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# pH Response Transcription Factor PacC Controls Salt Stress Tolerance and Expression of the P-Type $Na^+$-ATPase Ena1 in *Fusarium oxysporum*

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**Fungi possess efficient mechanisms of pH and ion homeostasis, allowing them to grow over a wide range of environmental conditions. In this study, we addressed the role of the pH response transcription factor PacC in salt tolerance of the vascular wilt pathogen *Fusarium oxysporum*. Loss-of-function $pacC^{+/-}$ mutants showed increased sensitivity to $Li^+$ and $Na^+$ and accumulated higher levels of these cations than the wild type. In contrast, strains expressing a dominant activating $pacC^c$ allele were more salt tolerant and had lower intracellular $Li^+$ and $Na^+$ concentrations. Although the kinetics of $Li^+$ influx were not altered by mutations in *pacC*, we found that $Li^+$ efflux at an alkaline, but not at an acidic, ambient pH was significantly reduced in $pacC^{+/-}$ loss-of-function mutants. To explore the presence of a PacC-dependent efflux mechanism in *F. oxysporum*, we cloned *ena1* encoding an orthologue of the yeast P-type $Na^+$-ATPase ENA1. Northern analysis revealed that efficient transcriptional activation of *ena1* in *F. oxysporum* required the presence of high $Na^+$ concentrations and alkaline ambient pH and was dependent on PacC function. We propose a model in which PacC controls ion homeostasis in *F. oxysporum* at a high pH by activating expression of *ena1* coordinately with a second $Na^+$-responsive signaling pathway.**

Fungi are a versatile class of organisms that have successfully occupied numerous ecological niches, including those of plant and animal pathogenesis. A striking property of fungi and a major determinant of their evolutionary success is their capacity to adapt to an extremely wide range of environmental conditions. This ability depends crucially on the presence of cellular sensory networks that monitor the environment and mediate changes in gene expression in response to shifts in the external conditions. We use the vascular wilt pathogen *Fusarium oxysporum* as a model to understand how environmental signals regulate gene expression in fungi and how these regulatory mechanisms determine fungal virulence.

A key factor in fungal growth and development is ambient pH. Fungi grow over a wide range of pH conditions and must thus be able to tailor gene expression to the particular pH of their growth environment. A conserved signaling cascade integrated by the products of the *pal* genes, whose terminal component is the zinc-finger transcription factor PacC/Rim101p, regulates gene expression in response to ambient pH (18). Upon shift to alkaline pH, an inactive PacC precursor is posttranscriptionally activated by proteolytic processing into a shorter functional form that activates genes preferentially expressed at alkaline pH and represses genes expressed under acidic growth conditions (18). The *pacC* orthologue of *F. oxysporum* was recently cloned, and the encoded protein was shown to regulate pH-dependent gene expression and to function as a negative regulator of virulence on plants (11). Thus, $pacC^{+/-}$ loss-of-function mutants of *F. oxysporum* mimic growth at acidic ambient pH and exhibit increased virulence, whereas $pacC^c$ strains expressing a dominant activating *pacC* allele mimic growth at alkaline pH and show significantly reduced virulence. At present, the downstream effector genes regulated by PacC in *F. oxysporum* remain largely unknown.

Yet another hallmark of fungal versatility is the capacity to grow over a wide range of salt concentrations. Generally, salt tolerance in living cells is conditioned by the capacity to maintain intracellular ion homeostasis. Fungi have developed extrusion systems to keep levels of intracellular sodium below concentrations toxic to the cell (9). In the best-studied system, that of *Saccharomyces cerevisiae*, the main $Na^+$ efflux system is encoded by the *ENA* genes, a tandem array of four to five genes encoding nearly identical proteins. *ENA1*, the most important and the best-studied component of this system, is essential for ion homeostasis and salt tolerance in yeast (15, 23). The ENA1 protein works as a P-type $Na^+$-ATPase but can also mediate $Li^+$ or $K^+$ efflux (8, 14, 15, 23). Expression of *ENA1* in *S. cerevisiae* is tightly regulated by $Na^+$ but also depends on alkaline ambient pH (for a review, see reference 20). Recent studies suggest that full expression of *ENA1* at alkaline pH requires *RIM101*, the *S. cerevisiae* orthologue of *PacC*, providing further evidence for a functional link between pH signaling and *ENA1* (17, 25).

In the present study we have addressed the role of *pacC* in salt tolerance of *F. oxysporum*. We provide evidence for the presence of a sodium efflux system based on an orthologue of the *S. cerevisiae ENA1* gene. We further show that full transcriptional activation of the *ena1* gene requires PacC and that both PacC and Ena1 play a fundamental role in the salt stress response of *F. oxysporum*.

