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3   **Growth at high pH, and sodium and potassium tolerance in above-**

4   **cytoplasmic pH media depend on ENA ATPases in *Ustilago maydis*.**

5

6   Running title: ENA ATPases in *Ustilago maydis*

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2   **ABSTRACT**

3

4   Potassium and Na^+ effluxes across the plasma membrane are crucial processes

5   for the ionic homeostasis of cells. In fungal cells, these effluxes are mediated by

6   cation/H^+ antiporters and ENA ATPases. We have cloned and studied the functions

7   of the two ENA ATPases of *Ustilago maydis*, UmEna1 and UmEna2. UmEna1 is a

8   typical K^+ or Na^+ efflux ATPase, whose function is indispensable for growth at pH

9   9.0, and for even modest Na^+ or K^+ tolerances above pH 8.0. UmEna1 locates to

10   the plasma membrane and has the characteristics of the low Na^+/K^+ discrimination

11   ENA ATPases. However, it still protects *U. maydis* cells in high Na^+ media because

12   Na^+ showed a low cytoplasmic toxicity. The UmEna2 ATPase is phylogenetically

13   distant from UmEna1 and is mainly located to the endoplasmic reticulum. The

14   function of UmEna2 is not clear but we found that it shares several similarities with

15   the *N. crassa* NcENA2, which suggests that endomembrane ENA ATPases may

16   exist in many fungi. The expression of *ena1* and *ena2* transcripts in *U. maydis* was

17   enhanced at high pH, and at high K^+ and Na^+ concentrations. We discuss that

18   there are two modes of Na^+ tolerance in fungi; the high Na^+ content mode involving

19   ENA ATPases with low Na^+/K^+ discrimination, as described here for *U. maydis*, and

20   the low Na^+ content mode involving Na^+-specific ENA ATPases, as in *Neurospora*

21   *crassa*.

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23

2

1   **INTRODUCTION**

2

3   Potassium is the most abundant cation in all types of living cells. Na^+, which is

4   fairly abundant in many natural environments, can partially substitute for K^+ but

5   becomes toxic above a certain Na^+/K^+ ratio (48). Therefore, the homeostatic

6   processes that regulate the steady state concentrations of K^+ and Na^+ in cells as

7   well as the systems that mediate the transport of these cations across the plasma

8   membrane and some endomembranes are crucial for maintaining cell viability.

9   Among all the transport processes involved, K^+ and Na^+ effluxes play an

10   indispensable role and, therefore, they take place in all types of living cells. For

11   instance, in animal cells an essential Na,K-ATPase that mediates Na^+ efflux and K^+

12   uptake consumes 20-30% of the produced ATP (33). Fungal and plant cells do not

13   have this animal-type Na,K-ATPase (6) but K^+ or Na^+ efflux ATPases, also called

14   ENA-ATPases, are present in every fungal species (12) and have also been

15   described in some bryophytes (15). ENA ATPases are phylogenetically close to but

16   functionally different from animal Na,K-ATPases. Unlike the latter, ENA ATPases

17   pump out almost every alkali cation and not exclusively Na^+, and they do not

18   mediate K^+ uptake (14). The cation promiscuity of ENA ATPases may be an

19   advantage in fungi because their membrane potential is very negative and they can

20   live in environments with high K^+ concentrations, such as plant tissues or plant

21   debris. In these environments, the energetic conditions that prevail for K^+ efflux are

22   similar to those prevailing for Na^+ efflux in Na^+ environments (12).

23

3

1   Fungal ENA ATPases are in most cases not essential in acidic environments

2   because when the external pH is lower than the citoplasmic pH, their function can

3   be replaced by electroneutral Na^+/H^+ and K^+/H^+ antiporters. In these antiporters,

4   which are universally present in eukaryotic cells, K^+ and Na^+ effluxes can be driven

5   by the ΔpH (18). Consistent with these facts, the substitution of the ScENA1

6   ATPase for the SOD2 antiporter of *S. pombe* (7) and the opposite substitution of

7   the SpSOD2 antiporter for the ENA1 ATPase of *S. cerevisiae* (28) do not reveal

8   any important functional advantage of the ATPase because these yeasts are

9   acidophilic. Furthermore, the expression of SpSOD2 in plant cells (23, 58)

10 apparently provides more benefits than the expression of ScENA1 (42). ENA

11 ATPases are indispensable for growth when the Na^+ and K^+ concentrations are

12 high in alkaline environments (10, 12), because in these environments the

13 transmembrane ΔpH would drive cation uptake instead of cation efflux if

14 electroneutral antiporters are functional. Electrogenic antiporters can mediate

15 membrane-potential driven cation effluxes when the external pH is high and, in

16 fact, they play a central role in bacteria growing at alkaline pH (44, 46). However,

17 fungal electrogenic K^+ or Na^+/H^+ antiporters have not been described.

18

19 It is paradoxical that although ENA ATPases are indispensable only at high pH, the

20 most extensive studies on these ATPases have been performed with *S. cerevisiae*

21 (50). This yeast is an acidophilic organism unable to grow at high pH, in which the

22 ENA ATPase is not essential for the above reasons. Disruptions of *ENA* genes

4

1 have also been attained in *S. pombe* and *Schwanniomyces occidentalis*, but these

2 disruptions do not resolve the uncertainties originated by the *S. cerevisiae* model.

3 *S. pombe* is also acidophilic and, moreover, it has an atypical ENA ATPase, Cta3

4 (43), which mediates K$^+$ efflux almost exclusively (12). In the case of *S.*

5 *occidentalis*, the double disruption of the two identified *ENA* genes was not

6 attained (9). Moreover, its genome has not been sequenced and the number of

7 *ENA* genes is unknown.

8

9 In addition to the pending questions about the reasons for the universal presence

10 of ENA ATPases in fungi (12) and their role in the growth of fungi at alkaline pH,

11 new questions have been raised by the discovery of ENA-ATPases in the parasites

12 *Leishmania* and *Trypanosoma* (12, 31), and in bryophytes (15). Therefore, further

13 studies on ENA ATPases are necessary, but the ENA ATPases of *S. cerevisiae*

14 cannot serve as models, nor can the expression of foreign ENA ATPases be

15 conveniently studied in *S. cerevisiae ena* mutants.

16

17 In the search for a new fungal model for studying ENA ATPases we selected

18 *Ustilago maydis*. *U. maydis* is a dimorphic basidiomycete plant pathogen (36), in

19 which some studies of alkali cation transport (13) and cellular pH responses (3, 39)

20 have already been carried out. In addition, it meets three important requirements;

21 it grows at high pH values, its genome sequence is available (38), and it is

22 amenable to easy molecular manipulations (35). Here we report the cloning and a

23 functional study of the two ENA ATPases of *U. maydis*, UmEna1 and UmEna2.

