 6).

The Pi-repressible *pho-2*$^+$ gene displays an expression pattern similar to those of *pho-4*$^+$ and *pho-5*$^+$ but in this case, activity of the encoded protein (alkaline phosphatase) can be monitored to evaluate if phosphorus regulation still functions in a *Δpar-1* background. As shown in Table 3, all strains display a Pi-dependent pattern for phosphatase activity, indicating that the phosphorus regulatory system is active. The concentration of Pi needed for repression, however, is much lower in the *Δpar-1* background. These results are intriguing because in *A. nidulans*, analogous acid-mimicking mutants lack alkaline phosphatase when grown under limiting Pi conditions. To our knowledge, alkaline phosphatase activities for *A. nidulans* grown with varied concentrations of Pi have not been reported so it remains unknown if the apparent differences between these fungi reflect unique features of phosphorus regulation, pH regulation or both.

Although Pi availability is often a function of ambient pH due to its effect on Pi solubility and ionic form, the fact remains that *pho-4*$^{+}$, *pho-5*$^{+}$ and *pho-2*$^{+}$ expression levels and their respective encoded enzymatic activities are dramatically reduced in the *Δpar-1* background compared to wild type. These results are consistent with the idea that at least some Pi-repressible genes, and especially those encoding high affinity Pi transporters, are responsive to both ambient pH and Pi concentration. Although such dual regulation was unexpected, we can envision growth conditions where coordinated control by pH and Pi may be required. For example, growth experiments indicate that at low Pi concentrations and very high ambient pH values (8.0), the pH regulatory system is required to sustain growth (Table 3). It is possible that under such conditions pH signaling may elicit greater expression of high affinity transporter genes than can be achieved via phosphorus regulation alone. That this response is primarily targeted for Pi uptake is inferred from the fact that an increased supply of Pi alone is sufficient to offset growth defects. However, examples have been reported of analogous dual regulation for sugar uptake and siderophore transport (18, 51), suggesting that pH regulation may be broadly involved in coordinating membrane transport processes.

The low affinity Pi transport system also is influenced by pH regulation but in ways that are distinct from the effects on high affinity transport. A *Δpar-1* mutation results in enhanced rather than diminished low affinity transport, and this effect is pH dependent rather than a constant acid-mimicking phenotype. Importantly, low affinity transport in a *par-1*$^{+}$ background does not appear to be pH responsive. Based on these findings it seems unlikely that a low-affinity Pi transporter gene is regulated directly by the ambient

pH-signaling pathway. Uptake studies support this idea because a transcriptional response would be expected to result in a change solely in Vmax. However, our results indicate that although Vmax may be altered, enhanced Pi transport is primarily a function of Km.

Several possible explanations exist for how *Δpar-1*, a defect in pH regulation, results in an altered Km for Pi transport. 1) Modification of a low affinity transport protein. In this case, the modification or lack of modification (e.g. phosphorylation, glycosylation) would occur only in the *Δpar-1* background and would be catalyzed by a pH responsive activity. Although possible, this explanation would not easily account for other phenotypes associated with the *Δpar-1* mutation that are unrelated to Pi transport. 2) Synthesis of an additional Pi transporter that is regulated in response to pH. This idea is not consistent with our data that indicate a single transport activity rather than a mixture of two activities with different Km values (biphasic curves). 3) An indirect or kinetic response, meaning that the transporter protein itself would not be altered in response to pH but other determinants of transport activity that impinge on Km and Vmax would be affected. Obvious candidates for such determinants include the transported substrates, the plasma membrane potential and the external and internal pH values. Because changes in most of these determinants could also lead to many, if not all, of the other phenotypes associated with the *Δpar-1* mutation we currently favor this indirect or kinetic model.