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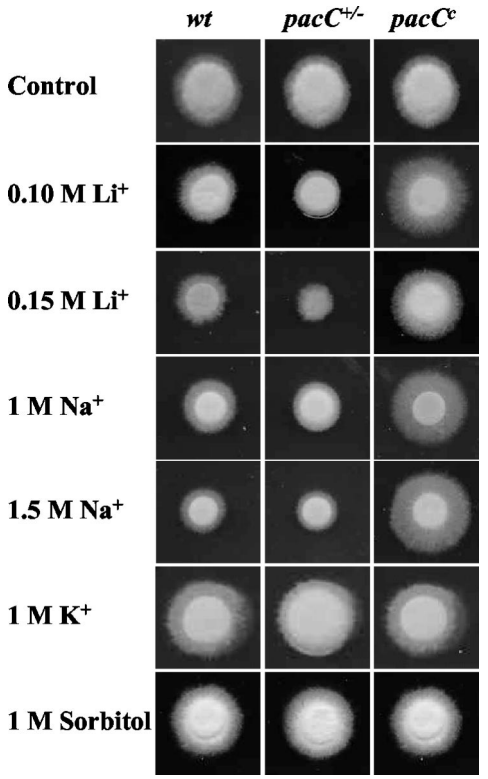



FIG. 1. *F. oxysporum pacC* mutants are affected in salt tolerance. (A) Wild-type strain 4287, loss-of-function mutant $pacC^{+/-}$, and dominant activating mutant $pacC^c$ were grown for 3 days on plates with SM buffered at pH 6.0 and supplemented with the indicated compounds.

## MATERIALS AND METHODS

**Fungal isolates and culture conditions.** *F. oxysporum* f.sp. *lycopersici* strain 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Almería, Spain, and stored at −80°C with glycerol as a microconidial suspension (13). Construction of the $pacC^{+/-}$ loss-of-function mutant and the merodiploid strain carrying a dominant activating $pacC^c$ allele was described previously (11). For microconidia production, cultures were grown in potato dextrose broth (Difco, Detroit, Mich.) at 28°C as described previously (13).

For phenotypic analysis of colony growth, a 5-μl drop containing $2.5 \times 10^5$ freshly obtained microconidia was transferred on 1.5% (wt/vol) agar plates of synthetic medium (SM) (13) containing 1% (wt/vol) glucose, 0.1% $NH_4NO_3$, and different concentrations of NaCl or LiCl. Media were buffered with 100 mM $Na_2HPO_4$ at pH 4; 50 mM $Na_2HPO_4$, 50 mM $NaH_2PO_4$, and 50 mM NaCl at pH 6; and 100 mM $NaH_2PO_4$ and 100 mM NaCl at pH 8.

**Determination of cation accumulation, influx, and efflux.** For determination of intracellular cation accumulation, microconidia were germinated in SM containing 1% (wt/vol) glucose and 0.1% $NH_4NO_3$, supplemented with 0.05, 0.1, or 0.15 mM LiCl or 0.5, 1, or 1.5 M NaCl. After 12 h, samples were filtered and processed for determination of intracellular ion content as previously reported (10, 21). Briefly, samples of cells were filtered, washed with 0.02 M $MgCl_2$, and treated with 0.2 M HCl, and the cations were analyzed by atomic absorption spectrophotometry.

For determination of lithium and rubidium influx (rubidium was used as a transport analog of potassium), microconidia were germinated in SM containing 1% (wt/vol) glucose and 0.1% $NH_4NO_3$ for 12 h. At time zero, 0.1 M LiCl or RbCl was added to the growth medium, and samples were taken at regular time intervals, filtered immediately, and processed for determination of intracellular ion content (21).

For determination of the lithium efflux rate, microconidia germinated in SM were supplemented with 0.1 M LiCl (wild-type and $pacC^{+/-}$ strains) or 0.3 M LiCl ($pacC^c$ strain). After 12 h, microconidia were filtered, washed with sterile 0.02 M $MgCl_2$, and resuspended in fresh SM buffered at pH 4.0 or 8.0 as described above. This medium was free of lithium and was supplemented with 0.05 M RbCl to trigger the efflux process. Samples were taken at regular time intervals, filtered, and processed for the determination of intracellular ion content (10, 21).