5

1

2   **MATERIALS AND METHODS**

3

4   **Bacterial and fungal strains, and growth conditions.** The *U. maydis*

5   strains FB1 (*a1b1*) and FB2 (*a2b2*) (5) were used throughout this study. The

6   *Escherichia coli* strain DH5α was routinely used for propagation of plasmids.

7   The *S. cerevisiae* strains used were W303.1A (*Mat* **a** *ade2 ura3 trp1 leu2*

8   *his3*), and its derivatives B31 (*Mat* **a** *ade2 ura3 trp1 ena1-4Δ::HIS3*

9   *nha1Δ::LEU2*) (8) and G19 (*Mat* **a** *ade2 ura3 trp1 ena1-4Δ::HIS3*) (45), in

10   which the Na$^+$ efflux systems ENA1-4 and NHA1 or only ENA1-4 are absent.

11   Fungal strains were normally grown either in the complex medium YPD (1%

12   yeast extract, 2% peptone, 2% glucose) or in the minimal SD medium (52).

13   Growth at variable K$^+$ and Na$^+$ concentrations was done on arginine

14   phosphate (AP) medium (49) supplemented with the indicated K$^+$ and Na$^+$

15   concentrations.

16

17   **Recombinant DNA techniques.** Manipulation of nucleic acids was performed by

18   standard protocols or, when appropriate, according to the manufacturers'

19   instructions. PCRs were performed in a Perkin-Elmer thermocycler, using the

20   Expand-High-Fidelity PCR System (Roche Molecular Biochemicals). Some of the

21   PCR fragments were first cloned into the PCR2.1-TOPO vector using the TOPO

22   TA Cloning Kit (Invitrogen). For expression in yeast cells, the genes were cloned

6

1   into the vector pYPGE15 (19). In all cases, most of the polylinker sequences

2   preceding the translation initiation codon were eliminated and a sequence

3   environment was created around it as similar as possible to (A/U)A(A/C)A(A/C)A

4   AUGUC(U/C) (29). DNA sequencing was performed in an automated ABI PRISM

5   3730 DNA analyzer (Applied Biosystems). DNA and total RNA were prepared

6   using the DNeasy and RNeasy Plant Kits (Qiagen), respectively. PCR

7   amplifications of mRNA fragments were carried out on double-stranded cDNA

8   synthesized from total RNA by using the cDNA Synthesis System Kit (GE

9   Healthcare). The full-length *ena1* and *ena2* cDNAs were obtained by RT-PCR from

10   RNA extracted from *U. maydis*, using specific primers which amplified DNA

11   fragments that contained the predicted START and STOP codons (Table 1). The

12   *ena1* and *ena2* genes were amplified from genomic DNA by PCR using the same

13   primers we used for the cDNAs.

14

15   **Real-time PCR assays.** The results reported in Table 2 were obtained in cells that

16   were grown in AP medium with 3 mM KCl and then transferred to the same

17   medium modified as follows: plus 10 mM tartaric acid and brought to pH 3.5 with

18   arginine; Ca^{2+} decreased to 0.5 mM and brought to pH 8.0 with arginine; plus 500

19   mM NaCl; plus 500 mM KCl; without K^+. All treatments were for 2 h, except K^+

20   starvation, which was for 4 h. Real-time PCR assays were performed as described

21   previously (25) except that the standard DNA solutions corresponded to the genes

22   studied in this report *ena1, ena2*, and actin genes of *U. maydis*. mRNA

23   preparations were treated with RNase-free DNase I (40 U in 100 μl; Roche) for 1 h

7

1   at 37 ºC. After treatment, mRNA was purified following the method described in the

2   RNeasy plant kit (Qiagen). PCR primers were designed to amplify the following

3   fragments (numbering as in databases): *ena1*, 2952-3106 (FM199940); *ena2*,

4   3233-3359 (FM199941); UmACT1-2: (5´-GTGCCCATCTACGAAGGTTACT-3´)

5   and UmACT1-1R: (5´-CGGCAGTGGTGGTGAAGGGGTAG-3´).

6

7   **Localization of UmEna1-GFP and UmEna2-GFP in *U. maydis* and NcENA2-**

8   **GFP in *Saccharomyces* yeast cells.** The *ena1-GFP* and *ena2-GFP* constructs

9   were in-frame fusions of the 3' end of the *ena1* and *ena2* ORFs to the *GFP* gene of

10   the plasmid pCU3. To generate these constructs, full-length *ena1* cDNA was

11   amplified using the NdeI-ENA1ATG and NdeI-ENA1Rev primers, which include the

12   *Nde*I restriction site. Full-length *ena2* cDNA was amplified using the BamHI-

13   ENA2ATG NdeI-ENA2Rev primers, which include the *Nde*I and *BamH*I restriction

14   sites (Table 1).

15

16   For expression in *U. maydis*, the *ena1* or *ena2* PCR fragments were cloned into

17   the *Nde*I and *Nde*I/*BamH*I sites of plasmid pCU3 (P*tef1*-dependent expression),

18   respectively, which are at the 5´end of *GFP* gene. These plasmids were linearized

19   with *Ssp*I and transformed into *U. maydis* to integrate the construct into the *cbx1*

20   locus by homologous recombination as described in (17). For expression in *S.*

21   *cerevisiae*, the *Ncena-2-GFP* fusion was cloned into the plasmid pYPGE15 (19).

22   This construct was transformed into the aforementioned B31 yeast mutant. To

23   visualize endoplasmic reticulum (ER), an ER-RFP fusion protein was produced as

8

1   described in (57) but using monomeric RFP as reporter and a hygromycin

2   resistance cassette as a selectable marker.

3

4   The GFP fluorescence signal in *U. maydis* and yeast cells was visualized in a

5   confocal ultraspectral Leica TCS-Sp2-AOBS-UV microscope (Leica Microsystems,

6   Mannheim, Germany).

7

8   **Disruption of *ena1* and *ena2* genes.** To obtain the Δ*ena1* mutant we constructed

9   a disruption plasmid ligating two DNA fragments of the *ena1* cDNA to the 5' and 3'

10 ends of the nourseothricin resistance cassette in pNEBNat(+), a *U. maydis*

11 integration vector (41). The 5´fragment of 1,157 bp was obtained by digesting the

12 *ena1* cDNA with *Spe*I and *BamH*I and was inserted between the *Spe*I and *Bgl*II

13 sites of pNEBNat(+) plasmid. The 3´fragment of 1,239 bp was obtained by

14 digesting the *ena1* cDNA with *Pvu*II and *Hind*III and was inserted between the

15 *EcoR*V and *Hind*III sites into the pNEBNat(+) plasmid. The plasmid with the two

16 insertions was linearized with *Ssp*I and transformed into the FB1 and FB2 *U.*

17 *maydis* wild type strains. Transformants were selected in the presence of

18 nourseothricin (Hans-Knöll-Institute, Jena, Germany) at 150 μg ml^{-1}.