When wild type *N. crassa* is grown at pH 7.5 the cytosolic pH is approximately 7.4 (28). Under such conditions cells would be dependent almost exclusively upon the

membrane potential as the energy source to drive H^+/Pi symport. Therefore a change in membrane potential would be expected to have a dramatic effect not only on Pi transport but also many other cellular processes. However, our results indicate that the membrane potential is unaffected by the *Δpar-1* mutation. It is possible that changes in the flux of other ions across the plasma membrane are triggered by a defect in pH signaling, and that these altered transport events are then effectively balanced by an influx of Pi (and sulfate) to maintain the membrane potential. Sodium would seem an ideal candidate because expression of *ena-1*$^+$ (plasma membrane Na^+-ATPase) is clearly dependent upon pH regulation. However we found no differences in sodium accumulation that could be attributed to the *Δpar-1* mutation.

If the *Δpar-1* mutation results in an increased relative efflux of protons, this could provide the basis for the observed effects on low affinity Pi transport via several different but interrelated mechanisms. An increased proton concentration outside the cell could affect Pi transport both by its effect on the ionic form of Pi and also by virtue of being a co-substrate. Although *Δpar-1* had no effect on the expression of *pma-1*$^+$ (plasma membrane H^+-ATPase) or on bulk acidification of the medium, we cannot rule out the possibility that increased proton efflux does occur but is restricted to the periplasmic space and does not readily equilibrate with the bulk medium.

It is also possible that the *Δpar-1* mutation leads to a change in the internal rather than external pH. This is an attractive hypothesis because Caddick et al. (8) demonstrated that in *A. nidulans*, internal pH varies as a function of external pH and that acid-mimicking and alkaline-mimicking mutations result in an internal pH more alkaline

and acidic, respectively, than wild type. It should be noted that total cell extracts were used for these studies so the differences in pH may reflect a change in various subcellular compartments rather than the cytosol. Therefore, although the reported pH differences are significant, caution is warranted for the interpretation of the results with respect to plasma membrane transport processes. However, if cytosolic pH is affected in these mutants and the same is true for *N. crassa,* we would expect the *Δpar-1* mutant to have a cytosolic pH more alkaline than wild type. We are actively investigating this possibility.

An effect on cytosolic pH might explain many of the *Δpar-1* phenotypes. Under alkaline growth conditions the pH gradient across the plasma membrane is minimal and perhaps even reversed compared to growth under more acidic conditions. As a consequence, proton-coupled transport processes are dependent entirely upon the membrane potential for the energy required to drive active transport. Pi and sulfate are likely to be highly sensitive to such conditions due to the fact that as anions, energy must be supplied not only for transport against their concentration gradient but also to overcome an electrical barrier (plasma membrane potential is negative inside). Increasing the cytosolic pH would help maintain a pH gradient that favors proton-coupled transport into the cell. In addition to affecting transport energetics, a change in cytosolic pH could also affect numerous aspects of transport activities (e.g. altered substrate binding) that could manifest as altered apparent Km and Vmax values. These possibilities raise many interesting questions regarding how cells coordinate a wide

range of physiological and regulatory processes to maintain pH and nutrient homeostasis.

Future work

The work described in this thesis has generated many new questions. Some of those we find most compelling and wish to explore are addressed below.

An initial goal of the work described here was to identify components of the low affinity Pi transport system. This remains an important goal, and perhaps even more so now that we have determined that the pH regulatory system affects this transport activity through an unexpected and novel mechanism. Towards this goal, we have identified a single candidate gene based on its sequence similarity to low-affinity Pi transporters in *S. cerevisae*. We have named this candidate gene *pho-6*$^{+}$. A *pho-6* gene replacement mutant has no obvious phenotype, but when combined with *pho-4* and *pho-5* null mutations, the triple mutant does have a more severe growth defect on Pi restrictive conditions than a *pho-4; pho-5* double mutant. This growth pattern is consistent with the idea that PHO-6 is a Pi transporter but also indicates that the low affinity transport system must have additional components.

