Potassium and Na\(^+\) effluxes across the plasma membrane are crucial processes for the ionic homeostasis of cells. In fungal cells, these effluxes are mediated by cation/H\(^+\) antiporters and ENA ATPases. We have cloned and studied the functions of the two ENA ATPases of *Ustilago maydis*, *U. maydis* Ena1 (UmEna1) and UmEna2. UmEna1 is a typical K\(^+\) or Na\(^+\) efflux ATPase whose function is indispensable for growth at pH 9.0 and for even modest Na\(^+\) or K\(^+\) tolerances above pH 8.0. UmEna1 locates to the plasma membrane and has the characteristics of the low-Na\(^+\)/K\(^+\)-discrimination ENA ATPases. However, it still protects *U. maydis* cells in high-Na\(^+\) media because Na\(^+\) showed a low cytoplasmic toxicity. The UmEna2 ATPase is phylogenetically distant from UmEna1 and is located mainly at the endoplasmic reticulum. The function of UmEna2 is not clear, but we found that it shares several similarities with *Neurospora crassa* ENA2, which suggests that endomembrane ENA ATPases may exist in many fungi. The expression of *ena1* and *ena2* transcripts in *U. maydis* was enhanced at high pH and at high K\(^+\) and Na\(^+\) concentrations. We discuss that there are two modes of Na\(^+\) tolerance in fungi: the high-Na\(^+\)-content mode, involving ENA ATPases with low Na\(^+\)/K\(^+\) discrimination, as described here for *U. maydis*, and the low-Na\(^+\)-content mode, involving Na\(^+\)-specific ENA ATPases, as in *Neurospora crassa*.
TABLE 1. Oligonucleotides used in this study

| Primer | 5’-3’ sequence |
|--------|----------------|
| ENA1-ATG | TCAGTTCAAGACGACAGTTCATC |
| ENA1-STOP | GCTACAGTGTGCTAAGAAGAAAG |
| Xbal-ENA2-ATG | GTCTCAGTAAATAACATGTTCAGGTCTAGG |
| ENA2-STOP | TGCCGACAGCAGACGACAGATAG |
| NdeI-ENA1ATG | CCATATGGTGCACAAGAAAGAAGAC |
| NdeI-ENA1Rev | CCGATGTTTACAATGGTCAGTGCTAGG |
| BamHI-ENA2ATG | GGGATCTTAAAAACATGTTCAGGTCTAGG |
| NdeI-ENA2Rev | CCGATGTTTACAATGGTCAGTGCTAGG |
| PC-13B8-1 | GGGACACTGTTGGAGAAGAACAG |
| PC-13B8-1R | GCCGGTGCAGACGAAGATGAT |
| PC-14G4-2 | CTTTCTAGCTCGTGGTCAAGCC |
| PC-14G4-2R | TTTTCTGCTAGTGGTCAAGCC |
| UmACT1-2 | GGGATCTTAAAAACATGTTCAGTGCTAGG |
| UmACT1-1R | GGGCACTGTTGGAGAAGAACAG |
| XbaNeENA2ATG | GGGATCTTAAAAACATGTTCAGTGCTAGG |
| NcENA2-STOP | TTTTCTGCTAGTGGTCAAGCC |
| BamNeENA2Rev | CCGATGTTTACAATGGTCAGTGCTAGG |

* In some of the primers, a restriction site sequence was included (underlined).

attained in S. pombe and Schwanniomyces occidentalis, but these disruptions do not resolve the uncertainties originated by the S. cerevisiae model. S. pombe is also acidophilic, and moreover, it has an atypical ENA ATPase, Cta3 (42), which mediates K⁺ efflux almost exclusively (12). In the case of S. occidentalis, the double disruption of the two identified ENA4 genes was not attained (7). Moreover, its genome has not been sequenced, and the number of ENA4 genes is unknown.

In addition to the pending questions about the reasons for the universal presence of ENA ATPases in fungi (12) and their role in the growth of fungi at alkaline pH, new questions have been raised by the discovery of ENA ATPases in the parasites Leishmania and Trypanosoma (12, 31) and in bryophytes (15). Therefore, further studies of ENA ATPases are necessary, but the ENA ATPases of S. cerevisiae cannot serve as models, nor can the expression of foreign ENA ATPases be conveniently studied using S. cerevisiae ena mutants.

In the search for a new fungal model for studying ENA ATPases, we selected Ustilago maydis. U. maydis is a dimorphic basidiomycete plant pathogen (36) for which some studies of ENA ATPases, Ena1 (UmEna1) and UmEna2.