19

20 The disruption plasmid for the Δ*ena2* mutant was constructed using fragments of

21 the *ena2* gene for flanking the hygromycin B resistance cassette in the plasmid

22 pNEBHyg(+) (20). The 600 bp 5´ fragment was obtained by the digesting the *ena2*

9

1   cDNA with *Sph*I and *BamH*I and was inserted between the *Sph*I and *BamH*I sites

2   of pNEBHyg(+). The 920 bp 3´ fragment was obtained by digesting *ena2* cDNA

3   with *Kpn*I and *EcoR*I and was inserted between the *Kpn*I and *EcoR*I sites of

4   pNEBHyg(+). The plasmid with the two insertions was linearized with *Sph*I and

5   *Nar*I and transformed into the FB1 and FB2 *U. maydis* wild type strains.

6   Transformants were selected in YPD medium supplemented with hygromycin B

7   (Sigma-Aldrich) at 50 μg ml^{-1}.

8

9   The double *Δena1 Δena2* mutant was constructed by transforming the *Δena1* strain

10 with the linearized DNA construct used for the disruption of *Δena2*. To recover the

11 hygromycinB-resistant transformants it was necessary to use regeneration agar (1

12 M Sorbitol, 1% yeast extract, 2% peptone, 2% sucrose and 1.5% agar) at pH 5.0

13 buffered with 20 mM MES. At other pH values the *Δena1 Δena2* mutant was never

14 recovered.

15

16 Integration of plasmids into the corresponding loci was verified by PCR and

17 Southern blot analyses in all cases. Southern blot analyses of the mutants were

18 carried out according to standard procedures. Genomic DNA from the *U. maydis*

19 mutants was digested with *BstX*I, *Sal*I, *EcoR*I*,* or *Nco*I and hybridized with a probe

20 that includes either the antibiotic resistance cassette, or fragments of the *ena1* or

21 *ena2* genes, which did not show hybridization bands in the corresponding

22 disrupted strains. The digoxigenin-labeled DNA probe was amplified by PCR.

1 Hybridization and detection was carried out according to the supplier's manual

2 (Roche Applied Science).

3

4 **Mating and virulence assays.** To test for mating, strains were co-spotted on

5 charcoal-containing PD (potato dextrose) plates that were sealed with parafilm and

6 incubated at 21 ºC for 48 hours (30). For virulence assays, the maize cultivar Early

7 Golden Bantam (Old Seeds, Madison Wisc.) was infected as described previously

8 (27). The infection was repeated twice.

9

10 **Na^+ and K^+ contents and intracellular distribution.** *U. maydis* cells were

11 collected by centrifugation, washed twice with K^+- and Na^+-free AP medium and

12 subsequently suspended in the same medium for 3 hours. Cell samples were

13 centrifuged and total internal ions were extracted with 0.1 M HCl and analyzed by

14 atomic emission spectrophotometry. The vacuolar Na^+/K^+ ratio was determined by

15 treating the cells with digitonin, which selectively permeabilizes the plasma

16 membrane (22, 34). Samples of cell suspensions incubated for 3 hours in K^+- and

17 Na^+-free AP medium were treated with 0.001% digitonin and 1 M sorbitol in the

18 same medium. At intervals, the cells were centrifuged at 8,000 g for 1 min and the

19 supernatants containing the ions released by the digitonin treatment were analyzed

20 by atomic emission spectrophotometry. The pellets were extracted with 0.1 M HCl

21 to determine the K^+ and Na^+ contents that had not been released by the digitonin

22 treatment. To ensure that during the digitonin treatment the vacuolar membranes

23 remained intact and able to maintain a ∆pH, the accumulation into the vacuole of

11

1   acridine orange (3, 6-bis (dimethylamino)acridine) was monitored (22). This

2   fluorescent dye penetrates the plasma membrane in an uncharged, neutral form

3   and then accumulates into acidic organelles where it is trapped in the protonated

4   form. Previous to the digitonin treatment, the cells were incubated in AP medium

5   with 100 μg ml^{-1} acridine orange for 20 min at room temperature. Then 0.001%

6   digitonin was added and, at intervals, 4 μl of the cell suspension was transferred to

7   a slide, covered with a cover glass, and observed with a Zeiss fluorescence

8   microscope under blue light. Images were recorded with a Leica DFC300FX color

9   camera connected to the microscope.

10

11   **Na$^+$ efflux in *U. maydis* and yeast cells.** *U. maydis* cells were grown overnight in

12   AP medium supplemented with 30 mM K$^+$ and then Na$^+$ loaded by suspending the

13   cells for 1 h in AP medium, pH 8.0 buffered with 10 mM TAPS, and containing

14   either 200 or 50 mM NaCl, for FB1 wild type and *Δena1 Δena2* mutant cells,

15   respectively. The cells thus loaded with Na$^+$ were spun down and suspended in AP

16   medium pH 8.0 in the presence of 50 mM K$^+$ and 10 mM Na$^+$. At intervals,

17   samples of the suspended cells were filtered, washed, and extracted with 0.1 M

18   HCl. For *S. cerevisiae*, B31 yeast transformants were grown overnight in AP

19   medium supplemented with 3 mM K$^+$. Cells were then Na$^+$ loaded in 10 mM TAPS

20   pH 8.0, 100 mM NaCl, 1 mM MgCl2, and 2% glucose for 1 hour. The Na$^+$-loaded

21   cells were spun down and suspended in TAPS pH8.0, 50 mM K$^+$, 2% glucose. At

22   intervals, samples of the suspended cells were filtered, washed, and HCl extracted.

12

1   In both *U. maydis* and in yeast cells, cation contents were determined from the

2   atomic emission spectrophotometric analyses of the extracts. For each experiment,

3   three independent repetitions were carried out.

4

5   **Protein alignments and generation of phylogenetic trees.** Protein sequence

6   alignments and phylogenetic trees were obtained using the Clustal X program (55).