It will be important to verify the function of PHO-6. One possible approach is to test whether *pho-6*$^{+}$ can complement a Pi transport defective yeast mutant. Alternatively, it may be possible to isolate *N. crassa* mutants that lack the remaining low affinity Pi transporter, provisionally *pho-7*, and then test strains bearing all combinations of transporter gene mutations for Pi auxotrophy and Pi transport activities. One strategy to isolate a *pho-7* mutant would be to mutagenize a *pho-4; pho-5; pho-6* triple mutant and

then select for the ability to grow under low Pi/high pH restrictive conditions. We would expect two classes of mutations from this selection. First, mutations that lead to increased PHO-7 transport activity. Second, null mutations in any of the seven *par* genes as documented here for *par-1* and *par-2* mutants. This second class would not be of primary interest and the mutations are likely to vastly outnumber the desired *pho-7* hypermorph mutations. However, we can select against *par* mutations by exploiting their sensitivity to LiCl. Any mutant that survives both the Pi restrictive selection and LiCl selection will be of interest. Subsequent mapping and cloning may be complicated but these efforts will be facilitated greatly by molecular markers that are scheduled to become available in the near future.

Many questions regarding ambient pH regulation remain unanswered. Although the mutants described in this thesis will serve as useful tools for further studies, it is important to isolate a constitutive *par-2* mutant. Such mutants have been described for *A. nidulans, S. cerevisiae* and *F. oxysporum* (5, 10, 30), and each bears mutations that effectively truncate the encoded transcription factors resulting in constitutive nuclear localization/transcriptional activation. Because we already have a strain in which *par-2* has been deleted, we could simply engineer a truncated version of the wild type gene and recombine it at the *his-3* locus. This mutant should produce the opposite effect on transcription and phenotypes as the null mutant. That is, is should lead to alkalinity mimicking rather than acidity mimicking phenotypes. This will be a terrific tool to distinguish between direct effects of pH regulation and those that are specific to any number of environmental conditions. Important questions to address using the *par-2*^{C}

mutant include: how does this mutation affect high affinity Pi transport and expression of Pi-repressible genes? Does it confer increased sensitivity to neomycin or increased ion tolerance? What is its effect on ion accumulation and pH homeostasis? Answers to these last questions are likely to help discern the molecular basis for how pH regulation affects low affinity Pi transport and sulfate transport, which may be representative of many other transport processes.

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APPENDIX A

Fries salts minus phosphate

4x Fries salts minus phosphate

		(1x Conc.)
Ammonium tartrate	20 g	27.16 mM
KCl	2.25 g	7.5 mM
NH_4NO_3	4 g	12.5 mM
$MgSO_4$ (anhydrous)	1 g	2.0 mM
Trace Elements + NaCl + $CaCl_2$	4 ml	see below for components
Biotin (5μg/ml in 50% EtOH)	4 ml	5 μg/L
Water	980 ml	
Total	1000 ml	

Add a few ml of chloroform as a preservative. Store at 4°C.

Trace Elements + NaCl + $CaCl_2$ for Fries medium

		(1x Conc.)
Boric Acid	57 mg	0.92 μM
$CuSO_4 \cdot 5H_2O$	396 mg	1.58 μM
$MnCl_2 \cdot 4H_2O$	72 mg	0.36 μM
Ammonium Molybdate $\cdot 4H_2O$	37 mg	0.03 μM
$FeCl_3 \cdot 6 H_2O$	0.98 g	3.6 μM
$ZnCl_2$	4.20 g	30.8 μM
NaCl	100 g	1.71 mM
$CaCl_2 \cdot 2 H_2O$	100 g	0.68 mM

Add water to make 1 liter Shake before using
Store at 4°C protected from light.

Biotin For Fries medium

Biotin		5 mg
Water		500 ml
Ethanol	(95% is OK)	500 ml

This is not exactly 50% ethanol, but it's not critical
Store in aliquots at -20°C.

APPENDIX B

Tris-Mes Buffer

Tris-Mes buffer is included in Fries-based media at 100 mM to maintain the pH of liquid and agar-solidified cultures at values ranging from 5.5 to 8.5. This buffer system lacks inorganic ions, which may affect salt-sensitive mutants. For pH values of 5.5-7, Mes is the primary buffer and is titrated to the desired pH with Tris. For pH values >7, Tris is the primary buffer and is titrated to the desired pH with Mes.