All experiments for determination of cation accumulation, influx, or efflux

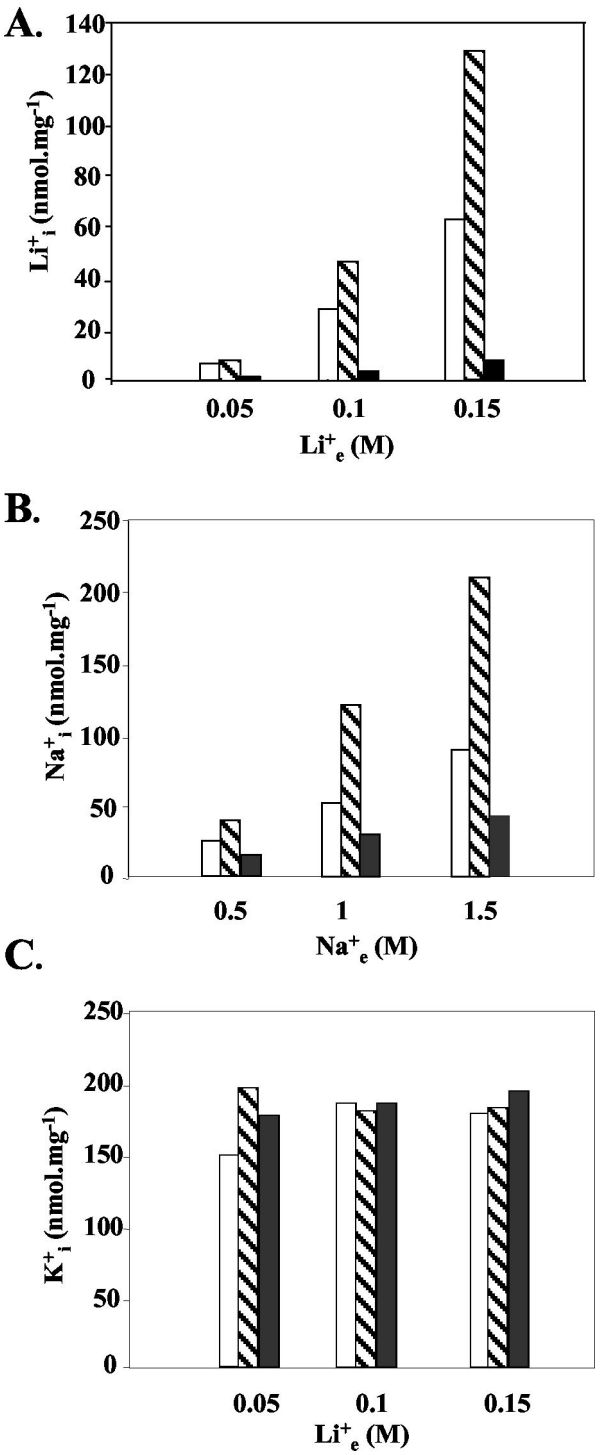



FIG. 2. Mutations in *pacC* affect ion homeostasis. Intracellular accumulation of cations was determined in germlings of the *F. oxysporum* wild-type strain (□), loss-of-function mutant $pacC^{+/-}$ (▨), or dominant activating mutant $pacC^c$ (■) grown for 12 h in the presence of the indicated concentrations of $Li^+$ (A and C) or $Na^+$ (B).

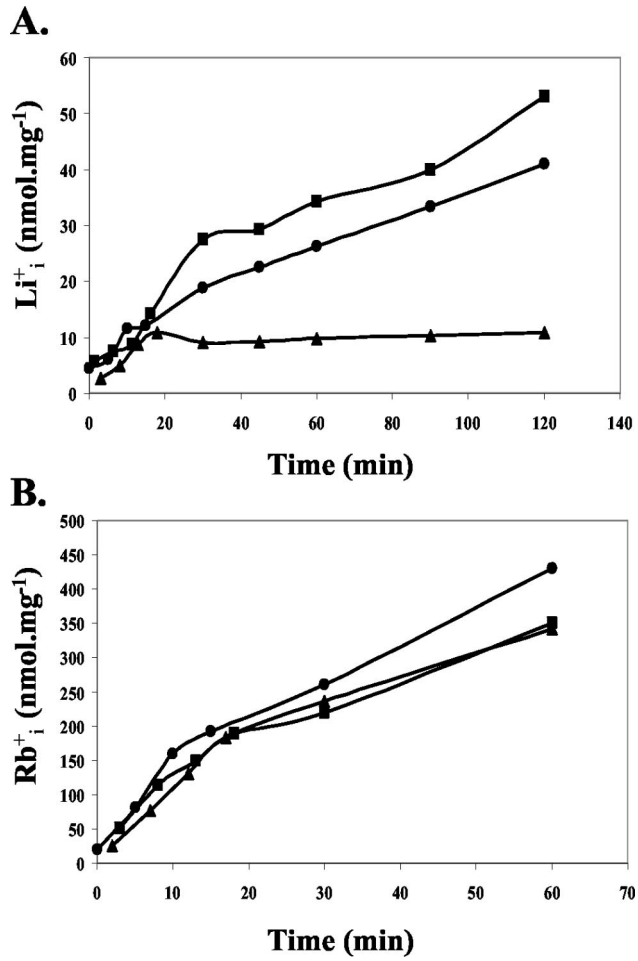



FIG. 3. Mutations in *pacC* do not affect kinetics of cation import. Kinetics of cation import was determined in germlings of the *F. oxysporum* wild-type strain (●), loss-of-function mutant *pacC*$^{+/-}$ strain (■), or dominant activating mutant *pacC*$^{c}$ strain (▲). Samples were taken at the indicated times after the addition of 0.1 M LiCl (A) or 0.1 M RbCl (B).