7

8   **Accession numbers.** *ena1*, FM199940; *ena2*, FM199941

9

10   **RESULTS**

11

12   **Basic description of *U. maydis* alkali cation tolerance.** *U. maydis* grew well in a

13   wide range of Na^+ or K^+ concentrations at pH values ranging from 3.5 to 9.0. In

14   YPD medium it grew up to 1.0 M Na^+ or 1.2 M K^+. Growth was also maintained at

15   low micromolar K^+ and Na^+ concentrations, where both cations were depleted

16   down to almost undetectable concentrations (13). In mineral AP medium the toxic

17   effect of Na^+ was almost independent from the K^+ concentration; for example,

18   growth rates at 150 mM Na^+ at either 4.5 or 0.5 mM K^+ were almost identical. At

19   4.5 mM K^+ growth rate was not affected by 500 mM Na^+ and only partially reduced

20   by 800 mM Na^+.

21

22   Analyses of cation contents of *U. maydis* cells growing at high Na^+ concentrations

23   revealed that they contained fairly high internal Na^+/K^+ molar ratios without any

13

1 apparent detrimental effect. These results raised the question of whether Na^+ was

2 sequestered into the vacuole as in plant cells (21, 54). To address this question we

3 determined the cytoplasmic Na^+/K^+ ratio by measuring the Na^+ and K^+ losses after

4 digitonin permeabilization of the plasma membrane. The accuracy of the results of

5 this approach relies on two conditions, that tonoplasts were not permeabilized and

6 that intact cells did not take up the K^+ released by permeabilized cells. To test the

7 integrity of the tonoplast we checked the capacity of the vacuoles to maintain a

8 ΔpH by acridine orange staining (2, 22). A significant effect of digitonin on the

9 tonoplast was found to start after 20 min of treatment. Therefore, in the

10 experiments that we report below the time of digitonin treatment was limited to 15

11 min so that not more than one vacuole out of 100 was unstained. To check that

12 during the experiments the K^+ released by permeabilized cells was not taken up by

13 intact cells we carried out parallel experiments in the presence and in the absence

14 of antimycin-A, which inhibits respiration and consequently K^+ uptake (47). The

15 presence of this inhibitor did not affect the results. Furthermore, the time courses of

16 the K^+ and Na^+ release showed a constant Na^+/K^+ ratio from the first sample taken,

17 with less than 10% of the cells permeabilized, up to the last sample, with probably

18 more than 80% of the cells permeabilized. This result also ruled out the possibility

19 that intact cells took up K^+.

20

21 *U. maydis* vacuoles were not stained by acridin orange in cells growing at high Na^+

22 concentrations (for example, 4.5 mM K^+/150 mM Na^+). This might be the result of

23 an excessive uptake of cations and alkalinization of the cells but the causes were

14

1   not investigated. Incubation of these cells in K^+- and Na^+-free medium for 3 h did

2   not change significantly their K^+ and Na^+ contents but fully restored the capacity of

3   the vacuoles to accumulate acridine orange (Fig. 1A). An additional advantage of

4   this incubation was that *U. maydis* cells adapted to keeping very low K^+ or Na^+

5   concentrations in the external medium (typically, 0.5 μM K^+ and 10 μM Na^+). Under

6   these conditions it was very simple to measure the K^+ and Na^+ released into the

7   external medium by the digitonin treatment because the treatment increased the

8   external concentrations very much while untreated cells kept them very low.

9

10   Our results with cells grown at different K^+ and Na^+ concentrations show that the

11   vacuole of *U. maydis* did not accumulate high amounts of Na^+. When cells grown

12   at 4.5 mM K^+, 150 mM Na^+ and subsequently K^+ and Na^+ starved for 3 h were

13   treated with digitonin the time courses of the K^+ and Na^+ releases to the external

14   medium showed a permanent increase at a constant Na^+/K^+ ratio of 1.4 (Fig. 1B).

15   At the same time, the ratio between the Na^+ and K^+ that remained in the cells

16   (vacuolar content of permeabilized cells plus the content of intact cells) after each

17   interval treatment decreased permanently (Fig. 1C). In three independent

18   experiments in cells grown at 4.5 mM K^+, 150 mM Na^+ the mean of the Na^+/K^+ ratio

19   was 1.5±0.2 (SD) while in cells grown at 10 mM K^+ 500 mM Na^+ the mean of the

20   ratio was 2.3±0.2. Taken together these experiments indicated that the Na^+/K^+ ratio

21   in the vacuole of actively growing cells was lower than the cytoplasmic Na^+/K^+ ratio

22   and that the latter could be as high as 2.3 without any detrimental effect.

15

1

2   *U. maydis* **has two ENA ATPases.** Computer-based searches in the genomic

3   sequence of *U. maydis* using as queries ENA ATPase sequences identified two

4   open reading frames that could encode Ena proteins. The corresponding genes,

5   *ena1* and *ena2*, were cloned by a standard PCR-based approach. These genes did

6   not contain introns and encode two proteins of 1,100 and 1,125 amino acids,

7   respectively. The study of the amino acid sequences of both ATPases showed that

8   their structure and functional characteristics corresponded to typical P-type

9   ATPases (33, 51) of group IID (4). Remarkably, the *ena1* and *ena2* genes did not

10   result from a recent duplication event because the phylogenetic distance between

11   the encoded pumps was larger than the phylogenetic distance between the

12   basidiomycete UmEna1 and the ascomycete NcENA1 pumps. The existence of

13   two or more ENA ATPases in two distant phylogenetic clusters was also found in

14   *Aspergillus*, *Neurospora*, and *Magnaporthe* (Fig 2). The *ena1* gene was located on

15   chromosome 3 and *ena2* on chromosome 1. Transcript expressions of *ena1* and

16   *ena2* as determined by real time PCR showed that the expression of both genes

17   was low under normal conditions and that almost a 100-fold induction occurred at

18   high Na^+ or K^+ concentrations, or at high pH (Table 2), very similar to previous

19   descriptions in other fungi (1, 9, 11, 24).

20

21   The *ena1* and *ena2* genes were then expressed in the mutant yeast strain B31,

22   which lacks the ENA ATPases and the NHA1 antiporter (10), using the yeast

23   expression vector pYPGE15, with the genes under the control of the *PGK1* gene

16

1   promoter (19). *ena1* but not *ena2* completely suppressed the defective growth of

2   B31 at high Na$^+$ and high K$^+$ (Fig. 3A). However, *ena2* suppressed the defect of

3   B31 only at the minimal Na$^+$ concentration at which the growth of B31 was

4   inhibited. Consistent with the pumping capacities of a Na-ATPase, UmEna1

5   mediated cellular Na$^+$ loss at pH 8.0 and 10 mM Na$^+$ in the external medium (Fig.