Tris: Tris(hydroxymethyl)aminomethane is a weak base (pKa 8.1)

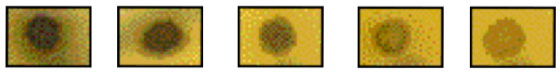
Mes: 2-(N-Morpholino)ethanesulfonic acid monohydrate is a weak acid (pKa 6.1)

10x Tris-Mes, amounts per L	**Tris**	**Mes**
pH 5.5 (1 M Mes - 150 mM Tris)	18.14 g	213.25 g
pH 6.5 (1 M Mes - 600 mM Tris)	72.68 g	213.25 g
pH 7.5 (1 M Tris – 900 mM Mes)	121.14 g	191.92 g

APPENDIX C

Detection of alkaline phosphatase activity in *N. crassa* colonies

1. Inoculate plates containing Fries, 100 mM Tris-Mes (pH 7.5), sorbose/fructose/glucose sugars, 1.5% Bacto-agar and the desired concentration of Pi (usually 0.05-20 mM) with 2-5 µl of a conidial suspension. Note that alkaline phosphatase is produced in strains grown at lower pH values (e.g. 5.5) if total P is low (50 µM Pi or 1 mM phosphorylethanolamine).
2. Incubate approximately 72 hrs at 30°C.
3. Prepare Alkaline Phosphatase staining solution:
   0.5 mg/ml α Naphthyl Acid Phosphate (Sigma CAS 81012-89-7)
   1 mg/ml Fast Blue BB Salts (Sigma CAS 5486-84-0)
   0.5 M Tris-HCl (pH 8.5)
   10 mM EDTA (pH 8.0)
4. Flood plates with approximately 20 ml of staining solution. Use a glass spreader to gently remove any bubbles from surface mycelia.
5. Leave the plates at room temp for up to 20 min (positive staining is usually detected within 10 min).
6. Discard the staining solution and gently rinse with water to reduce background.
7. Score plates for blue-purple stain intensity.



Alkaline phosphatase staining scored with an arbitrary scale. The *pho-2* null mutant serves as the negative control and is shown at far right.

APPENDIX D

Detection of acid phosphatase activity in *N. crassa* colonies

1. Inoculate plates containing Fries, 100 mM Tris-Mes (pH 5.5), sorbose/fructose/glucose sugars, 1.5% Bacto-agar and the desired concentration of Pi (usually 0.05 mM) with 2-5 μl of a conidial suspension.
2. Incubate approximately 72 hrs at 30°C.
3. Acid Phosphatase staining solution:
   0.5 mg/ml α Naphthyl Acid Phosphate (Sigma CAS 81012-89-7)
   1 mg/ml Fast Red TR Salts (Sigma CAS 89453-69-0)
   0.5 M Sodium acetate buffer (pH 4.8)
4. Flood plates with approximately 20 ml of staining solution. Use a glass spreader to gently remove any bubbles from surface mycelia.
5. Discard the staining solution and gently rinse with water to reduce background.
6. Score plates for reddish stain intensity.

APPENDIX E

Silanizing glassware

Background:

Silanization involves placing a thin layer of dimethyldichlorosilane onto glass surfaces to make them extremely hydrophobic, which discourages binding of cells and macromolecules to the glass. To tell whether a piece of glassware is adequately silanized or not, place a drop of water on an interior surface. If the water forms a bead, the glassware is silanized. If the water spreads across the glass, the item has not been silanized or needs to be re-silanized.

Supplies:

Dimethyldichlorosilane (DMDCS, also called dichlorodimethylsilane; VWR Cat. No. D0358-025ml)
This compound should be used with extreme caution as it can react with a number of common substances and is also highly flammable. When DMDCS contacts glass, HCl vapor is released. A similar reaction occurs when it interacts with water. Consequently, always handle this compound in a chemical hood. Wear latex gloves, a lab coat, and eye protection when dealing with either concentrated DMDCS or silanizing solution (2-5% DMDCS in chloroform). Do not mix concentrated DMDCS or silanizing solution with water as an explosive release of HCl gas may result. Avoid contact with skin and inhalation of fumes. If DMDCS comes into contact with exposed skin, wash the affected area(s) thoroughly with soap and water. Keep DMDCS away from open flame or excessive heat. If a DMDCS solution is accidentally ignited, use sand or CO_2 to put out the fire -- do not use water as this may result in an explosion.