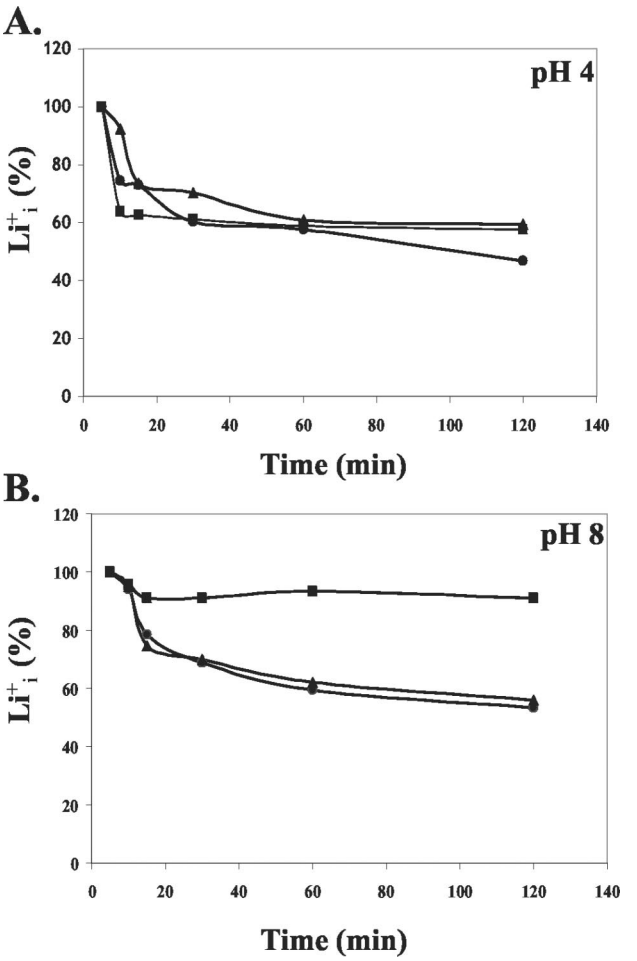



FIG. 4. Mutations in *pacC* affect cation efflux at alkaline but not at acidic ambient pH. Kinetics of cation export was determined in germlings of the *F. oxysporum* wild-type strain (●), loss-of-function mutant *pacC*$^{+/-}$ strain (■), or dominant activating mutant *pacC*$^{c}$ strain (▲). Microconidia were germinated for 12 h in SM containing 0.1 M $Li^+$ (wild type and *pacC*$^{+/-}$ mutant) or 0.3 M $Li^+$ (*pacC*$^{c}$ mutant), washed, and transferred to $Li^+$-free medium buffered at pH 4.0 (A) or 8.0 (B), and samples were taken at the indicated times.

were performed at least three times with similar results (the maximum standard deviations were <10%).

**Nucleic acid manipulations, cloning, and analysis of the *ena1* gene.** For Northern analysis, microconidia were germinated for 12 h in SM without added $Na^+$ and then transferred for different periods of time to SM buffered at the indicated pH values, with or without 0.5 M $Na^+$. Total RNA was extracted from mycelium as reported elsewhere (12), and Northern analysis and probe labeling was performed as described previously (13) by using the nonisotopic digoxigenin labeling kit (Roche Diagnostic S.L, Barcelona, Spain). Southern and Northern analyses were carried out as described above.

Genomic DNA of *F. oxysporum* isolate 4287 was extracted as previously reported (19) and used for PCR amplification on a Perkin-Elmer GeneAmp System 2400 with the primers ena3 (5′-TGACAAGCGACGATCTTTCTTCCG-3′) and ena4 (5′-GGTGATGCCCTTGTGCTTGAAGAC-3′) derived from an *F. oxysporum* expressed sequence tag clone. The following PCR conditions were used: 35 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. An initial denaturation step of 5 min at 94°C and a final elongation step at 72°C for 7 min were also performed. The amplified 250-bp DNA fragment was cloned into pGEM-T (Promega, Madison, Wis.), sequenced, and used to screen a lambda EMBL3 genomic library of *F. oxysporum* f.sp. *lycopersici* isolate 4287. Library screening, subcloning, and other routine procedures were performed as described in standard protocols (24). Sequencing of both DNA strands was performed at the Servicio Centralizado de Apoyo a la Investigación, University of Córdoba, by using the Dyedeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, Calif.) on an ABI Prism 377 genetic analyzer apparatus (Applied Biosystems, Foster City, Calif.). DNA and protein sequence databases were searched by using the basic local alignment search tool (BLAST) algorithm (2) at the National Center for Biotechnology Information (Bethesda, Md.).

## RESULTS

***PacC* controls salt tolerance and intacellular cation levels.** To test the hypothesis that *pacC* may be involved in the salt stress response of *F. oxysporum*, $2 \times 10^5$ freshly obtained microconidia of the wild-type strain, a *pacC*$^{+/-}$ loss-of-function or a *pacC*$^{c}$ dominant activating mutant (11) were inoculated on plates containing SM (pH 6.0) supplemented with different concentrations of salts or sorbitol. The results depicted in Fig. 1 show that the *pacC*$^{+/-}$ loss-of-function mutant displayed a clear $Li^+$ sensitivity phenotype and a somewhat more subtle $Na^+$ sensitivity phenotype, whereas the dominant activating *pacC*$^{c}$ allele conferred increased tolerance to these toxic cat-