6   3B). Under these conditions a Na$^+$ channel or an electroneutral Na$^+$/H$^+$ antiporter

7   would mediate Na$^+$ uptake driven by the membrane potential and ∆pH,

8   respectively. ENA ATPases may be specific for K$^+$ or Na$^+$, protecting only from high

9   concentrations of one of these cations, or non-specific, protecting from high

10 concentrations of either of them (12). The described results showed that UmEna1

11 belonged to the non-specific group (Fig. 3A).

12

13 **Effects of the disruption of *ena1* and *ena2*.** The function of the *U. maydis*

14 UmEna1 and UmEna2 ATPases was assessed by gene disruption. Initially, we

15 obtained the single and double disruptions in strain FB1, which is almost identical

16 to the strain whose genome has been sequenced (38). Later the disruptions were

17 also attained in a strain of the opposite mating type, FB2, suited to performing plate

18 mating assays and plant infection studies. The disruptions of the *ena1* and *ena2*

19 genes in either FB1 or FB2 produced identical results and only the results obtained

20 with strain FB1 are presented. Disruptions were checked by Southern blot

21 analyses, which proved that the *ena1* or *ena2* genes had been disrupted (data not

22 shown).

23

17

1   Consistent with the established function of ENA ATPases (12) and the previously

2   discussed functional expression of the UmEna1 and UmEna2 ATPases in yeast

3   cells (Fig. 3), clear defects of growth and Na$^+$/K$^+$ tolerance of the *U. maydis Δena1*

4   *Δena2* strain became evident at high pH values. At pH 5.0 the double *Δena1 Δena2*

5   mutant was as tolerant as the wild type strain (similar growth at 800 mM Na$^+$ or 1M

6   K$^+$ in YPD medium, data not shown). In contrast, at pH 8.0 the double *Δena1*

7   *Δena2* mutant was inhibited by Na$^+$ concentrations as low as 20 mM in mineral AP

8   medium (Fig. 4A). Remarkably, at pH 9.0 the *Δena1 Δena2* mutant failed to grow in

9   either YPD without Na$^+$ or K$^+$ addition (data not shown) or in 1 mM K$^+$ AP medium

10   (Fig. 4A). Most of the defects in the double *Δena1 Δena2* mutant were accounted

11   for by the *Δena1* mutation. The effects of the *Δena2* mutation regarding Na$^+$ or K$^+$

12   tolerance were almost undetectable both in the wild type and in the *Δena1* strain

13   (Fig. 4A). As expected from these results, the lack of the ENA ATPases abolished

14   Na$^+$ efflux at high pH values (Fig. 4B).

15

16   **UmEna1p and UmEna2p show different endosomal/plasma membrane**

17   **distribution.** Some eukaryotic Na$^+$/H$^+$ exchangers show dual endosomal/plasma

18   membrane distribution (18). Moreover, considering the phylogenetic divergence of

19   the UmENA1 and UmEna2 ATPases (Fig. 2) and our failure to detect a clear

20   function of UmEna2, the localization of UmEna2 could not be predicted. Therefore,

21   we checked the cellular locations of the UmEna1-GFP and UmEna2-GFP proteins

22   in *U. maydis* cells with the gene expression under the control of the transcriptional

18

1 elongation factor promoter (17). The GFP fusions did not affect the described

2 biological activities of the UmEna1 and UmEna2 ATPases. Expression of UmEna1-

3 GFP in the $\Delta ena1$ strain suppressed its sensitivity to high Na$^+$ or K$^+$ concentrations

4 (as in Fig. 4A) and expression UmEna2-GFP in the mutant yeast strain B31 weakly

5 suppressed its Na$^+$ sensitivity (as in Fig. 3A).

6

7 Microscopy analysis of *U. maydis* cells expressing UmEna1-GFP located the

8 protein mainly to the plasma membrane and to some vesicles, which might be in

9 transit to the plasma membrane (Fig. 5). In contrast UmEna2-GFP located around

10 the nucleus, in close proximity to the plasma membrane, and in internal vesicles.

11 Coexpression of UmEna2-GFP with an ER-RFP fusion demonstrated that

12 UmEna2-GFP localized to the ER and to other endomembranes that were not

13 investigated (Fig. 5).

14

15 **NcENA2 has similarities with UmEna2.** In a previous report, the function of the

16 *Neurospora crassa* ENA2 ATPase (previously called ph7) could not be established

17 (11). Interestingly, the phylogenetic divergence between NcENA1 and NcENA2

18 ATPases was similar to that between the UmEna1 and UmEna2 ATPases (Fig. 2).

19 Now, using a new construct in which the sequence context around the first in-frame

20 AUG was optimized for translation we found that NcENA2 weakly suppressed the

21 defect of the B31 mutant, exactly as shown for UmEna2 in Fig. 3A (data not

22 shown). Next, to investigate whether NcENA2 located to the plasma membrane or

23 to endomembranes we expressed the NcENA2-GFP protein in yeast cells. The

19

1 NcENA2-GFP signal localized to spots, which were neither in the tonoplast nor in

2 the plasma membrane. Although NcENA2 resembled UmEna2 in that both show a

3 similar functional expression in yeast cells and localized to endomembranes, they

4 might fulfill different functions because the microscopic images of NcENA2-GFP

5 did not correspond to a typical ER location (Fig. 6). *N. crassa* has a third ENA

6 ATPase (NcENA3 in Fig. 2) that might be a functional homologous of UmEna2.

7 This possibility was not tested because we have so far failed to clone NcENA3.

8

9 **Do ENA ATPases have functions other than cation pumping in the plasma**

10 **membrane?** ENA ATPases are universally present in fungi and many fungi have

11 *ENA* genes that encode phylogenetically distant ENA ATPases (see *U. maydis, A.*

12 *fumigatus,* and *N. crassa* ENA ATPases in Fig. 2), which apparently locate to

13 different membranes (Fig. 5 and 6). All these observations raised the question of

14 whether the functions of ENA ATPases may be more than the currently assigned

15 roles of Na^+ and K^+ pumping out of cytoplasm. Therefore, we selected several

16 physiological functions having no obvious relationship to ion transport to be tested

17 in the *Δena1* and *Δena2* strains.

18

19 First we tested the mating ability of the single and double mutants of the FB1 and

20 FB2 strains. All mixtures of sexually compatible strains developed positive Fuz

21 reactions, regardless of the *Δena1* or *Δena2* mutations (not shown). The virulence

22 capability of the *Δena* strains was also tested by inoculation of mixtures of sexually

20

1   compatible mutants (FB1 *Δena*1/ FB2 *Δena*1; FB1 *Δena*2/FB2 *Δena*2; FB1 *Δena*1

2   *Δena*2/FB2 *Δena*1 *Δena*2) or wild type strains (FB1/FB2) onto maize seedlings.