Chloroform - Avoid skin contact and inhalation of fumes. Chloroform can cause eye and respiratory tract irritation. Excessive exposure can result in organ damage.

Procedure:

Perform all steps in a standard chemical fume hood unless noted otherwise. Wear latex gloves, a lab coat, and eye protection.

1. Obtain a large glass bottle with a ground-glass stopper. This will serve as a 'permanent' storage container for the silanizing solution. Make sure that this bottle is completely dry.

2. Make up a 5% v/v solution of DMDCS in chloroform (other nonpolar solvents such as hexane, methylene chloride or toluene can also be used). Gently swirl to mix the solution. The solution can be stored in the glass-stoppered bottle in a chemical hood for an indefinite period of time. Make sure that an appropriate label is placed on the storage container so that its contents are known to all those who use the hood.

3. To silanize flasks or beakers:
    a) Fill with 5% DMDCS and swirl to coat the surface (~30 sec). Transfer solution to the next container to be treated and repeat or pour back into the storage bottle. Use a glass funnel to prevent spills if needed.
    b) Rinse with a bit of chloroform, discard in the organic waste.
    c) Rinse with methanol.
    d) Air dry overnight in the hood then dry in oven at ~80C for an hour or longer.
    e) Rinse with distilled water and dry before using or storing.

Disposal of DMDCS:
DMDSC can be de-chlorinated by the addition of methanol, liberating HCl. Add methanol in the fume hood then add aqueous sodium bicarbonate to neutralize the HCl. Dispose in the appropriate waste container (e.g. organic waste if added to DMDSC in chloroform, sink if added to the empty DMDSC bottle).

APPENDIX F

N. crassa Pi uptake assay

The following protocol describes uptake assays used for the purpose of evaluating kinetic parameters. The protocol is essentially the same when evaluating other properties such as the effects of inhibitors, but may require minor modifications in the volumes or substrate concentrations.

Michalis-Menten equation
$v=Vmax*S/(Km+S)$ where v is measured velocity (uptake rate) and S is Pi concentration.

Experiment Preparation

1. Prepare solid growth medium (2 mM Pi/Fries/1.5% sucrose pH 5.8)
2. Prepare liquid growth medium (10 mm Pi/Fries/100 mM TRIS-MES/ 1.5% sucrose)
3. Prepare 2X uptake solution (2X Fries/ 50 mM TRIS-MES/ minus Pi and sucrose)
4. Prepare 0.1 N NaOH (for nucleic acids measurement)
5. Prepare 1 mM K-phosphate buffer pH 6.5 for cell washing
6. Prepare diluted stock of isotope.
   185 ul H_20 15 μl ^{32}Pi * 10 μCi/μl * $2.22x10^6$ cpm/μCi = 3.33 X 10^8 cpm
   15 ul ^{32}Pi
   200 ul total $3.33x10^8$ cpm /200 ul = $1.65x10^6$ cpm/ul of diluted stock

   Adjust values as needed to account for the half life of ^{32}P
7. Prepare Pi substrate stock solutions (see substrate preparation)

Inoculation and Growth

1. Inoculate desired strain on 2 mM Pi/Fries/sucrose pH 5.8
2. Incubate 72 hrs a 30ºC
3. Incubate 48 hrs on lab bench (exposure to light increases conidiation)

Conidial Preparation:

1. Harvest 5 day old conidia in distilled H_2O (50 ml Falcon tube).
2. Remove mycelia by filtration through miracloth. Centrifuge conidia.
3. Suspend conidia in 25 ml H_2O. Spin to pellet. Discard supernant.
4. Repeat step 3 then suspend conidia in 25 ml H_2O.
5. Calculate cell density by light scattering (Abs $_{420}$ of 1 ~ $5x10^6$/ml).
6. Inoculate 10mM Pi/100mM TRIS-MES/Fries/1.5 %sucrose pH5.5 or pH 7.5 to 10^5 condia /ml.
7. Incubate at 30C for four (pH 5.5) or six hrs (pH 7.5) with shaking, 250 rpm.
8. Harvest germlings on 5 μm filters by vacuum filtration.
9. Wash cells immediately with 20 ml of H_2O, repeat wash.
10. Transfer filter to ice cold H_2O (Final germling density: $2x10^6$/ml)
11. Shake well to dislodge and suspend germlings. The membrane can be removed with forceps. Store on ice.
12. Remove 0.5 ml suspension and add to 0.5 ml H_2O for total nucleic measurement. (See Nucleic Acid Procedure)

Substrate preparation (examples)

Final Pi Conc	***Volume of cold Pi***	***diluted ^{32}Pi***	***H_2O***
5 mM	45 μl (500mM stock)	5 μl	50 μl
1 mM	9 μl (500mM stock)	5 μl	86 μl
0.5 mM	22.5 μl (100mM stock)	5 μl	77.5 μl
0.1 mM	45 μl (10 mM stock)	5 μl	50 μl

Calculated for final volume of 4.5 ml.

Pi uptake:

1. Mix 2.2 ml germlings and 2.2 ml of (2X uptake solution) in a 50 ml tube.
2. Pre-incubate the germlings at 30C with shaking for 10 minutes.
3. Add 100 μl of ^{32}Pi substrate. Mix quickly and continue to incubate.
4. At precisely 1, 2, 3, & 4min remove 1ml and immediately vacuum filter though pre-wetted glass fiber filters. Immediately wash 3x with 20 ml of 1mM Pi washing solution.
5. Transfer the filter to a scintillation vial containing 4 ml of 0.1N NaOH. Mix and set ~ 1 hr before counting. Include a blank and a control (5 μl of diluted ^{32}Pi).

APPENDIX G

Quantitation of *N. crassa* growth in liquid cultures

Nucleic acids & Dry weight

Doubling times calculated from dry weights and nucleic acids are identical so the methods can be used interchangeably. Measurement of nucleic acids is simpler in most cases and more sensitive for very short incubations. However, for incubations longer than 10-12 hr mycelia begin to clump making it impossible to ensure that aliquots are homogenous so dry weight of entire cultures is a more accurate measurement of growth.

Initial Set-up

1. Harvest conidia from 5 day old cultures in 25 ml H_20.
2. Remove mycelia by filtration through Miracloth.
3. Centrifuge conidia 5 min in clinical centrifuge at setting 5.
4. Discarded supernant and wash conidia with 25 ml H_2O, repeat once.
5. Suspend conidia in 20 ml H_20.
6. Calculate concentration by light scattering (Abs $_{420}$ of 1 ~ $5x10^6$/ml).
7. Inoculate 25 ml Fries medium containing 1.5% sucrose and required supplements to a final concentration of conidia of $2.5x10^6$/ml.
8. Incubate at 30C with shaking, 250 rpm.

Collection procedure and dry weight determination

1. At desired time point, transfer 1 ml of cells to a 1.5 ml tube for nucleic acid detrmination (see Nucleic Acid Procedure)
2. Filter remaining culture on a pre-weighed Whatman 540 filter.
3. Rinsed cells with H_20.
4. Placed cells plus filter in oven at 80ºC for 2 hours.
5. Record the weight of the dried cells plus filter.
6. Calculate and record dry weight of cells.

Nucleic Acids determination

1. Spin the 1 ml of cells for 3 min at 10000 rpm.
2. Discard supernant and wash with 1 ml of H_20.
3. Discard supernant and suspend cells in 1 ml 0.1 N NaOH.
4. Incubate minimum 2 hr at 65C.
5. Vortex to mix then spin for 3 min at 10000 rpm.
6. Determine absorbance at 260 nm and calculate total nucleic acids (A_{260} of 1 = 40 μg nucleic acids/ml). Most of the nucleic acids in a cell are RNA.

VITA

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