**MATERIALS AND METHODS**

**Bacterial and fungal strains and growth conditions.** U. maydis strains FB1 (a₁b₁) and FB2 (a₂b₂) (9) were used throughout this study. Escherichia coli strain DH5α was routinely used for the propagation of plasmids. The S. cerevisiae strains used were W303.1A (Mat a₁a₂ trpl 1 leu2 his3) and its derivatives B31 (Mat ade2 trpl 1 ena-1 URA3 his3 trpl 1 leu2 his3) (6) and G19 (Mat ade2 trpl 1 ena-1 URA3 his3 trpl 1 leu2 his3) (6), in which the Na⁺ efflux systems ENA1-4 and NHA1 or only ENA1-4 is absent. Fungal strains were normally grown either in complex yeast-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone, 1% dextrose) or in minimal SD medium (51). Growth at variable K⁺ and Na⁺ concentrations was done using arginine phosphate (AP) medium (48) supplemented with the indicated K⁺ and Na⁺ concentrations.

**Recombinant DNA techniques.** Manipulation of nucleic acids was performed by standard protocols or, when appropriate, according to the manufacturers’ instructions. PCRs were performed with a Perkin-Elmer thermocycler using the Expand-high-fidelity PCR system (Roche Molecular Biochemicals). Some of the PCR fragments were first cloned into the PCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen). For expression in yeast cells, the genes were cloned into vector pYPGE15 (20). In all cases, most of the polylinker sequences preceding the translation initiation codon were eliminated, and a sequence environment as similar as possible to (AU)₃(A/C/A)(A/C/A)ₙ(A/U/G/U/C)ₙ was created around it (29). DNA sequencing was performed using an automated ABI Prism 3730 DNA analyzer (Applied Biosystems). DNA and total RNA were prepared using the DNeasy and RNeasy plant kits (Qiagen), respectively. PCR amplifications of mRNA fragments were carried out on double-stranded cDNA synthesized from total RNA by using the cDNA synthesis system kit (GE Healthcare). The full-length ena1 and ena2 cDNAs were obtained by reverse transcription-PCR from RNA extracted from U. maydis using specific primers that amplified DNA fragments that contained the predicted start and stop codons (Table 1). The ena1 and ena2 genes were amplified from genomic DNA by PCR using the same primers that we used for the cDNAs.

**Real-time PCR assays.** The results reported in Table 2 were obtained using cells that were grown in AP medium with 3 mM KCl and then transferred into the same medium modified as follows: plus 10 mM tartaric acid and brought to pH 3.5 with arginine; with Ca²⁺ decreased to 0.5 mM and brought to pH 8.0 with arginine; plus 500 mM NaCl; plus 500 mM KCl; and without K⁺. All treatments were for 2 h, except K⁺ starvation, which was for 4 h. Real-time PCR assays were performed as described previously (26) except that the standard DNA solutions corresponded to the genes studied in this report, ena1, ena2, and actin genes of U. maydis. mRNA preparations were treated with RNase-free DNase I (40 U in 100 μl; Roche) for 1 h at 37°C. After treatment, mRNA was purified according to the instructions provided by the RNeasy plant kit (Qiagen). PCR primers UmACT1-2 (5’-GTGCCCACCATCAGGAACAGT-3’) and UmACT1-1R (5’-CCGCAATGGTGGTGAAAGGGTAG-3’) were designed to amplify the following fragments: ena1 at positions 2952 to 3106 (GenBank accession number FM199940) and ena2 at positions 3235 to 3359 (accession number FM199941).

**Table 2. Effect of growth conditions on U. maydis ena1 and ena2 transcript abundances**

| Transcript | YPD medium | AP plus 0.5 M Na⁺ | AP plus 0.5 M K⁺ | AP at pH 3.5 | AP at pH 8.0 | K⁺ starvation |
|------------|------------|------------------|-----------------|--------------|--------------|---------------|
| ena1       | 8.3        | 337              | 324             | 5.6          | 181          | 12.2          |
| ena2       | 1.1        | 85               | 124             | 0.7          | 153          | 4.3           |

*Cells were grown overnight in AP medium with 3 mM K⁺ and then transferred into the indicated media for 2 h. The given values are ratios with reference to actin transcript abundance.*
Localization of UmEna1-green fluorescent protein (GFP) and UmEna2-GFP in *U. maydis* and *Neurospora crassa* ENA2 (NcENA2)-GFP in Saccharomyces yeast cells. The ena1-GFP and ena2-GFP constructs were in-frame fusions of the 3' ends of the ena1 and ena2 open reading frames to the GFP gene of plasmid pCU3. To generate these constructs, full-length ena1 cDNA was amplified using primers Ndel-ENA1F and Ndel-ENA1R, which include the Ndel restriction site. Full-length ena2 cDNA was amplified using primers BamHI-ENA2ATG and Ndel-ENA2Rev, which include BamHI and Ndel restriction sites, respectively (Table 1).