3   Mixtures of mutants did not show any difference in virulence symptoms, such as

4   chlorosis, anthocyanin pigmentation, or tumor production when compared to wild

5   type mixtures (not shown).

6

7   Next we carried out growth tests in many different conditions and found a

8   surprising defect. Growth of *U. maydis* was slightly inhibited in YPD medium (1%

9   yeast extract, 2% peptone) as used in yeast research (52) at pH 4.0 or lower. In

10 contrast, the *Δena1 Δena2* double mutant strain was completely unable to grow in

11 YPD at pH 4.0 (Fig. 7), while the *Δena1* or *Δena2* single mutant strains grew

12 identically to the wild type strain. Different types of commercial peptones added to

13 AP medium reproduced the YPD effect, but vitamin-free casamino acid (Difco)

14 produced only a weak effect. The marked pH dependence of the toxic effect

15 suggested that the permeable form of a fatty acid might be involved but we failed to

16 find a fatty acid or a mixture of fatty acids that produce the inhibition. We also

17 tested whether the addition of NH_4^+ suppressed the inhibitory effect, finding that

18 concentrations up to 200 mM did not show any suppressive effect (data not

19 shown). The defective growth at pH 4.0 in YPD produced by the *Δena1 Δena2*

20 mutations in *U. maydis* was not produced by the equivalent *Δena1-4* mutation in *S.*

21 *cerevisiae* (Fig. 7).

22

21

1   **DISCUSSION**

2

3   **Low Na$^+$ toxicity in *U. maydis*.** It is normally assumed that K$^+$ is the most

4   abundant cellular cation and that cells growing in the presence of Na$^+$, as do

5   animal cells, exclude Na$^+$ to keep a high K$^+$ content. Under similar conditions plant

6   cells also sequester Na$^+$ in the vacuole to keep a low Na$^+$/K$^+$ ratio in the cytoplasm

7   (21, 54). The notion that Na$^+$ is low in the cytoplasm does not apply to *U. maydis*.

8   We observed good growth when the cytoplasmic Na$^+$/K$^+$ ratio was 2.3.

9

10   The inability of fungal cells to decrease the cytoplasmic Na$^+$ concentration by

11   accumulating it into the vacuole, as reported here for *U. maydis*, has been

12   previously reported for *S. cerevisiae* (40, 47, 56) and *Debaryomyces hansenii* (40).

13   These three species grow normally with a rather high Na$^+$ content, exhibiting low

14   cytoplasmic Na$^+$ toxicity. In *S. cerevisiae* a Na$^+$/K$^+$ ratio of 1 is completely non-toxic

15   (40) and in *D. hansenii* the Na$^+$/K$^+$ ratio can be as high as 4 without detrimental

16   effects (40). Similarly, *Hortaea werneckii* and *Aureobasidium pullulans* show

17   comparable K$^+$ and Na$^+$ contents when actively growing at 0.8 M NaCl (37). In

18   contrast, in *Neurospora crassa* (53)*, Candida albicans* (16), and *Candida tropicalis*

19   (26) low Na$^+$ contents are toxic, which suggests high cytoplasmic Na$^+$ toxicity.

20

21   In summary, there seem to be two types of fungi regarding Na$^+$ tolerance, those

22   tolerant to high Na$^+$ contents in the cytoplasm, which include *U. maydis*, and those

23   intolerant to high Na$^+$ contents.

22

1

2 **Role of UmEna1 in plasma membrane.** The functional expression of the UmEna1

3 ATPase in a Na$^+$-efflux defective strain of *S. cerevisiae* and the defects of the *U.*

4 *maydis Δena1* and *Δena1 Δena2* strains indicate that UmEna1 is a typical ENA

5 ATPase (12). Its main function is to pump Na$^+$ and K$^+$ out of the cytoplasm,

6 especially at high pH values, where the transcripts of these ATPases exhibit

7 maximal levels (Table 2; (1, 9, 11, 24)). At pH 8.0 UmEna1 was necessary even for

8 modest Na$^+$ or K$^+$ tolerances and, more remarkable still, UmEna1 was required for

9 growth at pH 9.0 even when Na$^+$ or K$^+$ concentrations were low (Fig. 4A). This

10 specific requirement of ENA ATPases for the growth of fungi in high pH media has

11 been suspected for a long time (12) but had not been demonstrated previously.

12

13 The basic explanation for the variable requirements of ENA ATPases in alkaline pH

14 media depending on the Na$^+$ and K$^+$ concentrations in the external media is that

15 the homeostasis of the K$^+$ and Na$^+$ levels in the cytoplasm depends on K$^+$ and Na$^+$

16 effluxes. Fungi possess K$^+$ or Na$^+$/H$^+$ antiporters (*U. maydis* has a *nha1* gene

17 which encodes a protein highly similar in sequence to the ScNHA1 antiporter;

18 unpublished results) and ENA ATPases (12), but fungal electrogenic antiporters

19 have not been reported. ATPases can function at any pH of the external medium

20 but this is not the case for electroneutral antiporters, which depend on an acidic

21 external medium to function optimally. At external pH values above the cytoplasmic

22 pH they may mediate Na$^+$ or K$^+$ efflux but only if the concentration of the

23 corresponding cation is lower in the external medium than in the cytoplasm.

23

1

2 UmEna1 is a pump of low Na^+/K^+ discrimination, like many of the so far studied

3 fungal ENA ATPases (12). The effectiveness of these ATPases in mediating Na^+

4 tolerance must necessarily be linked to a low cytoplasmic toxicity of Na^+ because

5 an ENA ATPase of low Na^+/K^+ discrimination cannot keep a low molar Na^+/K^+ ratio

6 in the cytoplasm. A low Na^+/K^+ ratio has to be maintained by *Neurospora crassa*

7 because it stops growing when Na^+ and K^+ contents reach a Na^+/K^+ ratio that is

8 much lower than 1 (53). In accordance with this requirement, *N. crassa* is furnished

9 with a Na^+-specific ENA ATPase that does not protect from high K^+ concentrations

10 (12). As already mentioned, *D. hansenii* (40), *U. maydis* (Fig. 1), and *S. cerevisae*

11 (40) are not affected by cytoplasmic molar Na^+/K^+ ratios of 4, 2, and 1,

12 respectively. Therefore, because their ENA ATPases do not discriminate between

13 Na^+ and K^+, they provide protection against high concentrations of any of these

14 cations (Fig. 3A; (1, 12)). The most plausible hypothesis that can be put forward at

15 this moment is that high Na^+ content fungi possess ENA ATPases of low Na^+/K^+

16 discrimination, and low Na^+ content fungi possess Na^+-specific ENA ATPases. This

17 further implies that the general idea that considers Na^+ as highly toxic in cytoplasm

18 needs to be revised, at least in fungi.