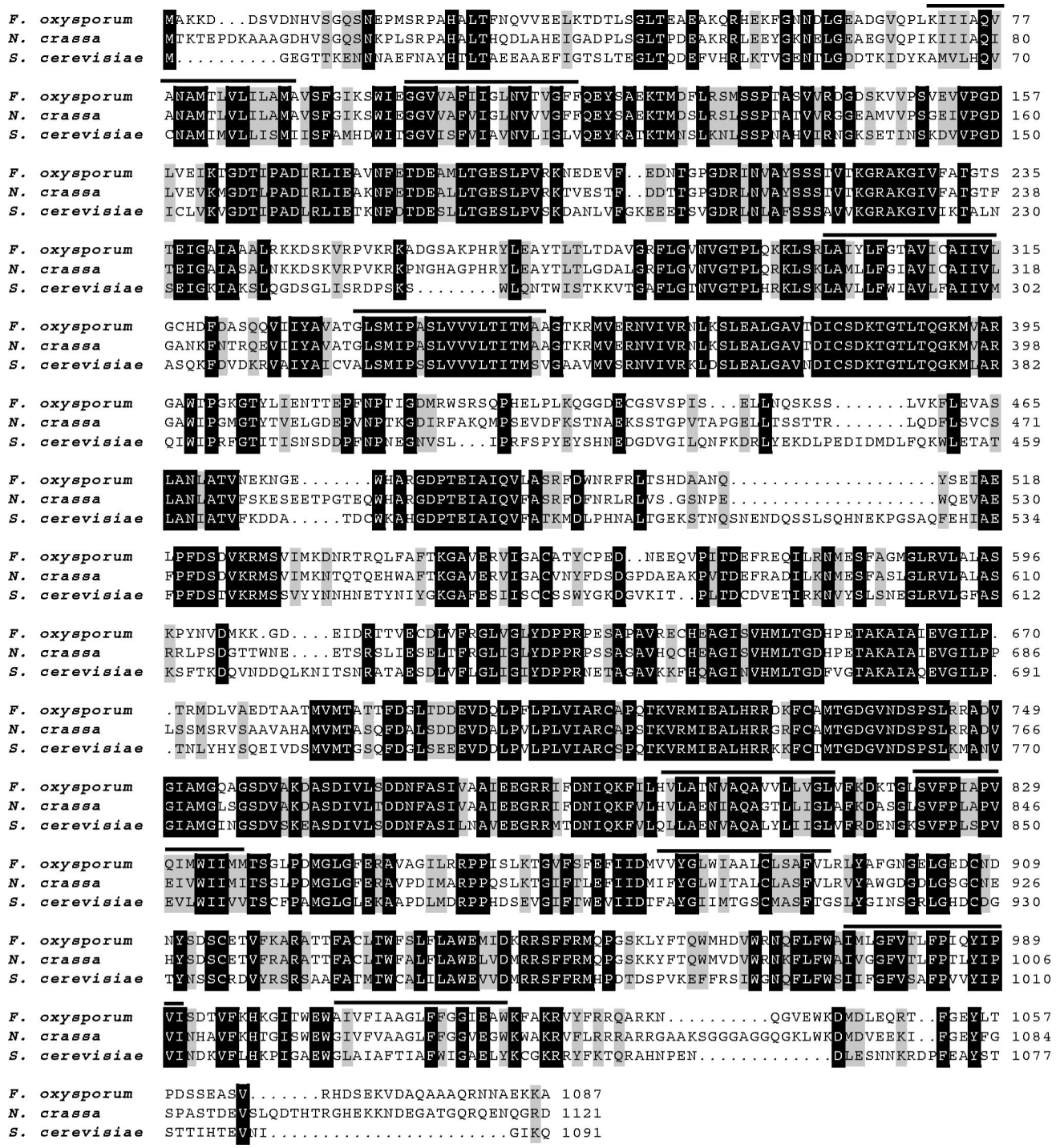
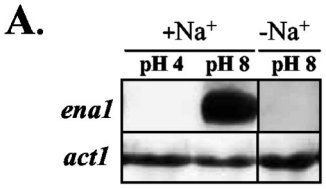



FIG. 5. Amino acid sequence alignment of the predicted *F. oxysporum ena1* gene product with fungal P-type $Na^+$-ATPases. Deduced Ena1 proteins from *F. oxysporum* (EMBL accession no. AY345588), *N. crassa* (AJ243520), and *S. cerevisiae* (U24069) are shown. Identical amino acids are highlighted on a shaded background. Dashes indicate gaps in the alignments. Nine predicted transmembrane domains are indicated by solid bars.

ions. Importantly, *pacC* mutations did not affect growth at high concentrations of the nontoxic cation $K^+$ or sorbitol, suggesting that PacC is specifically involved in the regulation of salt stress but not osmotic stress. Reintroduction of a functional *pacC* copy into the $pacC^{+/-}$ mutant restored the wild-type growth phenotype, suggesting that the increased $Li^+$ and $Na^+$ sensitivity of the mutant was caused solely by the absence of a functional *pacC* allele (data not shown).

To determine whether the differences in sensitivity to $Na^+$ or $Li^+$ observed in the three strains were due to differential accumulation of these cations, we measured the intracellular levels of $Li^+$ and $Na^+$ in the different strains grown in liquid