For expression in *U. maydis*, ena1 or ena2 PCR fragments were cloned into the Ndel and Ndel/BamHI sites of plasmid pCU3 (PflI-dependent expression), respectively, which are at the 5' ends of the GFP gene. These plasmids were linearized with SspI and transformed into *U. maydis* cells to integrate the construct into the chlB locus by homologous recombination as described previously (18). For expression in *S. cerevisiae*, the Ncena2-GFP fusion was cloned into plasmid pYGPE15 (20). This construct was transformed into the above-mentioned B31 yeast mutant. To visualize the endoplasmic reticulum (ER), an ER-red fluorescent protein (RFP) fusion protein was produced as described previously (56) but using monomeric RFP as a reporter and a hygromycin resistance cassette as a selectable marker.

The GFP fluorescence signal in *U. maydis* and yeast cells was visualized using a confocal ultraviolet Leica (Mannheim, Germany) TCS-Sp2-AOBS-UV microscope.

Disruption of the ena1 and ena2 genes. To obtain the ∆ena1 mutant, we constructed a disruption plasmid by ligating two DNA fragments of the ena1 cDNA to the 5' and 3' ends of the nourseothrin resistance cassette in pNEBHyg(+), a *U. maydis* integration vector (40). A 5' fragment of 1.157 bp was obtained by digesting ena1 cDNA with SpeI and BamHI and was inserted between the SpeI and BglII sites of plasmid pNEBHyg(+). A 3' fragment of 1.239 bp was obtained by digesting ena1 cDNA with PvuII and HindIII and was inserted into the EcoRV and HindIII sites in plasmid pNEBHyg(+). The plasmid with the two insertions was linearized with SspI and transformed into *U. maydis* wild-type strains FB1 and FB2. Transformants were selected in the presence of nourseothrin (Hans Knoll Institute, Jena, Germany) at 150 μg ml⁻¹.

The disruption plasmid for the ∆ena2 mutant was constructed using fragments of the ena2 gene for flanking the hygromycin B resistance cassette in plasmid pNEBHyg(+) (17). A 600-bp 5' fragment was obtained by digesting ena2 cDNA with SphI and BamHI and was inserted between the SphI and BamHI sites of pNEBHyg(+). A 920-bp 3' fragment was obtained by digesting ena2 cDNA with KpnI and EcoRI, and was inserted between the KpnI and EcoRI sites of pNEBHyg(+). The plasmid with the two insertions was linearized with SspI and transformed into *U. maydis* wild-type strains FB1 and FB2. Transformants were selected in YPD medium supplemented with hygromycin B (Sigma-Aldrich) at 50 μg ml⁻¹.

The ∆ena1 ∆ena2 double mutant was constructed by transforming the ∆ena1 strain with the linearized DNA construct used for the disruption of the ∆ena2 strain, to recover the hygromycin B-resistant transformants, it was necessary to use regeneration agar (1 M sorbitol, 1% yeast extract, 2% peptone, 2% sucrose, and 1.5% agar) at pH 5.0 buffered with 20 mM MES (morpholineethanesulfonic acid). At other pH values, the accumulation of acridine orange [3,6-bis(dimethylamino)acridine] in the vacuole was monitored (22). This fluorescent dye penetrates the plasma membrane in an uncharged, neutral form and then accumulates into acidic organelles, where it is trapped in the protonated form. Previous to the digitoxin treatment, the cells were incubated in AP medium with 100 μg ml⁻¹ acridine orange for 20 min at room temperature. Next, 0.001% digitoxin was added, and at intervals, 4 μl of the cell suspension was transferred onto a slide, covered with a cover glass, and observed with a Zeiss fluorescence microscope under blue light. Images were recorded with a Leica DFC300FX color camera connected to the microscope.

**RESULTS**

**Basic description of *U. maydis* alkali cation tolerance.** *U. maydis* grew well in a wide range of Na⁺ or K⁺ concentrations at pH values ranging from 3.5 to 9.0. In YPD medium, it grew in up to 1.0 M Na⁺ or 1.2 M K⁺. Growth was also maintained at low-micromolar K⁺ and Na⁺ concentrations, where both cations were depleted almost independently from the K⁺ concentration; for example, growth rates at 150 mM Na⁺ with either 4.5 or 0.5 mM K⁺ were almost identical. At 4.5 mM K⁺, the growth rate was affected by 500 mM Na⁺ and was only partially reduced by 800 mM Na⁺.