19

20 **Expression of UmEna2p and NcENA2p in endosomal membranes.** The

21 UmEna2 and UmEna1 ATPases are in different phylogenetic clusters of the ENA

22 phylogenetic tree. The same occurs with the ENA ATPases of *N. crassa*, and with

23 those of *Aspergillus fumigatus* and *Magnaporthe grisea*, although in the last two

24

1   species the ATPases have not been cloned and studied (Fig. 2). Because the

2   phylogenetic distance between UmEna1 and UmEna2, and between NcENA1 and

3   NcENA2 is greater than that between UmEna1 and NcENA1, it can be concluded

4   that a common ancestor of ascomycetous and basidiomycetous fungi already had

5   at least two types of ENA ATPases. The conservation of ENA ATPases in two

6   phylogenetic clusters in *U. maydis* and *A. fumigatus*, and in three clusters in *N.*

7   *crassa* and *M. grisea* (Fig. 2) suggests the existence of ENA ATPases with

8   different cellular functions. A similar suggestion can be derived from the different

9   membranes to which UmEna1 and UmEna2 locate (Fig. 5). The locations of

10   NcENA1 and NcENA2 have not been established. However, in *S. cerevisiae*

11   NcENA1 mediates rapid Na^+ effluxes (11) that are exclusively compatible with a

12   plasma membrane location, and the expression of NcENA2-GFP in yeast cells

13   strongly suggests that it locates to endomembranes (Fig. 6) resembling UmEna2.

14   NcENA2 and UmEna2 increased very slightly the Na^+ tolerance of the Na^+ efflux-

15   defective *S. cerevisiae* mutant. Such a weak effect cannot be directly attributed to

16   an increase in Na^+ efflux, which, if it actually occurred, would be very weak and

17   untestable, but supports that they are Na^+ ATPases. Furthermore, the expression

18   patterns of UmEna2 and NcENA2 (Table 2 and (11), respectively) and the

19   conservation of the typical motifs of ENA ATPases suggest that they pump Na^+

20   and K^+, as plasma membrane ENA ATPases do. In summary, taking all these

21   observations together, it seems that UmEna2 and NcENA2 mediate Na^+ or K^+

22   fluxes in the ER or in other endomembranes.

23

25

1   The *Δena2* mutation did not produce any detectable defect except for the lack of

2   growth in peptone at low pH values in the *Δena1* strain. Our failure to identify which

3   compound in peptone was toxic makes it impossible to predict the defective

4   function that produced the toxicity of peptones. However, as already discussed, the

5   defective function may occur in internal membranes. Because this defective

6   function only occurred in the double *Δena1 Δena2* mutant, UmEna1 must also be

7   involved in the function. To accomplish this, UmEna1 must cycle between

8   endomembranes and plasma membrane, as previously described for Na^+/H^+

9   exchangers (18). Consistent with this possibility, overexpression of the moss

10 PpENA1 ATPase in rice and barley produces changes in metabolite levels that are

11 difficult to predict based solely on the known function of this ATPase to pump Na^+

12 or K^+ out of the plasma membrane (15). Citric, isocitric, and aconitic acid levels

13 were consistently reduced in both species (32), which might indicate that

14 peroxisome function is affected.

15

16 It appears that ENA ATPases fulfill more functions than just that of cation pumping

17 across the plasma membrane. In *U. maydis* and *N. crassa*, different functions of

18 ATPases in different phylogenetic clusters may be shared to different degrees. The

19 same might occur in other fungi such as *Aspergillus* or *Magnaporthe* with ATPases

20 in different phylogenetic clusters (Fig. 2). In the case of *Saccharomyces,*

21 *Schwanniomyces, Zygosaccharomyces* (Fig. 2), and *Physcomitrella* (15) in which

22 two or more ENA ATPases in the same species are in the same phylogenetic

26

1   cluster, different functions might be carried out by the same ATPase or by

2   ATPases that are phylogenetically close.

3

4   **ACKNOWLEDGMENTS**

5

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11

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17

1   FIG 1. Molar Na^+/K^+ ratio in the cytoplasm of *U. maydis*. (A) Acridine orange

2   staining of *U. maydis* vacuoles after 15 min of treatment with digitonin. (B) Time

3   course of Na^+ and K^+ releases after digitonin permeabilization of the plasma

4   membrane. (C) Time course of Na^+ and K^+ contents of cells under digitonin

5   treatment.

6

7   FIG. 2. Phylogenetic tree of fungal ENA ATPases. Species and accession

8   numbers:

9   ScPMC1, *Saccharomyces cerevisiae*: P38929, Ca^{2+}-ATPase included as an

10 outgroup; UmEna2, *Ustilago maydis:* XP_756351; HwENA1 *Hortaea werneckii:*

11 ABD64570; NcENA2, *Neurospora crassa:* AJ243519; Magnap-4, *Magnaporthe*

12 *grisea:*   XP_001404752; Asperg-3, *Aspergillus fumigatus:* EAL87230; SoENA1,

13 *Schwanniomyces occidentalis,:* AAB86426; DhENA1, *Debaryomyces hansenii:*

14 AAK28385; DhENA2, *D. hansenii:* AAK52600; SoENA2, *S. occidentalis:*

15 AAB86427; ZrENA1, *Zygosaccharomyces rouxii*: BAA11411; ScENA1, *S.*

16 *cerevisiae*: P13587; Torul-1, *Torulaspora delbrueckii:* AAZ04389; Magnap-3, *M.*

17 *grisea,* XP_365372; Magnap-1, *M. grisea:* XP_360418; NcENA1, *N. crassa:*

18 AJ243520; FoENA1, *Fusarium oxysporum:* AAR01872; Asperg-2, *A. fumigatus:*

19 EAL85670; Asperg-1, *A. fumigatus:* EAL89843; UmEna1, *U. maydis:* XP_757891;

20 SpCTA3, *Schizosaccharomyces pombe:* NP_595246; Magnap-2, *M. grisea:*

21 XP_359699; NcENA3, *N. crassa:* XP_962099. * Indicates cloned pumps.

22

37

1   FIG 3. Functional expression of *ena1* and *ena2* cDNAs in *S. cerevisiae*. (A)

2   Suppression of the defective growth of the Na^+ efflux mutant B31 in the presence

3   of high Na^+ or K^+ concentrations; drops of serial dilutions of cell suspensions of the

4   wild type, and of the B31 strain transformed with the empty plasmid or with the

5   *ena1* or *ena2* cDNAs were inoculated in the indicated media, numbers indicate

6   concentrations (mM). (B) Time courses of Na^+ extrusion at pH 8.0 in B31

7   transformants loaded with Na^+; B31 transformed with the empty plasmid (open

8   triangles), *ena1* (open circles) and *ena2* (closed circles).