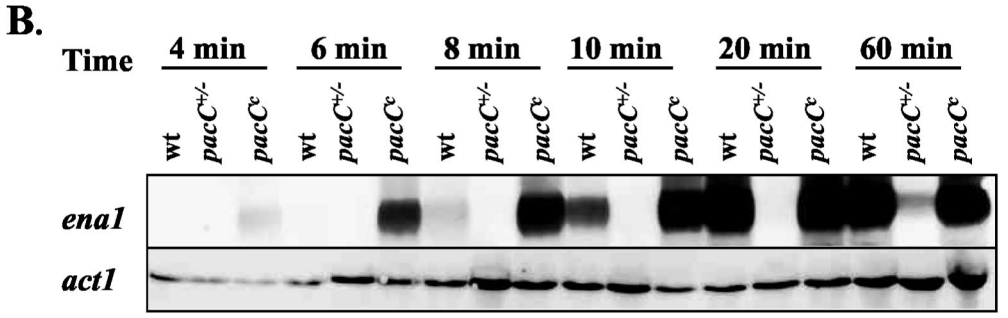




FIG. 6. Activation of *ena1* transcription requires high salt concentrations, alkaline ambient pH, and PacC. Transcript levels of *ena1* in the *F. oxysporum* wild-type strain, $pacC^{+/-}$ mutant, and $pacC^{c}$ mutant were analyzed by northern hybridization analysis. (A) Microconidia of the wild-type strain were germinated for 12 h in SM without added $Na^+$ and then transferred for 2 h to SM buffered at the indicated pH values, with or without 0.5 M $Na^+$. Total RNA was extracted, fractionated on an agarose gel, blotted onto a nylon membrane and hybridized with the *ena1* probe. As a loading control, a probe corresponding to the *F. oxysporum* actin gene (*act1*) was hybridized to the same RNA samples. (B) Microconidia of the wild-type strain, the $pacC^{+/-}$ mutant and the $pacC^{c}$ mutant were germinated as in panel A and transferred to SM buffered at pH 8.0 and containing 0.5 M $Na^+$. Total RNA was extracted from samples obtained at the indicated time points, fractionated, blotted, and hybridized with the *ena1* or *act1* probes.

medium (pH 6.0) containing increasing levels of LiCl or NaCl. The results (Fig. 2) confirmed that increased sensitivity to $Li^+$ and $Na^+$ of the $pacC^{+/-}$ mutant was related to higher internal levels of the two cations. Moreover, the increased tolerance observed in the $pacC^{c}$ dominant activating mutant correlated with a lower intracellular accumulation of $Li^+$ and $Na^+$. Importantly, the three strains contained similar amounts of internal $K^+$, ruling out the possibility that higher sensitivity or tolerance to $Li^+$ and $Na^+$ was caused by differences in the inhibition of $K^+$ uptake by the toxic cations (Fig. 2C).

***pacC* is required for efficient cation efflux at alkaline ambient pH.** Based on these initial results, we decided to explore the effect of PacC on cation fluxes. To determine $Li^+$ influx in the wild-type strain and the *pacC* mutants, mycelia were transferred to 0.1 M LiCl, and the time course of $Li^+$ uptake was monitored for several hours. During the first minutes, the three strains accumulated $Li^+$ at a very similar rate, suggesting that the kinetics of $Li^+$ influx was not altered in the *pacC* mutants (Fig. 3A). After a few minutes, the internal level of $Li^+$ in the salt-tolerant $pacC^{c}$ strain stabilized at ca. 10 nmol $mg^{-1}$ and did not increase further, whereas levels continued rising in the wild-type strain and reached even higher levels in the salt-sensitive $pacC^{+/-}$ mutant. Because it is generally assumed that most of the $Na^+$ and $Li^+$ influx in fungi under standard laboratory conditions takes place through the $K^+$ influx system (22), we studied the time course of influx of the $K^+$ transport analog $Rb^+$. We found that even after 1 h in the presence of 0.1 M RbCl, the kinetics of uptake and the internal levels of $Rb^+$ were highly similar in all of the strains (Fig. 3B). Taken together, these results support the view that mutations in *pacC* do not affect alkali cation influx, thus indicating that efflux of these toxic cations may be affected in the *pacC* mutants.

In fungi, two $Na^+$ and $Li^+$ efflux systems have been described, a sodium/proton antiporter working at acidic pH and a P-type sodium ATPase functioning at a higher pH (20). To test whether one or both of these efflux systems was affected in the *pacC* mutants, microconidia of the three strains were germinated for 12 h in liquid medium containing 0.1 M LiCl (wild type and the $pacC^{+/-}$ strain) or 0.3 M LiCl ($pacC^{c}$ strain), transferred to lithium-free medium buffered either at pH 4.0 or 8.0, and the time course of $Li^+$ efflux was determined. The results shown in Fig. 4 clearly indicate that, although at pH 4.0 the three strains extruded lithium with the same efficiency (Fig. 4A), at pH 8.0 the $pacC^{+/-}$ loss-of-function mutant displayed a strongly reduced $Li^+$ efflux compared to the wild type and the $pacC^{c}$ strain (Fig. 4B). These results are in agreement with the increased $Li^+$ accumulation and sensitivity of the $pacC^{+/-}$ loss-of-function mutant. Although we did not detect differences between the wild type and the $pacC^{c}$ strain in terms of kinetics of $Li^+$ efflux, the fact that absolute concentrations of internal $Li^+$ in the $pacC^{c}$ strain were significantly lower than those of the wild type (see Fig. 2A) suggests that $Li^+$ efflux was more efficient in the $pacC^{c}$ strain. In summary, our results indicate that PacC controls salt tolerance in *F. oxysporum* by regulating an efflux system functional at an alkaline pH but not at an acidic pH. In support of this view, differences in salt tolerance between the wild type and the *pacC* mutants were almost negligible when strains were grown on plates at pH 4.0 (results not shown).