Analyses of cation contents of *U. maydis* cells growing at high Na⁺ concentrations revealed that they contained fairly high internal Na⁺/K⁺ molar ratios without any apparent detrimental effect. These results raised the question of whether Na⁺ was sequestered into the vacuole as in plant cells (21, 53). To address this question, we determined the cytoplasmic Na⁺/K⁺ ratio by measuring the Na⁺ and K⁺ losses after digitoxin permeabilization of the plasma membrane. The accuracy of the results of this approach relies on two conditions, that tonoplasts were not permeabilized and that intact cells did not take up the K⁺ released by permeabilized cells. To test the integrity of the tonoplast, we checked the capacity of the vacuoles to maintain ΔpH by acridine orange staining (2, 22). A significant effect of digitoxin on the tonoplast was found to...
start after 20 min of treatment. Therefore, in the experiments that we report below, the time of digitonin treatment was limited to 15 min so that not more than 1 vacuole out of 100 was unstained. To check that the K⁺ released by permeabilized cells was not taken up by intact cells during the experiments, we carried out parallel experiments in the presence and in the absence of antimycin A, which inhibits respiration and, consequently, K⁺ uptake (46). The presence of this inhibitor did not affect the results. Furthermore, the time courses of the K⁺ and Na⁺ releases showed a constant Na⁺/K⁺ ratio from the first sample taken, with less than 10% of the cells permeabilized, up to the last sample taken, with probably more than 80% of the cells permeabilized. This result also ruled out the possibility that intact cells took up K⁺.

U. maydis vacuoles were not stained by acridine orange in cells growing at high Na⁺ concentrations (for example, 4.5 mM K⁺/150 mM Na⁺). This might be the result of an excessive uptake of cations and alkalization of the cells, but the causes were not investigated. Incubation of these cells in K⁺⁻ and Na⁺⁻-free medium for 3 h did not change their K⁺⁻ and Na⁺⁻ contents significantly but fully restored the capacity of the vacuoles to accumulate acridine orange (Fig. 1A). An additional advantage of this incubation was that U. maydis cells adapted to keeping very low K⁺ or Na⁺ concentrations in the external medium (typically, 0.5 µM K⁺ and 10 µM Na⁺). Under these conditions, it was very simple to measure the K⁺⁻ and Na⁺⁻ released into the external medium by the digitonin treatment because the treatment increased the external concentrations very much, while untreated cells kept them very low.

Our results with cells grown at different K⁺⁻ and Na⁺⁻ concentrations show that the vacuole of U. maydis did not accumulate large amounts of Na⁺. When cells grown at 4.5 mM K⁺⁻ and 150 mM Na⁺⁻ and subsequently K⁺⁻ and Na⁺⁻ starved for 3 h were treated with digitonin, the time courses of the K⁺⁻ and Na⁺⁻ releases into the external medium showed a permanent increase at a constant Na⁺⁻/K⁺⁻ ratio of 1.4 (Fig. 1B). At the same time, the ratio between the Na⁺⁻ and K⁺⁻ that remained in the cells (vacular content of permeabilized cells plus the content of intact cells) after each interval treatment decreased permanently (Fig. 1C). In three independent experiments with cells grown at 4.5 mM K⁺⁻ and 150 mM Na⁺⁻, the mean Na⁺⁻/K⁺⁻ ratio was 1.5 ± 0.2, while in cells grown at 10 mM K⁺⁻ and 500 mM Na⁺⁻, the mean ratio was 2.3 ± 0.2. Taken together, these experiments indicated that the Na⁺⁻/K⁺⁻ ratio in the vacuole of actively growing cells was lower than the cytoplasmic Na⁺⁻/K⁺⁻ ratio and that the latter could be as high as 2.3 without any detrimental effect.