9

10   FIG 4. Defects of *Δena1* and *Δena2* mutants. (A) Growth defects in AP medium at

11   different pH values and different Na^+ concentrations, as indicated; numbers

12   indicate concentrations (mM). (B) Na^+ extrusion from Na^+-loaded cells in AP

13   medium at pH 8.0, 50 mM KCl, 10 mM NaCl.

14

15   FIG. 5. Localization of UmEna1-GFP and of UmEna2-GFP fusion proteins in *U.*

16   *maydis Δena1* or *Δena2* mutants, respectively. (A, B, C) Images of the *Δena1* strain

17   expressing the UmEna1-GFP fusion protein; (A) GFP fluorescence, (B) DIC image,

18   (C) merge of the GFP signal and the DIC image. (D, E, F, G) Images of the *Δena2*

19   strain expressing the UmEna2-GFP and ER-RFP fusion proteins; (D) DIC image,

20   (E) GFP fluorescence, (F) RFP fluorescence, (G) merge of the GFP and RFP

21   signals.

22

38

1 FIG. 6. Localization of the NcENA2-GFP fusion proteins in B31 yeast cells. (A, B,

2 C) Images of the NcENA2-GFP fusion protein; (A) GFP fluorescence, (B) DIC

3 image, (C) merge of the GFP signal and the DIC image.

4

5 FIG. 7. Defective growth of the *U. maydis Δena1 Δena2* strain in YPD pH 4.0. The

6 wild type strain of *U. maydis* (FB1), and the wild type and *Δena1-4* (G19) strains of

7 *S. cerevisiae* were used as controls.

8

1   Table1. Oligonucleotides used in this study. In some of the primers a restriction site

2   sequence was included (underlined).

3   _____

4   Primer name   5´-3´sequence

5   _____

6   ENA1-ATG   TCAGTTCAAGACAGCAGGTTCATC

7   ENA1-STOP   GCTACAGTGTGCTATGAAAGAAAG

8   XbaI-ENA2-ATG   GTCTAGATAAACAATGGTTCACGGTCATGGCT

9   ENA2-STOP   TGGCAGGACGGGGAGACGCAATAC

10 NdeI-ENA1ATG   CCATATGGTGCACAAGAAAGAAGACAAG

11 NdeI-ENA1Rev   CCATATGTTTCACCATCGTTTTCTCGGTCGAGG

12 BamHI-ENA2ATG   GGGATCCTAAACAATGGTTCACGGTCATGGCT

13 NdeI-ENA2Rev   CCATATGTCTAGCAGTGGCGGCCGACTTTTC

14 PC-13B8-1   GGACACCTGGGGGAAGAACAAG

15 PC-13B8-1R   GGCCGGTGCAGACGAAGATGAT

16 PC-14G4-2   CTTTCATTGCCGTGGTCGAGCTGT

17 PC-14G4-2R   TTTTCGCTCAGTTGCTTCTTCGCC

18 UmACT1-2   GTGCCCATCTACGAAGGTTACT

19 UmACT1-1R   CGGCAGTGGTGGTGAAGGGGTAG

20 XbaNcENA2-ATG   GTCTAGAAAACAATGGGGACAGAGATCGAACTT

21 NcENA2-STOP   TTCTTACACACCCTCTCCACCAACC

22 Bam-NcENA2Rev   CGGATCCTGTACACACCCTCTCCACCAACC

23 _____

40

TABLE 2. Effect of growth conditions on *U. maydis ena1* and *ena2* transcript abundance[a]

_____

Transcript	YPD	AP 0.5 M Na$^+$	AP 0.5 M K$^+$	AP pH 3.5	AP 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

_____

[a] Cells were grown overnight in AP medium with 3 mM K$^+$ and then transferred to the indicated media for 2 h. The given values are ratios with reference to actin transcript abundance
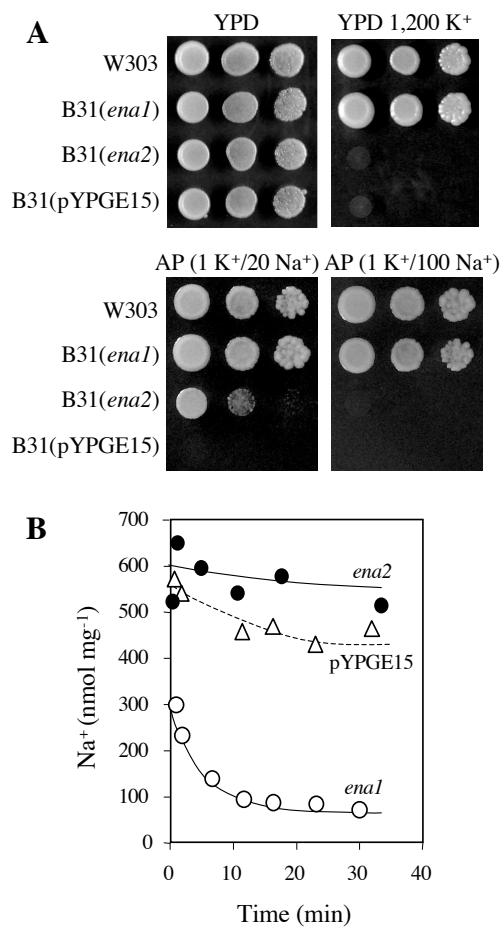
41

A

B

C

Fig 1

Fig. 2

A

	YPD	YPD 1,200 K$^+$
W303		
B31(*ena1*)		
B31(*ena2*)		
B31(pYPGE15)		

	AP (1 K$^+$/20 Na$^+$)	AP (1 K$^+$/100 Na$^+$)
W303		
B31(*ena1*)		
B31(*ena2*)		
B31(pYPGE15)		

B

Fig3

A

1 K⁺/20 Na⁺   1 K⁺/20 Na⁺

pH 6.5   pH 8.0

1 K⁺

| Δena1 Δena2 | FB1 |
| Δena2 | Δena1 |

pH 9.0

B

Fig. 4

Fig. 5

Fig. 6

YPD pH 6.5   YPD pH 4.0

| Δena1 Δena2 | FB1 |
| G19 | W303 |

Fig. 7