**PacC controls transcriptional activation of *ena1* encoding a P-type $Na^+$-ATPase of *F. oxysporum*.** The results shown in Fig. 4 suggest that, like *S. cerevisiae*, *F. oxysporum* has at least two different $Na^+$ ($Li^+$) efflux systems: one functioning at pH 4.0 that is still present in the $pacC^{+/-}$ loss-of-function mutant and the other one working at pH 8.0 that is not present in the mutant. Because the main alkaline $Na^+$ and $Li^+$ efflux system in yeast and filamentous fungi is based on P-type $Na^+$-ATPases encoded by the *ENA* genes (1, 3, 6, 7, 15), we decided to clone

the *ENA1* orthologue in *F. oxysporum.* PCR amplification with degenerate primers derived from highly conserved regions of the *Neurospora crassa ena1* gene produced a fragment of the expected size (250 bp) that was cloned and sequenced. After we confirmed its homology with *N. crassa ena1*, we used the fragment to probe a λEMBL3 genomic library of *F. oxysporum* isolate 4287. Sequencing of a hybridizing genomic clones revealed the presence of an open reading frame of 3,261 nucleotides encoding a predicted protein of 1,087 amino acids. Sequence alignment of *F. oxysporum ena1* with fungal *ena1* genes in the databases suggested the presence of three introns 215, 51, and 49 nucleotides in length, respectively. The 5′-flanking sequence of the *ena1* gene contains four copies of the PacC consensus binding sequence 5′-GCCARG-3′ (26) at positions −549, −474, −382, and −321. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AY345588. The deduced *F. oxysporum* Ena1 protein has 70 and 46% amino acid identity with the Ena1 proteins of *N. crassa* and *S. cerevisiae*, respectively, and contains nine putative transmembrane regions that are highly conserved between fungal P-type $Na^+$-ATPases (Fig. 5). Southern analysis of genomic DNA digested with different restriction enzymes suggested that *ena1* is present as a single copy in the *F. oxysporum* genome. Moreover, a BLAST search of the *F. graminearum* complete genome sequence with *ena1* only produced one significant match (results not shown).

To study the mechanisms controlling expression of *ena1* in *F. oxysporum*, Northern hybridization analysis was performed with total RNA from mycelia of the wild-type strain transferred for 2 h to SM buffered at different pH values and containing either 0.5 M $Na^+$ or no added $Na^+$. No *ena1* transcript was detected in mycelia grown in the absence of $Na^+$ (Fig. 6A). In contrast, *ena1* expression was strongly induced in the presence of $Na^+$ at pH 8.0 but not at pH 4.0. Thus, a combination of $Na^+$ and alkaline pH is required to trigger the expression of *ena1* gene.

We next studied the kinetics of *ena1* activation in the wild type and the *pacC* mutants upon transfer of mycelia to SM buffered at pH 8.0 containing 0.5 M $Na^+$. In the wild-type strain, the *ena1* transcript was detectable after 10 min and increased further until 20 min after transfer (Fig. 6B). In the $pacC^{c-}$ mutant, *ena1* transcript levels increased much earlier and reached higher levels than in the wild-type strain. In both strains, transcript levels remained high until 60 min after transfer and declined slightly after 90 min (data not shown). In contrast, in the $pacC^{+/-}$ mutant *ena1* expression was induced much later, only 60 to 90 min after the transfer, and remained at much lower levels. These results suggest that PacC positively controls transcriptional activation of *ena1* in response to $Na^+$ at alkaline pH.