**U. maydis has two ENA ATPases.** Computer-based searches of the genomic sequence of U. maydis using ENA ATPase sequences as queries identified two open reading frames that could encode Ena proteins. The corresponding genes, *ena1* and *ena2*, were cloned by a standard PCR-based approach. These genes did not contain introns and encode two proteins of 1,100 and 1,125 amino acids, respectively. The study of the amino acid sequences of both ATPases showed that their structures and functional characteristics corresponded to typical P-type ATPases (33, 50) of group IID (4). Remarkably, the *ena1* and *ena2* genes did not result from a recent duplication event because the phylogenetic distance between the encoded pumps was larger than the phylogenetic distance between the basidiomycete UmEna1 and the ascomycete NeENA1 pumps. The existence of two or more ENA ATPases in two distant phylogenetic clusters was also found for *Aspergillus*, *Neurospora*, and *Magnaporthe* (Fig. 2). The *ena1* gene was located on chromosome 3, and the *ena2* gene was located on chromosome 1. Transcript expressions of *ena1* and *ena2* as determined by real-time PCR showed that the levels of expression of both genes were low under normal conditions and that almost a 100-fold induction occurred at high Na⁺⁻ or K⁺⁻ concentrations or at a high pH (Table 2), which is very similar to previous descriptions of other fungi (1, 7, 11, 25).

The *ena1* and *ena2* genes were then expressed in mutant yeast strain B31, which lacks the ENA ATPases and the NHA1 antiporter (8), using yeast expression vector pYPGE15 with the genes under the control of the PGKI gene promoter (20). *ena1* but not *ena2* completely suppressed the defective growth of B31 at high Na⁺⁻ and high K⁺⁻ concentrations (Fig. 3A). However, *ena2* suppressed the defect of B31 only at the minimal Na⁺⁻ concentration at which the growth of B31 was inhibited. Consistent with the pumping capacities of an Na⁺-ATPase, UmEna1 mediated the cellular Na⁺⁻ loss at pH 8.0 with 10 mM Na⁺⁻ in the external medium (Fig. 3B). Under these conditions, an Na⁺⁻ channel or an electroneutral Na⁺⁻/H⁺ antiporter would mediate Na⁺⁻ uptake driven by the membrane potential and ΔpH, respectively. ENA ATPases may be specific for K⁺⁻ or Na⁺⁻, protecting cells only from high concentrations of one of
these cations, or nonspecific, protecting cells from high concentrations of either of them (12). The results showed that UmEna1 belonged to the nonspecific group (Fig. 3A).

**Effects of the disruption of ena1 and ena2.** The function of the UmEna1 and UmEna2 ATPases was assessed by gene disruption. Initially, we obtained the single and double disruptions in strain FB1, which is almost identical to the strain whose genome has been sequenced (36). Later, the disruptions were also attained in a strain of the opposite mating type, FB2, whose genome has been sequenced (36). Later, the disruptions in strain FB1, which is almost identical to the strain accounted for by the two ena1 and ena2 cDNAs were inoculated into the indicated media. Numbers indicate concentrations (mM). (B) Time courses of Na⁺ extrusion at pH 8.0 in B31 transformants loaded with Na⁺. B31 was transformed with the empty plasmid (open triangles), ena1 (open circles), and ena2 (closed circles).

![Phylogenetic tree of fungal ENA ATPases](image)

**FIG. 2.** Phylogenetic tree of fungal ENA ATPases. Species are as follows: ScPMC1, *Saccharomyces cerevisiae* Ca²⁺-ATPase included as an outgroup (GenBank accession number P58929); UmEna2, *Ustilago maydis* (accession number XP_756351); HwENA1, *Hortaea werneckii* (accession number ABD64570); NeENA2, *Neurospora crassa* (accession number AJ243519); Magnap-4, *Magnaporthe grisea* (accession number XP_001404752); Asperg-3, *Aspergillus fumigatus* (accession number AAR01872); DhENA1, *Dipodascus hansenii* (accession number AAB86427); DhENA2, *D. hansenii* (accession number AAK52600); SoENA1, *Schwanniomyces occidentalis* (accession number AAK28385); SoENA2, *S. occidentalis* (accession number EAL87230); SoENA3, *Zygosaccharomyces rouxii* (accession number EAL85670); Asperg-1, *A. fumigatus* (accession number EAL85670); Asperg-2, *A. fumigatus* (accession number EAL89843); UmEna1, *U. maydis* (accession number XP_757891); SpCTA3, *Schizosaccharomyces pombe* (accession number XP_359699); NeENA1, *N. crassa* (accession number XP_356372); NeENA2, *N. crassa* (accession number AAB86427); NeENA3, *N. crassa* (accession number XP_962099). An * indicates cloned pumps.

![Functional expression of ena1 and ena2 cDNAs in *S. cerevisiae*.](image)

**FIG. 3.** Functional expression of *ena1* and *ena2* cDNAs in *S. cerevisiae*. (A) Suppression of the defective growth of Na⁺ efflux mutant strain B31 in the presence of high Na⁺ or K⁺ concentrations; drops of serial dilutions of cell suspensions of the wild type and of the B31 strain transformed with the empty plasmid or with the *ena1* or *ena2* cDNAs were inoculated into the indicated media. Numbers indicate concentrations (mM). (B) Time courses of Na⁺ extrusion at pH 8.0 in B31 transformants loaded with Na⁺. B31 was transformed with the empty plasmid (open triangles), *ena1* (open circles), and *ena2* (closed circles).
proteins in *U. maydis* cells with gene expression under the control of the transcriptional elongation factor promoter (18). The GFP fusions did not affect the above-described biological activities of the UmEna1 and UmEna2 ATPases. The expression of UmEna1-GFP in the Δena1 strain suppressed its sensitivity to high Na\(^+\) or K\(^+\) concentrations (Fig. 4A), and the expression of UmEna2-GFP in mutant yeast strain B31 weakly suppressed its Na\(^+\) sensitivity (Fig. 3A).

Microscopy analysis of *U. maydis* cells expressing UmEna1-GFP located the protein mainly to the plasma membrane and to some vesicles, which might be in transit to the plasma membrane (Fig. 5). In contrast, UmEna2-GFP was located around the nucleus, in close proximity to the plasma membrane, and in internal vesicles. The coexpression of UmEna2-GFP with an ER-RFP fusion demonstrated that UmEna2-GFP localized to the ER and to other endomembranes that were not investigated (Fig. 5).

**NcENA2 has similarities with UmEna2.** In a previous report, the function of the NcENA2 ATPase (previously called ph7) could not be established (11). Interestingly, the phylogenetic divergence between the NcENA1 and NcENA2 ATPases was similar to that between the UmEna1 and UmEna2 ATPases (Fig. 2). Now, using a new construct in which the sequence context around the first in-frame AUG was optimized for translation, we found that NcENA2 weakly suppressed the defect of the B31 mutant, exactly as shown for UmEna2 in Fig. 3A (data not shown). Next, to investigate whether NcENA2 was located to the plasma membrane or to endomembranes, we expressed the NcENA2-GFP fusion protein in yeast cells. The NcENA2-GFP signal localized to spots that were neither in the tonoplast nor in the plasma membrane. Although NcENA2 resembled UmEna2 in that both proteins show similar levels of functional expression in yeast cells and localized to endomembranes, they might fulfill different functions because the microscopic images of NcENA2-GFP did not correspond to a typical ER location (Fig. 6). *N. crassa* has a third ENA ATPase (NcENA3) (Fig. 2) that might be a functional homolog of UmEna2. This possibility was not tested because we have so far failed to clone NcENA3.

**Do ENA ATPases have functions other than cation pumping in the plasma membrane?** ENA ATPases are universally present in fungi, and many fungi have ENA genes that encode phylogenetically distant ENA ATPases (for example, *U. maydis*, *Aspergillus fumigatus*, and *N. crassa* ENA ATPases) (Fig. 2), which apparently locate to different membranes (Fig. 5 and 6). All these observations raised the question of whether the functions of ENA ATPases may be more than the currently...
assigned roles of Na\(^+\) and K\(^+\) pumping out of cytoplasm. Therefore, we selected several physiological functions with no obvious relationship to ion transport to be tested in the \(\text{ena}1\) and \(\text{ena}2\) strains.

First, we tested the mating abilities of the single and double mutants of the FB1 and FB2 strains. All mixtures of sexually compatible strains developed positive Fuz reactions regardless of the \(\text{ena}1\) or \(\text{ena}2\) mutation (not shown). The virulence capability of the \(\text{ena}\) strains was also tested by the inoculation of mixtures of sexually compatible mutants (FB1 \(\text{ena}1/\text{FB2}\), FB1 \(\text{ena}2/\text{FB2}\), and FB1 \(\text{ena}1\) \(\text{ena}2/\text{FB2}\) \(\text{ena}1\) \(\text{ena}2\)) wild-type strains (FB1/FB2) onto maize seedlings. Mixtures of mutants did not show any difference in virulence symptoms such as chlorosis, anthocyanin pigmentation, or tumor production compared to those of wild-type mixtures (not shown).