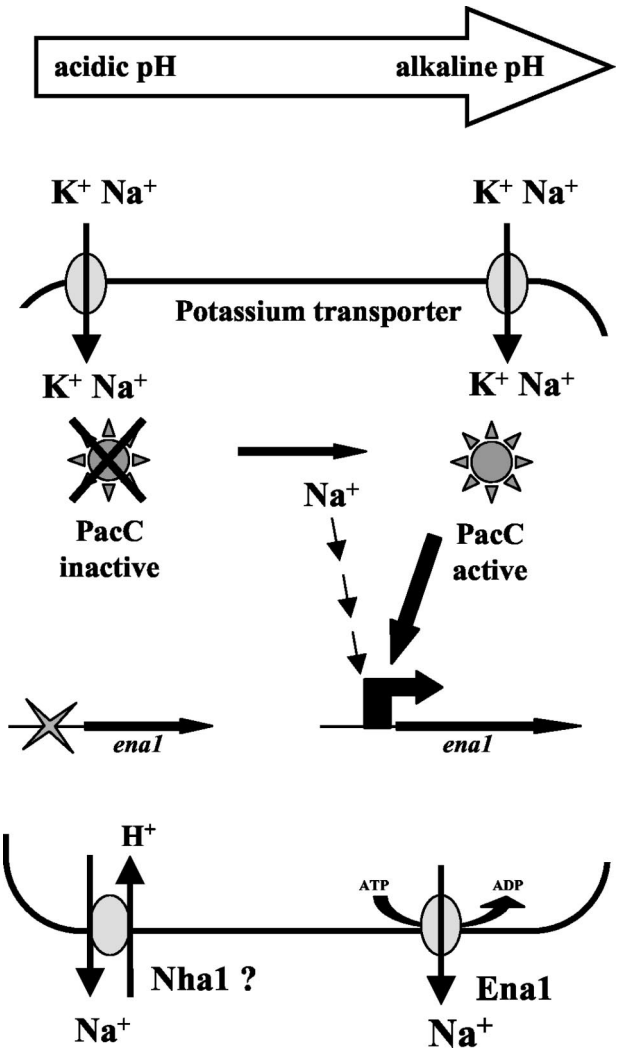



FIG. 7. Model for the role of PacC in the control of ion homeostasis in *F. oxysporum*. High external concentrations of $Na^+$ result in an increased influx of the cation, elevating intracellular $Na^+$ levels beyond a threshold. This triggers transcriptional activation of the *ena1* gene encoding a P-type $Na^+$-ATPase via an unknown signaling mechanism. In addition, expression of *ena1* also requires binding of the activated form of PacC transcription factor to its cognate binding sites in the promoter. Proteolytic activation of PacC occurs predominantly at alkaline ambient pH; therefore, both high $Na^+$ concentrations and high pH are required for transcriptional activation of *ena1*. Conversely, at acidic pH an alternative, PacC-independent $Na^+$ efflux system is active, possibly based on a $Na^+/H^+$ antiporter orthologous to Nha1 of *S. cerevisiae*. Besides activation of *ena1*, PacC may have additional regulatory effects on ion homeostasis.

## DISCUSSION

The transcription factor PacC regulates expression of alkaline- and acid-expressed genes in *F. oxysporum* (11). We show here that this factor plays an essential role in salt tolerance of *F. oxysporum* because strains that lack PacC are highly sensitive to $Na^+$ and $Li^+$. Our data strongly suggest that salt sensitivity in these mutants is caused by a defect in the $Na^+$ and $Li^+$ efflux process. Conversely, dominant activating $pacC^c$ mutants show increased salt tolerance, correlating with rapid and increased expression of a sodium efflux system in this strain. In a previous study, we proposed the existence of an $Na^+$-($Li^+$)-ATPase in *F. oxysporum* as the main system involved in the efflux of these cations (10). We identify here *ena1*, an *F. oxysporum* orthologue of the *ENA1* genes from yeast and *N. crassa* (3, 6, 7, 15, 23). We show that expression of *ena1* requires a combination of high $Na^+$ concentrations and alkaline pH. Although the presence of $Na^+$ alone is sufficient to induce expression of P-type $Na^+$-ATPases in certain cases (1), our results are in

agreement with most previous studies demonstrating that full expression of *ENA* genes requires a combination of $Na^+$ and high pH (1, 3).

The changes in salt tolerance and intracellular $Na^+$ and $Li^+$ levels observed in the *pacC* mutants suggested a functional link between ambient pH, PacC, and *ena1* expression in *F. oxysporum*. Our data support a positive role of PacC in the regulation of *ena1*, since $pacC^{+/-}$ loss-of-function mutants show strongly delayed and reduced expression of *ena1*, whereas $pacC^c$ strains expressing a dominant activating *pacC* allele induce *ena1* expression more rapidly and to higher levels than did the wild type. Our results are similar to those reported in *S. cerevisiae*, where the *pacC* orthologue *RIM101* was shown to control the expression of *ENA1* (17, 25). Further supporting this view, we found that the 5′-flanking sequence of the *ena1* gene of *F. oxysporum* contains four copies of the PacC consensus binding sequence 5′-GCCARG-3′. In Fig. 7 we present a model summarizing the regulation of *ena1* expression by PacC in *F. oxysporum.* According to this model, PacC activates *ena1* expression at alkaline ambient pH coordinately with a second factor that responds to high $Na^+$ levels. Conversely, at an acidic ambient pH the $Na^+$ efflux process would be mediated by a $Na^+/H^+$ antiporter acting independently of PacC. In *S. cerevisiae*, a $Na^+/H^+$ antiporter system encoded by the *NHA1* gene mediates $Na^+$ tolerance at acidic pH values (4, 5, 16). We have detected the existence in *F. oxysporum* of an orthologue of *NHA*, a plasma membrane $Na^+/H^+$ antiporter from fungi and plants (Z. Caracuel et al., unpublished data).

The results of the present study suggest a major role for PacC in salt tolerance of *F. oxysporum*. Although most of the changes in $Na^+$ sensitivity and accumulation observed in the *pacC* mutants could be explained by differences in *ena1* expression with the consequent effects on cation efflux, such a function for *ena1* in *F. oxysporum* remains to be demonstrated. Moreover, a role of additional regulatory mechanisms controlled by PacC cannot be ruled out. Thus, the fact that even after prolonged times of exposure, absolute concentrations of internal $Li^+$ in the $pacC^{+/-}$ and $pacC^c$ mutants stabilized at significantly higher and lower levels than in the wild-type strain, respectively, suggests that mutations in *pacC* may not only affect cation efflux but also intracellular cation sensing and homeostasis, although the underlying mechanisms remain to be elucidated.

## ACKNOWLEDGMENTS

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