Next, we carried out growth tests under many different conditions and found a surprising defect. The growth of \(U.\ maydis\) was slightly inhibited in YPD medium (1% yeast extract, 2% peptones added to AP medium reproduced the YPD effect, respectively to the wild-type strain. Different types of commercial peptones added to AP medium reproduced the YPD effect, but vitamin-free Casamino Acids (Difco) produced only a weak effect. The marked pH dependence of the toxic effect suggested that the permeable form of a fatty acid might be involved, but we failed to find a fatty acid or a mixture of fatty acids that produced the inhibition. We also tested whether the addition of NH\(_4\)\(^+\) suppressed the inhibitory effect, finding that concentrations of up to 200 mM did not show any suppressive effect (data not shown). The defective growth at pH 4.0 in YPD medium produced by the \(\text{ena}1\) \(\text{ena}2\) mutations in \(U.\ maydis\) was not produced by the equivalent \(\text{ena}1-4\) mutation in \(S.\ cerevisiae\) (Fig. 7).

**DISCUSSION**

**Low Na\(^+\) toxicity in \(U.\ maydis\).** It is normally assumed that K\(^+\) is the most abundant cellular cation and that cells growing in the presence of Na\(^+\), as do animal cells, exclude Na\(^+\) to keep a high K\(^+\) content. Under similar conditions, plant cells also sequester Na\(^+\) in the vacuole to keep a low Na\(^+\)/K\(^+\) ratio in the cytoplasm (21, 53). The notion that the concentration of Na\(^+\) is low in the cytoplasm does not apply to \(U.\ maydis\). We observed good growth when the cytoplasmic Na\(^+\)/K\(^+\) ratio was 2.3.

The inability of fungal cells to decrease the cytoplasmic Na\(^+\) concentration by accumulating it into the vacuole, as reported here for \(U.\ maydis\), was previously reported for \(S.\ cerevisiae\) (39, 46, 55) and \(Debaryomyces\ hansenii\) (39). These three species grow normally with a rather high Na\(^+\) content, exhibiting low cytoplasmic Na\(^+\) toxicity. In \(S.\ cerevisiae\), an Na\(^+\)/K\(^+\) ratio of 1 is completely nontoxic (39), and in \(D.\ hansenii\), the Na\(^+\)/K\(^+\) ratio can be as high as 4 without detrimental effects (39). Similarly, \(Hortaea\ verneckii\) and \(Aureobasidium\ pullulans\) show comparable K\(^+\) and Na\(^+\) contents when actively growing at 0.8 M NaCl (37). In contrast, in \(Neurospora\ crassa\) (52), \(Candida\ albicans\) (16), and \(Candida\ tropicalis\) (24), low Na\(^+\) contents are toxic, which suggests high cytoplasmic Na\(^+\) toxicity.

In summary, there seem to be two types of fungi regarding Na\(^+\) tolerance, those tolerant to high Na\(^+\) contents in the cytoplasm, which include \(U.\ maydis\), and those intolerant to high Na\(^+\) contents.

**Role of UmEna1 in the plasma membrane.** The functional expression of the UmEna1 ATPase in an Na\(^+\) efflux-defective strain of \(S.\ cerevisiae\) and the defects of the \(U.\ maydis\) \(\text{ena}1\) and \(\text{ena}2\) strains indicate that UmEna1 is a typical ENA ATPase (12). Its main function is to pump Na\(^+\) out of the cytoplasm, especially at high pH values, where the transcripts of these ATPases exhibit maximal levels (Table 2) (1, 7, 11, 25). At pH 8.0, UmEna1 was necessary even for modest Na\(^+\) or K\(^+\) tolerances, and more remarkable still, UmEna1 was required for growth at pH 9.0 even when Na\(^+\) or K\(^+\) concentrations were low (Fig. 4A). This specific requirement of ENA ATPases for the growth of fungi in high-pH media has been suspected for a long time (12) but had not been demonstrated previously.

The basic explanation for the variable requirements of ENA ATPases in alkaline-pH media depending on the Na\(^+\) and K\(^+\) concentrations in the external media is that the homeostasis of the K\(^+\) and Na\(^+\) levels in the cytoplasm depends on K\(^+\) and Na\(^+\) effluxes. Fungi possess K\(^+\) or Na\(^+\)/H\(^+\) antiporters (\(U.\ maydis\) has an \(nhdA\) gene, which encodes a protein that is highly similar in sequence to the ScNHDA1 antiporter) (our unpublished results) and ENA ATPases (12), but fungal electroneutral antiporters have not been reported. ATPases can function at any pH of the external medium, but this is not the case for electroneutral antiporters, which depend on an acidic external medium to function optimally. At external pH values above the cytoplasmic pH, they may mediate Na\(^+\) or K\(^+\) efflux but only if the concentration of the corresponding cation is lower in the external medium than in the cytoplasm.

UmEna1 is a pump of low Na\(^+\)/K\(^+\) discrimination, like many of the fungal ENA ATPases studied so far (12). The effectiveness of these ATPases in mediating Na\(^+\) tolerance must necessarily be linked to a low cytoplasmic toxicity of Na\(^+\), because an ENA ATPase of low Na\(^+\)/K\(^+\) discrimination cannot keep a low molar Na\(^+\)/K\(^+\) ratio in the cytoplasm. A low Na\(^+\)/K\(^+\) ratio has to be maintained by \(Neurospora\ crassa\) because it stops growing when Na\(^+\) and K\(^+\) contents reach an Na\(^+\)/K\(^+\) ratio that is much lower than 1 (52). In accordance with this requirement, \(N.\ crassa\) is furnished with an Na\(^+\)-specific ENA ATPase that does not protect cells from high K\(^+\)
concentrations (12). As mentioned above, D. hansenii (39), U. maydis (Fig. 1), and S. cerevisiae (39) are not affected by cytoplasmic molar \( \text{Na}^+/\text{K}^+ \) ratios of 4, 2, and 1, respectively. Therefore, because their ENA ATPases do not discriminate between \( \text{Na}^+ \) and \( \text{K}^+ \), they provide protection against high concentrations of any of these cations (Fig. 3A) (1, 12). The most plausible hypothesis that can be put forward at this moment is that high-\( \text{Na}^+ \)-content fungi possess ENA ATPases of low \( \text{Na}^+/\text{K}^+ \) discrimination and that low-\( \text{Na}^+ \)-content fungi possess \( \text{Na}^+ \)-specific ENA ATPases. This further implies that the general idea that considers \( \text{Na}^+ \) to be highly toxic in the cytoplasm needs to be revised, at least for fungi.

**Expression of UmEna2p and NeENA2p in endosomal membranes.** The UmEna2 and UmENA1 ATPases are in different phylogenetic clusters of the ENA phylogenetic tree. The same occurs with the ENA ATPases of *N. crassa* and with those of *Aspergillus fumigatus* and *Magnaporthe grisea*, although in the latter two species, the ATPases have not been cloned and studied (Fig. 2). Because the phylogenetic distances between UmENA1 and UmENA2 and between NeENA1 and NeENA2 are greater than that between UmENA1 and NeENA2, it can be concluded that a common ancestor of ascomycetous and basidiomycetous fungi has already had at least two types of ENA ATPases. The conservation of ENA ATPases in two phylogenetic clusters in *U. maydis* and *A. fumigatus* and in three clusters in *N. crassa* and *M. grisea* (Fig. 2) suggests the existence of ENA ATPases with different cellular functions. A similar suggestion can be derived from the different membranes to which ENA ATPases with different cellular functions. A similar suggestion can be derived from the different membranes to which ENA ATPases with different cellular functions. A similar suggestion can be derived from the different membranes to which ENA ATPases with different cellular functions. A similar suggestion can be derived from the different membranes to which ENA ATPases with different cellular functions.

**Expression of UmENA1 and UmENA2 and between NcENA1 and NcENA2** and *Aspergillus fumigatus* and the expression of NcENA2-GFP in yeast cells strongly are exclusively compatible with a plasma membrane location, as mentioned above, the defective function may occur in internal membranes. Because the defective function occurred only in the *\( \alphaena1 \* \alphaena2 \) double mutant, UmENA1 must also be involved in the function. To accomplish this, UmENA1 must cycle between endomembranes and the plasma membrane, as previously described for Na\(^+\)/H\(^+\) exchangers (19). Consistent with this possibility, the overexpression of the *Physcomitrella patens* ENA1 ATPase in rice and barley produces changes in metabolite levels that are difficult to predict based solely on the known function of this ATPase to pump Na\(^+\) or K\(^+\) out of the plasma membrane (15). Citric, isocitric, and aconitic acid levels were consistently reduced in both species (32), which indicates that peroxisome function is affected.

It appears that ENA ATPases fulfill more functions than just that of cation pumping across the plasma membrane. In *U. maydis* and *N. crassa*, different functions of ATPases in different phylogenetic clusters may be shared to different degrees. The same might occur in other fungi, such as *Aspergillus* or *Magnaporthe*, with ATPases in different phylogenetic clusters (Fig. 2). In the case of *Sacharomyces*, *Schwanniomyces*, *Zygosaccharomyces* (Fig. 2), and *Physcomitrella* (15), in which two or more ENA ATPases in the same species are in the same phylogenetic cluster, different functions might be carried out by the same ATPase or by ATPases that are phylogenetically close.

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